# CYTOKINES AS PLAYERS OF NEURONAL PLASTICITY AND SENSITIVITY TO ENVIRONMENT IN HEALTHY AND PATHOLOGICAL BRAIN

EDITED BY: Silvia Alboni and Laura Maggi

PUBLISHED IN: Frontiers in Cellular Neuroscience

frontiers Research Topics

frontiers

***Frontiers Copyright Statement***

*© Copyright 2007-2016 Frontiers Media SA. All rights reserved.*

*All content included on this site, such as text, graphics, logos, button icons, images, video/audio clips, downloads, data compilations and software, is the property of or is licensed to Frontiers Media SA ("Frontiers") or its licensees and/or subcontractors. The copyright in the text of individual articles is the property of their respective authors, subject to a license granted to Frontiers.*

*The compilation of articles constituting this e-book, wherever published, as well as the compilation of all other content on this site, is the exclusive property of Frontiers. For the conditions for downloading and copying of e-books from Frontiers' website, please see the Terms for Website Use. If purchasing Frontiers e-books from other websites or sources, the conditions of the website concerned apply.*

*Images and graphics not forming part of user-contributed materials may not be downloaded or copied without permission.*

*Individual articles may be downloaded and reproduced in accordance with the principles of the CC-BY licence subject to any copyright or other notices. They may not be re-sold as an e-book.*

*As author or other contributor you grant a CC-BY licence to others to reproduce your articles, including any graphics and third-party materials supplied by you, in accordance with the Conditions for Website Use and subject to any copyright notices which you include in connection with your articles and materials.*

*All copyright, and all rights therein, are protected by national and international copyright laws.*

*The above represents a summary only. For the full conditions see the Conditions for Authors and the Conditions for Website Use.*

**ISSN 1664-8714**
**ISBN 978-2-88919-768-2**
**DOI 10.3389/978-2-88919-768-2**

## About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

## Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

## Dedication to Quality

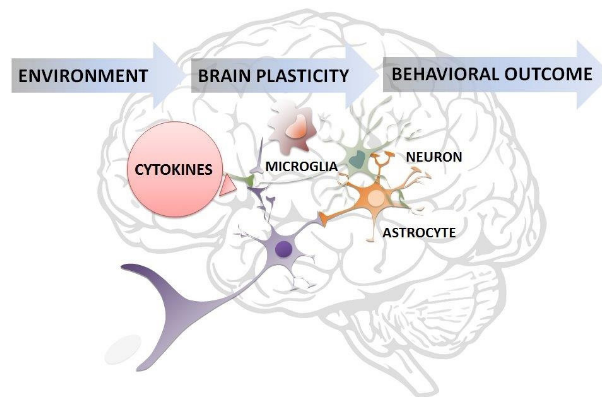
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

## What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

# CYTOKINES AS PLAYERS OF NEURONAL PLASTICITY AND SENSITIVITY TO ENVIRONMENT IN HEALTHY AND PATHOLOGICAL BRAIN

Topic Editors:
**Silvia Alboni,** University of Modena and Reggio Emilia, Italy
**Laura Maggi,** Sapienza University of Rome, Italy




Schematic representation of the cytokines role in mediating the effects of the environment on neuronal plasticity and behavioral outcomes. Throughout the brain, cytokines play a key role in the crosstalk between the neurons and glia cells. Central cytokines levels are sensitive to environmental challenge and translate peripheral stimuli in to central molecular and cellular responses underling changes in brain plasticity and behavior.
Image by Silvia Alboni and Laura Maggi.

It is now accepted that immune molecules are not only present within the brain during pathology but they exert physiological functions in the "healthy" brain as well. Increasing evidence points to a neuro-modulatory role of cytokines and chemokines (CHEMOtactic cytoKINES) in basal transmission and plasticity processes where signaling between peri-synaptic astrocytes, microglia and neurons plays an important role. Nevertheless, the exact mechanisms as to how cytokines, and in particular chemokines, participate in the molecular and cellular processes thought to subserve memory formation, plasticity processes and responsiveness to environmental stimuli remain to be clarified.

Interestingly, in in vitro preparations, molecules like TNF-$\alpha$, interleukin (IL)-1$\beta$, IL-6, CX3CL1, CXCL12, CCL2 and CCL3 are implicated in synaptic formation and scaling, in modulation of glutamatergic transmission, in plasticity and neurogenesis, in particular in the hippocampus. The hippocampus is an extremely plastic structure, one of the main neurogenic niches in the adult brain, that exhibits a marked sensibility to environmental stimuli. Indeed exposure of mice to environmental enrichment (EE) modifies learning and memory abilities increasing neurogenesis and neuronal plasticity whether exposure to severe stressful experiences diminishes neurotrophic

support, impairs neurogenesis, plasticity and cognition. In the hippocampus cytokines play a key role in mediating both positive as well as negative effects of the environment affecting neuronal plasticity also in stress related pathologies, such as depression. It has been reported that mice lacking type 1 receptor for IL-1 display impaired hippocampal memory and LTP that are restored by EE; moreover negative effects on neuronal plasticity (and thus behavior) induced by stress exposure can be prevented by blocking IL-1 activity. In addition, mice lacking IL-6 have improved cognitive functions whereas the absence of microglia-driven CX3CR1 signaling increases hippocampal plasticity and spatial memory occluding the potentiating effects of EE.

However, the factors mediating the effect of environmental stimuli on behavior and plasticity has been only partially identified. Interestingly, it has been suggested that chemokines can play a key role in the flexibility of hippocampal structure and may modulate neuronal signaling during behavior. The question is how cytokines may translate environmental stimuli in plasticity and behavioral changes.

This research topic is proposed to explore the role of cytokines, and more in particular chemokines, in the modulation of neuronal activity as a fundamental step for the correct brain wiring, function and susceptibility to environment.

We encourage the submission of original research reports, review articles, commentaries, perspectives or short communications, in the following (but not limited to) topics:
- Role of cytokines and chemokines in neuronal plasticity
- Immune molecules and responsiveness to environment
- Role of chemokine in the flexibility of hippocampal structure

**Citation:** Alboni, S., Maggi, L., eds. (2016). Cytokines as Players of Neuronal Plasticity and Sensitivity to Environment in Healthy and Pathological Brain. Lausanne: Frontiers Media.
doi: 10.3389/978-2-88919-768-2

# Table of Contents

06 ***Editorial: Cytokines as Players of Neuronal Plasticity and Sensitivity to Environment in Healthy and Pathological Brain***
Silvia Alboni and Laura Maggi

08 ***Fractalkine regulation of microglial physiology and consequences on the brain and behavior***
Rosa Chiara Paolicelli, Kanchan Bisht and Marie-Ève Tremblay

18 ***Defective microglial development in the hippocampus of* Cx3cr1 *deficient mice***
Francesca Pagani, Rosa C. Paolicelli, Emanuele Murana, Barbara Cortese, Silvia Di Angelantonio, Emanuele Zurolo, Eva Guiducci, Tiago A. Ferreira, Stefano Garofalo, Myriam Catalano, Giuseppina D'Alessandro, Alessandra Porzia, Giovanna Peruzzi, Fabrizio Mainiero, Cristina Limatola, Cornelius T. Gross and Davide Ragozzino

32 ***CX3CL1 is up-regulated in the rat hippocampus during memory-associated synaptic plasticity***
Graham K. Sheridan, Anita Wdowicz, Mark Pickering, Orla Watters, Paul Halley, Niamh C. O'Sullivan, Claire Mooney, David J. O'Connell, John J. O'Connor and Keith J. Murphy

47 ***IFN-γ differentially modulates memory-related processes under basal and chronic stressor conditions***
Darcy Litteljohn, Eric Nelson and Shawn Hayley

61 ***Hippocampal Lipocalin 2: An emotional link between neurons and glia***
Oliver von Bohlen und Halbach and Hermona Soreq

62 ***Interleukin-18 alters protein expressions of neurodegenerative diseases-linked proteins in human SH-SY5Y neuron-like cells***
Elina M. Sutinen, Minna A. Korolainen, Jukka Häyrinen, Irina Alafuzoff, Steven Petratos, Antero Salminen, Hilkka Soininen, Tuula Pirttilä and Johanna O. Ojala,

78 ***The inflammatory molecules IL-1β and HMGB1 can rapidly enhance focal seizure generation in a brain slice model of temporal lobe epilepsy***
Angela Chiavegato, Emanuele Zurolo, Gabriele Losi, Eleonora Aronica and Giorgio Carmignoto

87 ***Increased astrocyte expression of IL-6 or CCL2 in transgenic mice alters levels of hippocampal and cerebellar proteins***
Donna L. Gruol, Khanh Vo and Jennifer G. Bray

101 ***CXCL12 chemokine and its receptors as major players in the interactions between immune and nervous systems***
Alice Guyon

**111** ***Brain-derived neurotrophic factor: A bridge between inflammation and neuroplasticity***
Francesca Calabrese, Andrea C. Rossetti, Giorgio Racagni, Peter Gass, Marco A. Riva and Raffaella Molteni

**118** ***Inflammation and neuronal plasticity: A link between childhood trauma and depression pathogenesis***
Annamaria Cattaneo, Flavia Macchi, Giona Plazzotta, Begni Veronica, Luisella Bocchio-Chiavetto, Marco Andrea Riva and Carmine Maria Pariante

**130** ***Cellular and molecular mechanisms of immunomodulation in the brain through environmental enrichment***
Gaurav Singhal, Emily J. Jaehne, Frances Corrigan and Bernhard T. Baune

frontiers in Cellular Neuroscience

EDITORIAL
published: 12 January 2016
doi: 10.3389/fncel.2015.00508

# Editorial: Cytokines as Players of Neuronal Plasticity and Sensitivity to Environment in Healthy and Pathological Brain

*Silvia Alboni*[1*†] *and Laura Maggi*[2*†]

[1] Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy, [2] Department of Physiology and Pharmacology, Sapienza University, Rome, Italy

**Keywords: cytokine, chemokines, microglia, neurons, astrocytes, neuronal plasticity, environment, behavior**

**The Editorial on the research topic**

**Cytokines as Players of Neuronal Plasticity and Sensitivity to Environment in Healthy and Pathological Brain**

In this e-Book, we collected recent evidence on the role of cytokines (including chemokines) in the interplay between environmental stimulation and central responses in both physiological and pathological conditions. The e-Book includes original studies and review articles focused on cytokine function during brain development as well as in the mature brain.

Cytokines, together with neurotransmitters and hormones, are signaling molecules playing a key role in the maintenance of neuro-immune-endocrine system homeostasis. The cytokine systems (constituted by cytokines, their receptors and regulators of their activity) are expressed throughout the brain and their expression is regulated during brain development until aging. Brain cells, including neurons as well as glia cells, can release and/or be responsive to cytokines, therefore these molecules can enable communication between different cell types. Under physiological conditions, cytokines typically participate in brain development and plasticity by translating environmental into molecular signals. However, once the allostatic equilibrium is compromised, cytokine systems, if over- or chronically-activated, may participate in mediating toxic effects in the brain. Indeed, a central role for cytokines in neuropsychiatric as well as neurodegenerative disorders is now well recognized.

The major source of cytokines release in the brain is microglia cells that are actively involved in adult brain homeostasis and in neural loss and synaptic maturation during development. The contribution of Pagani et al. addresses the role of fractalkine (CX3CL1) signaling in the developmental profile of morphological features and physiological properties of microglia using mice lacking the fractalkine receptor (who is expresses only in microglia within the healthy brain). Sheridan et al. investigated the role for fractalkine in synaptic plasticity showing that the levels of hippocampal fractalkine increases after a memory task and that the chemokine regulates glutamate-mediated neurotransmission tone. A comprehensive review on the role of fractalkine in regulating microglia properties, brain plasticity and behavior is provided by Paolicelli et al.. Another cytokine known to modulate memory-related processes is interferon (IFN)-γ. One of the article included in this e-book describe the effects induced by the lack of IFN-γ on memory function under basal or stressful conditions (Litteljohn et al.). This study emphasizes the importance of considering the "brain state" (healthy or disease) when the modulation of neurobehavioral processes by the cytokine systems it is evaluated. Interestingly, in the commentary of von Bohlen and Halbach, the astrocytic

OPEN ACCESS

***Edited and reviewed by:***
*Egidio D'Angelo,*
*University of Pavia, Italy*

****Correspondence:***
*Silvia Alboni*
*silvia.alboni@unimore.it;*
*Laura Maggi*
*laura.maggi@uniroma1.it*

*†These authors have equally contributed to this work.*

***Received:*** *02 October 2015*
***Accepted:*** *18 December 2015*
***Published:*** *12 January 2016*

***Citation:***
*Alboni S and Maggi L (2016) Editorial: Cytokines as Players of Neuronal Plasticity and Sensitivity to Environment in Healthy and Pathological Brain. Front. Cell. Neurosci. 9:508. doi: 10.3389/fncel.2015.00508*

secreted lipocalin 2 is described as a mediator of the dialogue between astrocytes and neuron that in turn affects synaptic plasticity.

Neuroimmune factors are particularly relevant to a number of neurophatologies. They play important pro-survival and/or pro-death roles by regulating targets in specific brain regions. Sutinem et al. reported that in pathological conditions, such as Alzheimer's disease, the cytokine interleukin (IL)-18 seems potentially involved in driving protein changes relevant for the pathogenesis, whereas Chiavegato et al. described the role of IL-1 beta and the high mobility group B1 (HMGB1) in preventing seizure-like discharges in models of focal epilepsy.

Cytokines affect brain functions through different molecular mechanisms. Groul et al. show that up-regualtion of astrocytic CCL2 and Il-6 differentially affect the levels of specific proteins in cerebellum and hippocampus. The review of Guyon described how the stimulation of the receptor CXCR4 by the chemokine CXCL12 regulates the synaptic release of glutamate and γ-aminobutyric acid (GABA). Calabrese et al. proposed the neurotrophin brain-derived neurotrophic factor (BDNF) as a bridge between increased levels of pro-inflammatory cytokines and impaired neuroplasticity while the contribution of Cattaneo et al. is centered on epigenetics mechanisms that, by altering inflammation-immune systems and neuronal plasticity, may increase vulnerability to develop psychiatric disorders following early life stressful events.

Finally, in their review, Singhal et al. analyze the neuroimmune mechanisms associated with neurobiological and behavioral changes following different environmental stimulations. In particular, they described how environmental enrichment (mainly physical exercise) affects neuroimmune targets and behavior.

Overall, the work presented herein provides an insight into important aspects of the role of neuroimmune factors on brain activity and behavior. It represent a small windows on a complex panorama that still remain to be fully elucidated, especially with regard to the interplay between cytokine action, environmental stimulation and neuronal outcome.

## ACKNOWLEDGMENTS

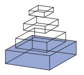
As Editors, we would like to thank all the Authors and Reviewers that contributed to this Research Topic.

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Copyright © 2016 Alboni and Maggi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 13 May 2014
doi: 10.3389/fncel.2014.00129



# Fractalkine regulation of microglial physiology and consequences on the brain and behavior

**Rosa Chiara Paolicelli[1], Kanchan Bisht[2,3] and Marie-Ève Tremblay[2,3]***

[1] Division of Psychiatry Research, University of Zurich, Zurich, Switzerland
[2] Axe Neurosciences, Centre de Recherche du CHU de Québec, Québec, Canada
[3] Department of Molecular Medicine, Université Laval, Québec, Canada

**Edited by:**
Laura Maggi, Sapienza, Italy

**Reviewed by:**
Etienne Audinat, Université Paris Descartes, France
Martin Fuhrmann, German Center for Neurodegenerative Diseases (DZNE), Germany

***Correspondence:**
Marie-Ève Tremblay, Axe Neurosciences, Centre de Recherche du CHU de Québec, 2705, boulevard Laurier, T2-33, Québec (QC) G1V 4G2, Canada
e-mail: tremblay.marie-eve@crchuq.ulaval.ca

Neural circuits are constantly monitored and supported by the surrounding microglial cells, using finely tuned mechanisms which include both direct contact and release of soluble factors. These bidirectional interactions are not only triggered by pathological conditions as a S.O.S. response to noxious stimuli, but they rather represent an established repertoire of dynamic communication for ensuring continuous immune surveillance and homeostasis in the healthy brain. In addition, recent studies are revealing key tasks for microglial interactions with neurons during normal physiological conditions, especially in regulating the maturation of neural circuits and shaping their connectivity in an activity- and experience-dependent manner. Chemokines, a family of soluble and membrane-bound cytokines, play an essential role in mediating neuron-microglia crosstalk in the developing and mature brain. As part of this special issue on *Cytokines as players of neuronal plasticity and sensitivity to environment in healthy and pathological brain*, our review focuses on the fractalkine signaling pathway, involving the ligand $CX_3CL1$ which is mainly expressed by neurons, and its receptor $CX_3CR1$ that is exclusively found on microglia within the healthy brain. An extensive literature largely based on transgenic mouse models has revealed that fractalkine signaling plays a critical role in regulating a broad spectrum of microglial properties during normal physiological conditions, especially their migration and dynamic surveillance of the brain parenchyma, in addition to influencing the survival of developing neurons, the maturation, activity and plasticity of developing and mature synapses, the brain functional connectivity, adult hippocampal neurogenesis, as well as learning and memory, and the behavioral outcome.

**Keywords: microglia, neurons, CX3CR1, fractalkine, development, synapses, neurogenesis, behavior**

## INTRODUCTION

Among the many strategies used by cells to communicate one with another, the repertoire of chemokines constitutes one of the most tightly regulated systems. Often, a particular cell type is uniquely expressing the ligand for a receptor that is selectively found on another cell type, thereby conferring a high degree of specificity to the ensuing signaling (Rossi and Zlotnik, 2000; Zlotnik and Yoshie, 2000; Allen et al., 2007). This is the case of fractalkine, a chemokine which signals directly from the producing neurons to their effector microglia, the only cells expressing its cognate receptor $CX_3CR1$ in the healthy brain (Nishiyori et al., 1998; Schwaeble et al., 1998; Maciejewski-Lenoir et al., 1999; Hughes et al., 2002).

Fractalkine, also known as $CX_3CL1$, is the only member of the ∂ ($CX_3C$) chemokine family, characterized by the presence of 3 amino acidic residues ($X_3$) localized between 2 cysteine residues, thus forming a disulphide bond, a CX3C motif, and also a transmembrane domain (Pan et al., 1997). The full-length fractalkine protein consists of 397 amino acids encoded by the *Cx3cl1* gene mapped on the chromosome 16 in human (Bazan et al., 1997; Pan et al., 1997) and of 395 amino acids encoded by the neurotactin gene mapped on the chromosome 11 in mouse (Rossi et al., 1998). Fractalkine is constitutively expressed at high levels by neurons, mostly in forebrain structures such as the hippocampus, amygdala, cerebral cortex, globus pallidus, striatum and thalamus, but also in the olfactory bulb, with almost no expression in the cerebellum, at the mRNA and protein levels in adult mouse *in situ* (Tarozzo et al., 2003). In the brainstem, a few scattered cells immunoreactive for fractalkine were initially depicted by Tarozzo and colleagues, while significant expression of the protein was subsequently observed *in situ* (Heinisch and Kirby, 2009; Ruchaya et al., 2012). Besides this neuronal expression, fractalkine mRNA and protein was also shown to be constitutively expressed by astrocytes, albeit at lower levels, in adult mouse, rat and human brain *in situ* (Hulshof et al., 2003; Sunnemark et al., 2005).

Fractalkine is a unique chemokine in that it exists in two different forms: a membrane-bound protein tethered to neuronal

membranes by a mucine-like stalk (approximately 95 kDa), and a soluble factor released upon cleavage of its N-terminal chemokine domain (approximately 70 kDa) (Garton et al., 2001). Membrane-bound fractalkine has been proposed to act as an adhesion molecule, whereas the diffusible form works as an extracellular chemoattractant promoting cellular migration. This function is shared with other members of the chemokines family, commonly acting as "**chemo**tactic cyto**kines**" during innate and adaptive immunity. The name "chemokines" is precisely derived from this ability to mediate attraction of their responsive cells (Bazan et al., 1997; Comerford and McColl, 2011; Pan et al., 2011; Zlotnik and Yoshie, 2012).

Chemokine receptors belong to the family of G-protein coupled receptors (GPCR), showing the presence of 7 transmembrane helices connected by several intra- and extracellular loops, as well as N-terminal extracellular and C-terminal intracellular domains. The N-terminal extracellular domain is considered important for chemokine binding and receptor activation, while the C-terminal end is coupled to G-proteins, and is important for receptor signaling upon chemokine binding. Based on their primary amino acid sequence and the respective ligands that they bind, chemokine receptors are also classified into four subfamilies, i.e., CXCR, CCR, CR and CX3CR (Proudfoot et al., 2010). The fractalkine receptor $CX_3CR1$ is a $G_i$-protein coupled receptor encoded by the *Cx3cr1* gene, previously named V28, located on the chromosome 3 in human (Combadiere et al., 1995) and the chromosome 9 in mouse (Combadiere et al., 1998). The subunit protein Gi inhibits the production of cAMP, triggering a variety of intracellular second messengers including phosphoinositide 3-kinase (PI3K), protein kinase B (AKT) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), which are well-known for mediating a wide range of cellular functions, including apoptosis, proliferation, transcription and migration (Al-Aoukaty et al., 1998; Chandrasekar et al., 2003). $CX_3CR1$ is ubiquitously expressed by monocytes, dendritic cells, and natural killer cells throughout the body (Imai et al., 1997; Combadiere et al., 1998; Harrison et al., 1998; Jung et al., 2000). Since these cells rarely infiltrate the brain parenchyma during normal physiological conditions, resident microglia are considered the only source of $CX_3CR1$ expression and thus the only recipient of fractalkine signaling in the healthy brain (Jung et al., 2000; Mizutani et al., 2012).

In recent years, microglia were demonstrated to originate from yolk-sac derived progenitors infiltrating the brain during early embryonic development, with no subsequent contribution to their renewal from bone-marrow derived myeloid cells (Ginhoux et al., 2010; Mizutani et al., 2012; Kierdorf et al., 2013). Microglia were also shown to be extremely dynamic in their once presumed "resting" state, continuously surveying the brain parenchyma and contacting pre- and post-synaptic elements with their highly motile processes (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009; Tremblay et al., 2010). Their physiological roles discovered so far comprise the elimination of supernumerary neurons and the maturation of synapses in the developing brain (Hoshiko et al., 2012; Cunningham et al., 2013; Lenz et al., 2013; Ueno et al., 2013), the regulation of neuronal and synaptic activity (Li et al., 2012; Pascual et al., 2012), the elimination of apoptotic newborn neurons generated in excess during adult hippocampal neurogenesis (Sierra et al., 2010), and the activity- and experience-dependent remodeling of neuronal circuits. Neuronal circuit plasticity is required for learning and memory processes in the developing and mature brain, where microglia contribute to both the formation and elimination of synapses (Tremblay et al., 2010, 2012; Paolicelli et al., 2011; Schafer et al., 2012; Parkhurst et al., 2013).

The $CX_3CR1$-GFP knock-in mouse line where the fractalkine receptor gene has been replaced by a green fluorescent protein (GFP) reporter (Jung et al., 2000), represents one of the most important tools for studying microglial involvement in the healthy brain. All microglial cells abundantly express GFP, resulting in an exceptional fluorescent labeling of their complex arborization, from cell body to distal processes (Tremblay et al., 2010). Since the $CX_3CR1$-GFP homozygous mice ($CX_3CR1^{KO/KO}$) are completely devoid of microglial $CX_3CR1$, and therefore of fractalkine signaling, comparing the heterozygous ($CX_3CR1^{KO/+}$) used for imaging with the homozygous mice also provides a strategy for dissecting the molecular determinants of neuron-microglia communication in a non-invasive manner (Tremblay, 2011; Wolf et al., 2013). The $CX_3CR1$-GFP heterozygous mice may be partially deficient in fractalkine signaling. Nevertheless, microglial morphology, dynamic surveillance (Nimmerjahn et al., 2005; Wake et al., 2009), dendritic spine turnover (Parkhurst et al., 2013), and microglial interactions with synaptic elements (Wake et al., 2009; Tremblay et al., 2010) were comparable *in vivo* between these $CX_3CR1$-GFP heterozygous mice and the Iba1-GFP mice from Hirasawa and colleagues where $CX_3CR1$ is not deleted (Hirasawa et al., 2005).

In the past few years, an extensive literature largely based on the $CX_3CR1$-GFP line revealed that fractalkine signaling influences a broad spectrum of microglial physiological properties. Within this perspective, our focused review is dedicated to the emerging roles of fractalkine signaling in the regulation of microglial motility, as much as its consequences on neuronal survival, synaptic pruning, maturation, function and plasticity, hippocampal neurogenesis, the brain functional connectivity, learning and memory, and on the behavioral outcome (see **Figure 1** for a schematic overview).

## MODULATORY EFFECTS OF FRACTALKINE ON MICROGLIAL PROPERTIES

Considering the crucial roles conferred by chemokines in the immune system, the $CX_3CL1$-$CX_3CR1$ axis was similarly expected to induce effector responses that are tightly adjusted to the homeostatic needs of the neuronal circuitry in the brain. Establishing microglial interactions with their dedicated neuronal subsets might be one example. The first evidence for such an important function comes from *in vitro* observations by Maciejewski-Lenoir and colleagues that fractalkine exerts a strong, dose-dependent migratory effect on cultured microglia derived from newborn rats (Maciejewski-Lenoir et al., 1999). Fractalkine application rapidly triggered changes in microglial activity, on a time scale of minutes, as revealed by the elevation

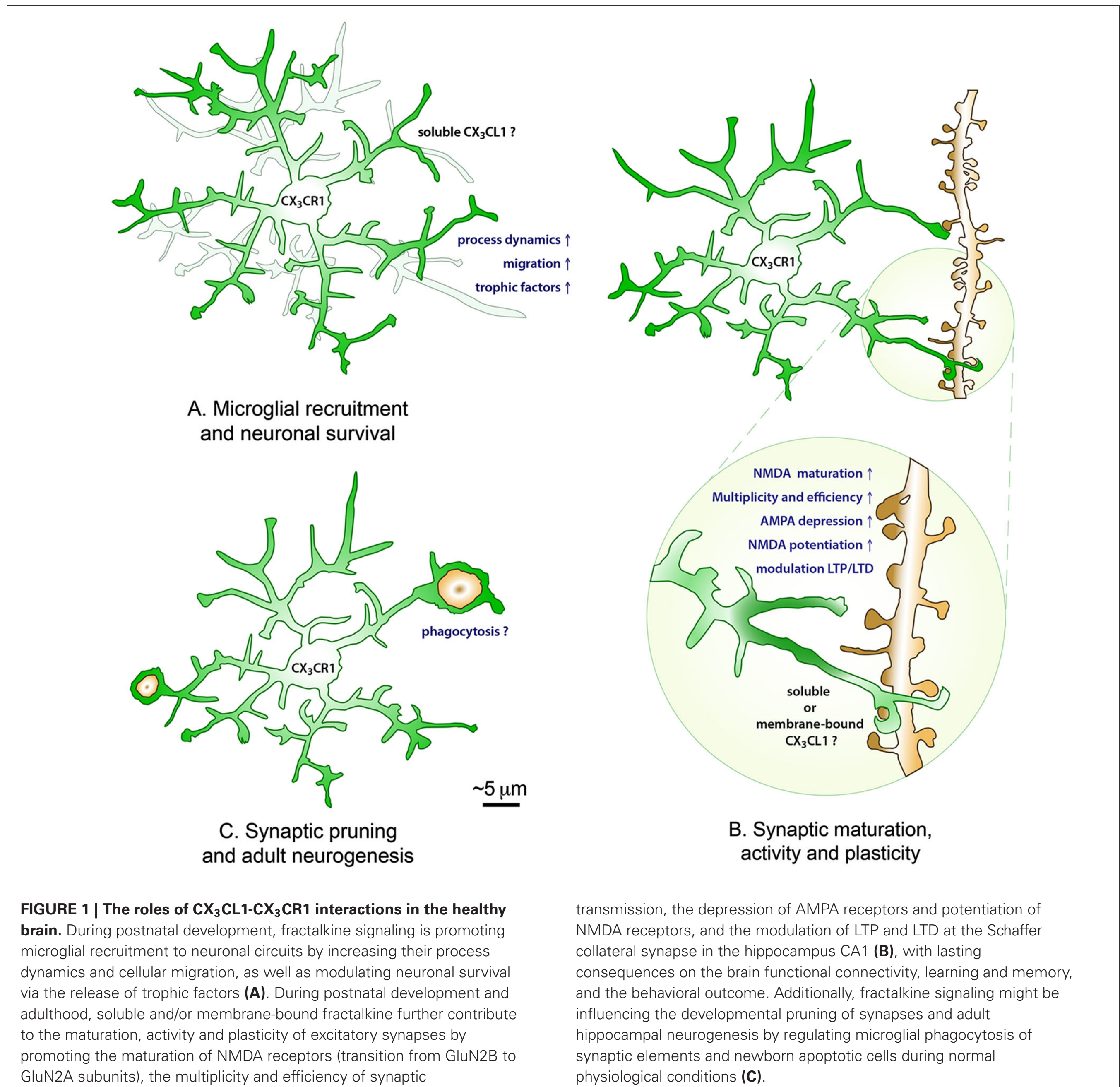



**FIGURE 1 | The roles of $CX_3CL1$-$CX_3CR1$ interactions in the healthy brain.** During postnatal development, fractalkine signaling is promoting microglial recruitment to neuronal circuits by increasing their process dynamics and cellular migration, as well as modulating neuronal survival via the release of trophic factors **(A)**. During postnatal development and adulthood, soluble and/or membrane-bound fractalkine further contribute to the maturation, activity and plasticity of excitatory synapses by promoting the maturation of NMDA receptors (transition from GluN2B to GluN2A subunits), the multiplicity and efficiency of synaptic transmission, the depression of AMPA receptors and potentiation of NMDA receptors, and the modulation of LTP and LTD at the Schaffer collateral synapse in the hippocampus CA1 **(B)**, with lasting consequences on the brain functional connectivity, learning and memory, and the behavioral outcome. Additionally, fractalkine signaling might be influencing the developmental pruning of synapses and adult hippocampal neurogenesis by regulating microglial phagocytosis of synaptic elements and newborn apoptotic cells during normal physiological conditions **(C)**.

of their intracellular calcium mobilization, which triggered a cascade of signaling leading to their cytoskeletal rearrangement and resulting in their migration. This response of microglia was diminished with antibodies against $CX_3CR1$ or pharmacological inhibitors of the $G_i$ subunit protein, supporting the functional involvement of $CX_3CL1$-$CX_3CR1$ interactions (Maciejewski-Lenoir et al., 1999).

Following these pioneering observations, Liang and colleagues further investigated the effects of $CX_3CR1$ signaling on microglial dynamics *ex vivo*, by using time-lapse confocal imaging of retinal explants from adult mice. In these experiments, microglial processes were found to be significantly slower in the $CX_3CR1$-GFP homozygous mice ($CX_3CR1^{KO/KO}$), either during basal condition or in response to focal laser-induced injury, compared with heterozygous controls ($CX_3CR1^{KO/+}$). Not only was microglial motility reduced in the $CX_3CR1$ deficient microglia, but also their migration towards the site of injury, which was determined over minutes (Liang et al., 2009). Similarly, Zhang and colleagues have provided evidence that fractalkine signaling is implicated in the regulation of microglial migration *in vitro*, using microglia freshly isolated from the retina of newborn rats, co-cultured with a photoreceptor cell line. In this study, microglial migration

quantified in a transwell chemotaxis assay was also found to be reduced following treatment with neutralizing anti-$CX_3CR1$ antibodies. Conversely, the addition of recombinant full-length or soluble fractalkine resulted in an increased proportion of microglial cells moving towards the photoreceptors (Zhang et al., 2012).

In the developing hippocampus, Paolicelli and colleagues also reported a slower increase of microglial density in the $CX_3CR1^{KO/KO}$ mice versus $CX_3CR1^{KO/+}$ littermates, between postnatal days (P)8 and 28 in the CA1 region (Paolicelli et al., 2011). A similar situation was recently described by Audinat and colleagues in the somatosensory cortex, where microglial infiltration normally occurs over the first postnatal days. In this study, microglial numbers were found to be significantly diminished among the centers of the barrel cortex which represent the whiskers, when comparing the $CX_3CR1^{KO/KO}$ mice with wild-type littermates at P5, but this effect was transient as similar levels of microglial density were reached by P9 (Hoshiko et al., 2012). More recently, microglial colonization of the motor cortex duding early postnatal development was also shown to be impaired in the absence of fractalkine signaling, resulting in microglial accumulation within the subcortical white matter of the $CX_3CR1^{KO/KO}$ mice at P5 (Ueno et al., 2013). Considering that $CX_3CL1$ expression is normally upregulated in the brain over the course of embryonic and postnatal maturation (Mody et al., 2001), these observations support a chemoattractant function of fractalkine signaling during normal development, particularly aimed at recruiting microglial cells to the relevant neuronal circuits, requiring specialized functional intervention during a period of intense activity-dependent remodeling.

## CONSEQUENCES OF FRACTALKINE ON NEURONAL CIRCUIT FUNCTION AND PLASTICITY

### SURVIVAL OF NEURONS

During normal development, microglia also contribute to the elimination of supernumerary neurons in various brain regions, including the cerebral cortex, hippocampus and cerebellum. The proposed mechanisms comprise the release of superoxide ions triggering apoptosis, the phagocytosis of non-apoptotic neural precursors and newborn neurons, and the release of trophic factors promoting neuronal survival (Marin-Teva et al., 2004; Wakselman et al., 2008; Cunningham et al., 2013; Ueno et al., 2013). Fractalkine signaling was recently involved in the latter mechanism. In particular, apoptotic neurons immunopositive for the cleaved-caspase 3 or detected with a TUNEL labeling of DNA fragmentation were more frequently encountered within the motor cortex of $CX_3CR1^{KO/KO}$ mice versus wild-type controls at P5 (Ueno et al., 2013). In this study by Ueno and colleagues, the insulin-like growth factor 1 (IGF-1) was further identified as a microglial factor preventing neuronal apoptosis. Interfering with IGF-1 signaling significantly increased the number of dying neurons in wild-type mice, using either intraventricular injection of a peptide blocking autophosphorylation of the IGF-1 receptor (H-1356), or a small hairpin (sh)RNA silencing gene expression of IGF-1. In the $CX_3CR1^{KO/KO}$ mice, mRNA expression of the IGF-1 binding protein 5 (IGFBP5) was also increased, and the free levels of IGF-1 were decreased, while injection of IGFBP5 promoted neuronal apoptosis (Ueno et al., 2013). These observations propose that $CX_3CL1$-$CX_3CR1$ interactions could serve to regulate the number of neurons during normal development, notably by promoting microglial release of trophic factors, even though the signaling cascade mediating microglial release of IGF-1 upstream of fractalkine signaling remains to be elucidated.

In a complementary manner, microglia were recently shown to promote neurogenesis and oligodendrogenesis in the developing rat brain, by releasing pro-inflammatory cytokines. Microglial release of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)α and interferon (IFN)γ was found to regulate subventricular zone (SVZ) neurogenesis between P1 and P10. Systemic injection of the anti-inflammatory antibiotic minocycline, which is commonly used to reduce microglial activation, was accompanied by reduced levels of pro-inflammatory cytokines, and numbers of proliferating cells, neuronal and oligodendrocytic progenitors. In the somatosensory cortex, between P6 and P8, minocycline was recently shown to increase neuronal death and microglial activation (Arnoux et al., 2014), but in this study by Shigemoto-Mogami and colleagues, the effects of minocycline on reducing microglial activation were confirmed by a strong reduction in activation markers such as CD11b and CD68. Interestingly, IGF-1 levels were not affected, thus suggesting that this signaling pathway is not regulating the effects of microglia on neurogenesis in the SVZ during the first postnatal days (Shigemoto-Mogami et al., 2014).

### MICROGLIAL PRUNING OF SYNAPSES

In the healthy brain, microglial cells also participate to the remodeling of neuronal circuits, an activity- and experience-dependent process required for learning and memory, and proper behavioral outcome, that is particularly exacerbated during postnatal development, but still persists throughout adulthood and normal aging. So far, microglial contacts with pre-synaptic axon terminals and post-synaptic dendritic spines, sometimes accompanied by their phagocytic engulfment and elimination, have been documented in the cerebral cortex, hippocampus and thalamus using a combination of cutting-edge microscopy techniques: transcranial two-photon *in vivo* imaging (cerebral cortex), electron microscopy with immunostaining and 3D reconstructions (cerebral cortex, hippocampus, thalamus), and stimulated-emission depletion confocal microscopy (hippocampus) (reviewed in Tremblay, 2011; Kettenmann et al., 2013). Using these techniques, phagocytic inclusions identified as synaptic elements, based on their ultrastructural features (i.e., 40 nm synaptic vesicles and post-synaptic densities) or immunoreactivity for the synaptic markers vesicular glutamate transporter 2 (Vglut2), post-synaptic density protein 95 (PSD95), and synaptosomal-associated protein 25 (SNAP25), were visualized inside of microglial cell bodies and processes (Tremblay et al., 2010; Paolicelli et al., 2011; Schafer et al., 2012).

The involvement of fractalkine signaling in mediating the elimination of synapses during normal development was particularly addressed by comparing the $CX_3CR1^{KO/KO}$ mice with $CX_3CR1^{KO/+}$ littermates in the hippocampus CA1 during the first postnatal weeks. In the study by Paolicelli and colleagues, the

decrease in microglial number was also found to be accompanied by a transient increase in dendritic spine density on the pyramidal neurons apical dendrites of the same region, thus indicating a possible role for fractalkine signaling in mediating microglial pruning of dendritic spines (Paolicelli et al., 2011). However, it remains to be elucidated whether fractalkine signaling exclusively instructs the recruitment of microglial cells to the proximity of maturing dendrites, or, in addition, also contributes to directly regulating microglial pruning itself.

In parallel, the work of Schafer and colleagues has shown that microglial pruning of synaptic elements is determined during postnatal development by the microglial complement 3 receptor (C3R), since microglial phagocytosis of axon terminals was significantly reduced *in situ* in the visual thalamus of C3R knockout mice at P5 and P20, resulting in a sustained impairment of synaptic density until P32–P35 at least (Stevens et al., 2007; Schafer et al., 2012). The subsequent work of Linnartz and colleagues additionally revealed that the classical complement cascade becomes recruited by the changes in neuronal glycocalyx *in vitro*. In particular, desialylated (i.e., lacking the terminal sugar residue sialic acid) neurites were found to be preferentially phagocytosed by microglial cells in primary culture via a CR3-dependent mechanism (Linnartz et al., 2012), but the molecular relationships between fractalkine signaling and the classical complement pathway remain unknown at this early stage of investigation in the field.

## FUNCTIONAL MATURATION OF SYNAPSES

A role for fractalkine signaling in modulating the postnatal development of neuronal circuits was also lately proposed by the work of Hoshiko and colleagues. In this study, the delayed infiltration of microglial cells in the barrel cortex was accompanied by defects of synaptic maturation. Electrophysiological analyses in acute thalamocortical slices from $CX_3CR1^{KO/KO}$ mice versus wild-type littermates revealed that $CX_3CR1$ deletion affects the functional maturation of postsynaptic glutamate receptors which normally occurs at thalamocortical synapses in the first 2 postnatal weeks. During the first postnatal week, NMDA receptors contain predominantly GluN2B subunits, replaced by GluN2A subunits by the end of the second postnatal week to increase the efficiency of synaptic transmission. Considered as an index of synaptic maturation, this switch is associated with faster kinetics of the NMDARs-mediated excitatory postsynaptic currents (EPSC). This transition from GluN2B to GluN2A subunits was precisely delayed in the $CX_3CR1$ knockout mice. In particular, the decay time of the NMDAR-mediated responses was found to be significantly reduced at P9-10 in the $CX_3CR1^{KO/KO}$ mice compared with wild-type littermates, using single-cell recordings. However, the defect was transient, without any remaining difference in the decay time of NMDAR-mediated responses at P27–P33 (Hoshiko et al., 2012).

In addition, Zhan and colleagues recently reported differences in the regulation of EPSCs in the $CX_3CR1^{KO/KO}$ mice, by using single-cell recording at the Schaffer collateral synapse in acute hippocampal slices (Zhan et al., 2014). Both the miniature EPSCs (mEPSC) and spontaneous EPSCs (sEPSCs) were investigated. Since mEPSCs are recorded in presence of tetrodotoxin (TTX), a sodium channel blocker that prevents action potential-induced currents, they constitute a measure of post-synaptic currents induced by random release of synaptic vesicles. On the other hand, sEPSCs reflect currents triggered by an action potential, and confer a measure of synaptic efficiency. The amplitude of sEPSC arising from the action potential-dependent release of synaptic vesicles normally increases over the course of postnatal development, as functional synapses become increasingly formed and neuronal networks properly interconnected, a process named synaptic multiplicity. On the contrary, the amplitude of mEPSCs remains relatively constant across the same age interval. The ratio between sEPSCs and mEPSCs increases as neural circuits develop, and can be considered as an index of neural circuit maturation (Hsia et al., 1998). In littermate wild-type mice, the amplitude of sEPSCs was found to be significantly larger than mEPSCs both at P15 and P40. In $CX_3CR1$ knockout mice, however, no significant difference was observed between action potential- (sEPSCs) and non-action potential-dependent (mEPSCs) currents at P15 and later at P40, suggesting reduced synaptic multiplicity and less efficient post-synaptic transmission when microglial function is compromised. With the previous observations, these recent findings support a crucial role for fractalkine signaling in regulating the maturation of excitatory synapses during normal development.

## SYNAPTIC TRANSMISSION

In line with these observations, several studies have recently revealed modulatory effects of fractalkine signaling on the electrophysiological properties of excitatory synapses in acute hippocampal slices. Ragozzino and colleagues have first reported that fractalkine negatively modulates the AMPA receptors-dependent component of glutamatergic transmission at the Schaffer collaterals synapse. In this study, the application of fractalkine induced a sustained, dose-dependent reduction of the evoked EPSC amplitude in the CA1 region of acute hippocampal slices from P14–P22 mice and rats. This depression of EPSC was not observed in slices from $CX_3CR1^{KO/KO}$ mice, and specifically depended on AMPA receptors function, being elicited by AMPA during the silencing of endogenous synaptic transmission with TTX and bicuculline (an antagonist of $GABA_A$ receptors), and suppressed by the AMPA and kainate receptors blocker 6,7-dinitroquinoxaline-2,3-dione (DNQX). In contrast, the NMDA receptors blocker D-amino-5-phosphonovaleric acid (D-APV) produced no effects (Ragozzino et al., 2006). Bertollini and colleagues similarly observed that fractalkine induces a sustained but reversible depression of field excitatory postsynaptic potentials (fEPSP), reflecting the postsynaptic response to the stimulation of Schaffer collaterals recorded in a population of neurons, in the CA1 region of acute slices derived from 21–29-day old mice. These effects were absent in slices from $CX_3CR1^{KO/KO}$ mice, and further suppressed in wild-type mice after application of a $CX_3CR1$ blocking antibody (Bertollini et al., 2006). Nevertheless, the mechanisms by which neuron to microglia crosstalk might contribute to these effects of fractalkine signaling on glutamatergic synapses function still remain unknown.

In this context, recent studies have suggested a possible involvement of microglial release of adenosine, not only in

mediating the depression of AMPA receptors function, but also the potentiation of NMDA receptors function. A role for the purine nucleoside adenosine was first proposed by the work of Piccinin and colleagues, showing that fractalkine causes a reversible depression of EPSCs in acute hippocampal slices from 14–20 days old mice, an effect that is prevented by treatment with adenosine deaminase, an enzyme that irreversibly converts adenosine to the related nucleoside iosine, and abolished by the specific AR3 antagonist MRS1523, contrarily to the AR1 (DPCPX) and AR2 (SCH58261) antagonists. These observations were corroborated by additional findings that fractalkine induces EPSC depression in acute slices from AR1 and AR2 knockout mice, but not in slices from AR3 knockout mice (Piccinin et al., 2010). More recently, Scianni and colleagues further proposed that fractalkine-induced release of adenosine increases the amplitude of fEPSPs resulting from the stimulation of Schaffer collaterals, by acting on its NMDA dependent component specifically. In this study, the effects of fractalkine on the fEPSP were found to be abolished by the selective antagonist of NMDA receptors 5,7-dichlorokynurenic acid (DCKA), the specific AR2 blocker SCH58261, and in acute slices from AR2 knockout mice, thus suggesting a role for AR2 in mediating these effects. Upon fractalkine stimulation, a significant increase in the concentration of D-serine, a co-agonist of NDMA receptors, was also measured by mass spectrometry analysis in the extracellular medium of microglia and astrocyte primary cultures. Microglial (or astrocytic) release of D-serine might therefore potentiate NMDA receptors function downstream of fractalkine signaling (Scianni et al., 2013).

### SYNAPTIC PLASTICITY

How fractalkine-induced alterations of postsynaptic glutamatergic responses modulate the propensity for long-term synaptic plasticity was also recently investigated. In particular, Maggi and colleagues have used fEPSP recordings at the Schaffer collaterals synapse to reveal that fractalkine significantly inhibits long-term potentiation (LTP) when administered during the critical period for induction, in acute slices from 6–8 weeks old wild-type mice. This impairment of LTP was however absent in slices from $CX_3CR1^{KO/KO}$ mice, and additionally found to require the activation of AR3 receptors as it was prevented by the selective antagonist MRS1523 (Maggi et al., 2009), in agreement with the previous findings. Nevertheless, subsequent studies using $CX_3CR1^{KO/KO}$ mice to explore the effects of fractalkine signaling on long-term plasticity, in acute slices from the hippocampus CA1 region, reported a complete absence of LTP (Rogers et al., 2011), or a more sustained LTP than in wild-type littermates, when LTP was induced by a weak stimulation protocol (Maggi et al., 2011). This apparent discrepancy warranting further investigation might result from differences in the ages, diet and housing conditions of the animals, or electrophysiological preparation and stimulation protocols between studies. Lastly, Paolicelli and colleagues also examined the influence of fractalkine signaling on the induction of long-term depression (LTD) in the $CX_3CR1^{KO/KO}$ mice compared to wild-type littermates, reporting no difference between genotypes during adulthood (P40), despite a significant enhancement of LTD in the $CX_3CR1^{KO/KO}$ at P13 (Paolicelli et al., 2011), thus suggesting the possibility that only LTP could be modulated by fractalkine signaling in the mature healthy brain.

### ADULT HIPPOCAMPAL NEUROGENESIS

Neurogenesis continues throughout life in the hippocampus, where it was demonstrated to be necessary for synaptic plasticity, including the induction of LTP and LTD, as well as classical eye blink and fear conditioning, memory retention in spatial learning tasks, and encoding of overlapping input patterns (Sierra et al., 2014). Contributing to these processes, microglia have been shown to regulate the neurogenic cascade during normal physiological conditions, by their phagocytic elimination of apoptotic newborn cells, an efficient process that is undeterred by increased age or inflammatory challenge (Sierra et al., 2010). Conversely, microglial physiological properties such as their proliferation, migration and phagocytosis were recently found to be modulated by soluble factors, such as vascular endothelial growth factor (VEGF) released by neuronal progenitor cells (NPCs) when implanted in the striatum of adult mice *in vivo*, as supported by the work of Mosher and colleagues proposing that microglia are not only influencing neurogenesis, but may also be regulated by newborn cells (Mosher et al., 2012).

With respect to the involvement of fractalkine signaling, Bachstetter and colleagues first revealed that the density of proliferating, newly generated cells is significantly reduced in the subgranular zone (SGZ) of 4 month old $CX_3CR1^{KO/KO}$ mice compared with $CX_3CR1^{KO/+}$ littermates. Conversely, chronic treatment with fractalkine was found to promote neurogenesis in aged (22 months old) but not young (3 months old) or middle-aged rats (12 months old), while an antagonist of $CX_3CR1$ produced opposite effects in young, middle-aged and old rats (Bachstetter et al., 2011). In a follow-up study, comparing $CX_3CR1^{KO/KO}$ and $CX_3CR1^{KO/+}$ mice with wild-type littermates further revealed that adult hippocampal neurogenesis is regulated by fractalkine signaling in a gene-dose dependent manner, with intermediate levels of neurogenesis measured in the heterozygous mice (Rogers et al., 2011). Supporting these results, Maggi and colleagues also described a reduced adult hippocampal neurogenesis in the SGZ of 13 to 14 weeks old $CX_3CR1^{KO/KO}$ mice, versus wild-type littermates, and further revealed that neurogenesis is positively regulated by chronic environmental enrichment, even in the absence of $CX_3CR1$ (Maggi et al., 2011). Nevertheless, the cellular and molecular mechanisms which are mediating these effects downstream of fractalkine signaling remain unknown.

### FUNCTIONAL CONNECTIVITY

Lastly, Zhan and colleagues have recently shown that $CX_3CR1^{KO/KO}$ mice display a lasting impairment of synaptic connectivity into adolescence, in the hippocampus CA1 region. In particular, quantifying at the ultrastructural level the density of multi-synaptic boutons, i.e., axon terminals making synapses with 2 dendritic spines, revealed a significant reduction in the knockout mice compared to wild-type littermates at P40. These data support the hypothesis that in the absence of fractalkine signaling, compromised neuron-microglia interactions are affecting the efficiency of synaptic transmission (Zhan et al.,

2014) despite a normalized density of dendritic spines which was observed by the same group in adult animals (Paolicelli et al., 2011). In addition, local field potentials (LFPs) were investigated by Zhan and colleagues by performing *in vivo* recordings at P40. LFPs inform about the overall levels of electrical activity measured in a certain volume, resulting from the sum of synaptic activity. As a measure of long-range connectivity, coherence spectra of the LFPs measured locally were calculated, under the premise that high coherence values reflect strongly connected structures. By implanting electrodes within different brain regions simultaneously, a significant decrease in the coherence between the hippocampus and the prefrontal cortex was found in $CX_3CR1^{KO/KO}$ adult mice, compared to wild-type littermates, thus reflecting a decrease in functional connectivity. Interestingly, the coherence between the hippocampus and the prefrontal cortex was also investigated in behaving mice, during bouts of social investigation, revealing a significant increase in coherence following the onset of social investigation in wild-type mice, but not in $CX_3CR1$ knockouts. In line with these results, the global functional connectivity assessed by functional magnetic resonance imaging (fMRI) showed a significant reduction in the connectivity across brain regions, becoming particularly evident for distant regions, in the $CX_3CR1^{KO/KO}$ mice versus wild-type controls (Zhan et al., 2014). These recent findings are complementing one another in supporting a fundamental role for $CX_3CL1$-$CX_3CR1$ interactions in mediating the development and plasticity of neuronal circuits, at the molecular, cellular and neuronal circuit levels.

## CONSEQUENCES ON LEARNING, MEMORY, AND BEHAVIOR

At the behavioral level, Rogers and colleagues also reported deficits in different forms of learning and memory in 3 month old $CX_3CR1^{KO/KO}$ mice, in parallel with the alteration of LTP. In particular, motor learning was found to be compromised in the $CX_3CR1$ knockouts, compared to wild-type littermates, using standard rotarod training (Rogers et al., 2011). Locomotor and exploratory activity was however similar between the two genotypes, when assessed in the open field test, and no difference was observed in anxiety behavior, measured by the elevated plus maze. In addition, associative learning and memory was altered in a standard fear-conditioning paradigm: after a similar freezing behavior in the training session, knockout mice failed to display associative learning (i.e., reduced freezing) when tested 24 h later in the same environment (Rogers et al., 2011). On the contrary, mice exposed to conditioning in a novel environment displayed a similar freezing behavior across genotypes, probably underlying hippocampal-specific deficits in cognition, considering that context-specific types of associative memory predominantly depend on the hippocampus (Sousa et al., 2006; Crawley, 2008). The $CX_3CR1$ knockouts also displayed deficits when performing the water maze test, thus further supporting a role for fractalkine signaling in modulating hippocampal-dependent learning and memory. These effects could be mediated by microglial release of the pro-inflammatory cytokine IL-1β since intrahippocampal infusion of its antagonist IL-1ra significantly reversed the deficits in cognitive function measured in the $CX_3CR1$ knockouts (Rogers et al., 2011).

However, Maggi and colleagues revealed that the $CX_3CR1^{KO/KO}$ mice are not significantly different from aged-matched wild-type controls in their ability to learn the water maze task, when they are tested at 13 to 14 weeks of age, even though they fail to perform better following prolonged exposure to an enriched environment, compared with standard housing conditions. Since environmental enrichment also failed to increase LTP, as well as adult hippocampal neurogenesis in parallel experiments, it has been proposed that $CX_3CR1$ deficiency could increase hippocampal plasticity as well as spatial memory, thereby blunting the potentiating effects of the environmental enrichment (Maggi et al., 2011). Here again, the discrepancy between studies is unclear, possibly resulting from differences in the animals or behavioral paradigms and analyses.

Interestingly, social interaction was also found to be altered in the $CX_3CR1^{KO/KO}$ mice, both early in life and into adulthood. Impaired social behavior was observed in juvenile mice, displaying no preference for their own mother over an inert stimulus, as assessed by the homing test. However, no impairment in performing the novel object recognition test was observed in the same mice. Similarly, adult $CX_3CR1$ knockout mice tested in a standard three-chamber apparatus failed to display significant interest towards a sex-matched social stimulus, compared to wild-type controls (Zhan et al., 2014). No deficit in responding to social olfactory cues was reported in these mice, suggesting that the impairment observed in social behavior was due to reduced social motivation, rather than difficulties with the discrimination of social cues. Additionally, increased grooming behavior was reported in adult $CX_3CR1$ knockout mice, when tested in a novel cage for 10 min, suggesting a propensity for increased repetitive behavior, particularly triggered under stressful conditions (Zhan et al., 2014).

## CONCLUSION

In recent years, a combination of imaging, electrophysiology, and behavioral analyses performed in the $CX_3CR1^{KO/KO}$ mice, versus $CX_3CR1^{KO/+}$ or wild-type littermates, have revealed that $CX_3CL1$-$CX_3CR1$ signaling crucially regulates the development and plasticity of neuronal circuits, with functional consequences on the brain connectivity, adult hippocampal neurogenesis, learning and memory, and the behavioral performance. Since microglia are the only cell type expressing $CX_3CR1$ in the healthy brain, they might be crucially involved in every one of these processes shown to be influenced by fractalkine signaling during normal physiological conditions: the survival of newborn neurons, the maturation and elimination of synapses, the regulation of synaptic transmission, long-term synaptic plasticity, and adult hippocampal neurogenesis. Nevertheless, the microglial effector functions which are precisely recruited, the molecular mechanisms acting downstream of $CX_3CL1$-$CX_3CR1$ interactions, much as the respective contributions of soluble versus membrane-bound fractalkine to these essential processes of normal physiology remain to be elucidated.

## ACKNOWLEDGMENTS

This work was supported by an EMBO postdoctoral fellowship to Rosa Chiara Paolicelli, and grants from the Banting Research Foundation, Scottish Rite Charitable Foundation of Canada, and Natural Sciences and Engineering Research Council of Canada, as well as a start-up fund from the Université Laval and Centre de recherche du CHU de Québec to Marie-Ève Tremblay.

## REFERENCES

Al-Aoukaty, A., Rolstad, B., Giaid, A., and Maghazachi, A. A. (1998). MIP-3alpha, MIP-3beta and fractalkine induce the locomotion and the mobilization of intracellular calcium and activate the heterotrimeric G proteins in human natural killer cells. *Immunology* 95, 618–624. doi: 10.1046/j.1365-2567.1998.00603.x

Allen, S. J., Crown, S. E., and Handel, T. M. (2007). Chemokine: receptor structure, interactions and antagonism. *Annu. Rev. Immunol.* 25, 787–820. doi: 10.1146/annurev.immunol.24.021605.090529

Arnoux, I., Hoshiko, M., Sanz Diez, A., and Audinat, E. (2014). Paradoxical effects of minocycline in the developing mouse somatosensory cortex. *Glia* 62, 399–410. doi: 10.1002/glia.22612

Bachstetter, A. D., Morganti, J. M., Jernberg, J., Schlunk, A., Mitchell, S. H., Brewster, K. W., et al. (2011). Fractalkine and CX 3 CR1 regulate hippocampal neurogenesis in adult and aged rats. *Neurobiol. Aging* 32, 2030–2044. doi: 10.1016/j.neurobiolaging.2009.11.022

Bazan, J. F., Bacon, K. B., Hardiman, G., Wang, W., Soo, K., Rossi, D., et al. (1997). A new class of membrane-bound chemokine with a CX3C motif. *Nature* 385, 640–644. doi: 10.1038/385640a0

Bertollini, C., Ragozzino, D., Gross, C., Limatola, C., and Eusebi, F. (2006). Fractalkine/CX3CL1 depresses central synaptic transmission in mouse hippocampal slices. *Neuropharmacology* 51, 816–821. doi: 10.1016/j.neuropharm.2006.05.027

Chandrasekar, B., Mummidi, S., Perla, R. P., Bysani, S., Dulin, N. O., Liu, F., et al. (2003). Fractalkine (CX3CL1) stimulated by nuclear factor kappaB (NF-kappaB)-dependent inflammatory signals induces aortic smooth muscle cell proliferation through an autocrine pathway. *Biochem. J.* 373, 547–558. doi: 10.1042/bj20030207

Combadiere, C., Ahuja, S. K., and Murphy, P. M. (1995). Cloning, chromosomal localization and RNA expression of a human beta chemokine receptor-like gene. *DNA Cell Biol.* 14, 673–680. doi: 10.1089/dna.1995.14.673

Combadiere, C., Salzwedel, K., Smith, E. D., Tiffany, H. L., Berger, E. A., and Murphy, P. M. (1998). Identification of CX3CR1. A chemotactic receptor for the human CX3C chemokine fractalkine and a fusion coreceptor for HIV-1. *J. Biol. Chem.* 273, 23799–23804. doi: 10.1074/jbc.273.37.23799

Comerford, I., and McColl, S. R. (2011). Mini-review series: focus on chemokines. *Immunol. Cell Biol.* 89, 183–184. doi: 10.1038/icb.2010.164

Crawley, J. N. (2008). Behavioral phenotyping strategies for mutant mice. *Neuron* 57, 809–818. doi: 10.1016/j.neuron.2008.03.001

Cunningham, C. L., Martinez-Cerdeno, V., and Noctor, S. C. (2013). Microglia regulate the number of neural precursor cells in the developing cerebral cortex. *J. Neurosci.* 33, 4216–4233. doi: 10.1523/jneurosci.3441-12.2013

Davalos, D., Grutzendler, J., Yang, G., Kim, J. V., Zuo, Y., Jung, S., et al. (2005). ATP mediates rapid microglial response to local brain injury in vivo. *Nat. Neurosci.* 8, 752–758. doi: 10.1038/nn1472

Garton, K. J., Gough, P. J., Blobel, C. P., Murphy, G., Greaves, D. R., Dempsey, P. J., et al. (2001). Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine (CX3CL1). *J. Biol. Chem.* 276, 37993–38001. doi: 10.1074/jbc.M106434200

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., et al. (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* 330, 841–845. doi: 10.1126/science.1194637

Harrison, J. K., Jiang, Y., Chen, S., Xia, Y., Maciejewski, D., McNamara, R. K., et al. (1998). Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. *Proc. Natl. Acad. Sci. U S A* 95, 10896–10901. doi: 10.1073/pnas.95.18.10896

Heinisch, S., and Kirby, L. G. (2009). Fractalkine/CX3CL1 enhances GABA synaptic activity at serotonin neurons in the rat dorsal raphe nucleus. *Neuroscience* 164, 1210–1223. doi: 10.1016/j.neuroscience.2009.08.075

Hirasawa, T., Ohsawa, K., Imai, Y., Ondo, Y., Akazawa, C., Uchino, S., et al. (2005). Visualization of microglia in living tissues using Iba1-EGFP transgenic mice. *J. Neurosci. Res.* 81, 357–362. doi: 10.1002/jnr.20480

Hoshiko, M., Arnoux, I., Avignone, E., Yamamoto, N., and Audinat, E. (2012). Deficiency of the microglial receptor CX3CR1 impairs postnatal functional development of thalamocortical synapses in the barrel cortex. *J. Neurosci.* 32, 15106–15111. doi: 10.1523/jneurosci.1167-12.2012

Hsia, A. Y., Malenka, R. C., and Nicoll, R. A. (1998). Development of excitatory circuitry in the hippocampus. *J. Neurophysiol.* 79, 2013–2024.

Hughes, P. M., Botham, M. S., Frentzel, S., Mir, A., and Perry, V. H. (2002). Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, during acute and chronic inflammation in the rodent CNS. *Glia* 37, 314–327. doi: 10.1002/glia.10037.abs

Hulshof, S., van Haastert, E. S., Kuipers, H. F., van den Elsen, P. J., De Groot, C. J., van der Valk, P., et al. (2003). CX3CL1 and CX3CR1 expression in human brain tissue: noninflammatory control versus multiple sclerosis. *J. Neuropathol. Exp. Neurol.* 62, 899–907.

Imai, T., Hieshima, K., Haskell, C., Baba, M., Nagira, M., Nishimura, M., et al. (1997). Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell* 91, 521–530. doi: 10.1016/s0092-8674(00)80438-9

Jung, S., Aliberti, J., Graemmel, P., Sunshine, M. J., Kreutzberg, G. W., Sher, A., et al. (2000). Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol. Cell. Biol.* 20, 4106–4114. doi: 10.1128/mcb.20.11.4106-4114.2000

Kettenmann, H., Kirchhoff, F., and Verkhratsky, A. (2013). Microglia: new roles for the synaptic stripper. *Neuron* 77, 10–18. doi: 10.1016/j.neuron.2012.12.023

Kierdorf, K., Erny, D., Goldmann, T., Sander, V., Schulz, C., Perdiguero, E. G., et al. (2013). Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. *Nat. Neurosci.* 16, 273–280. doi: 10.1038/nn.3318

Lenz, K. M., Nugent, B. M., Haliyur, R., and McCarthy, M. M. (2013). Microglia are essential to masculinization of brain and behavior. *J. Neurosci.* 33, 2761–2772. doi: 10.1523/jneurosci.1268-12.2013

Li, Y., Du, X. F., Liu, C. S., Wen, Z. L., and Du, J. L. (2012). Reciprocal regulation between resting microglial dynamics and neuronal activity in vivo. *Dev. Cell* 23, 1189–1202. doi: 10.1016/j.devcel.2012.10.027

Liang, K. J., Lee, J. E., Wang, Y. D., Ma, W., Fontainhas, A. M., Fariss, R. N., et al. (2009). Regulation of dynamic behavior of retinal microglia by CX3CR1 signaling. *Invest. Ophthalmol. Vis. Sci.* 50, 4444–4451. doi: 10.1167/iovs.08-3357

Linnartz, B., Kopatz, J., Tenner, A. J., and Neumann, H. (2012). Sialic acid on the neuronal glycocalyx prevents complement C1 binding and complement receptor-3-mediated removal by microglia. *J. Neurosci.* 32, 946–952. doi: 10.1523/jneurosci.3830-11.2012

Maciejewski-Lenoir, D., Chen, S., Feng, L., Maki, R., and Bacon, K. B. (1999). Characterization of fractalkine in rat brain cells: migratory and activation signals for CX3CR-1-expressing microglia. *J. Immunol.* 163, 1628–1635.

Maggi, L., Scianni, M., Branchi, I., D'Andrea, I., Lauro, C., and Limatola, C. (2011). CX(3)CR1 deficiency alters hippocampal-dependent plasticity phenomena blunting the effects of enriched environment. *Front. Cell Neurosci.* 5:22. doi: 10.3389/fncel.2011.00022

Maggi, L., Trettel, F., Scianni, M., Bertollini, C., Eusebi, F., Fredholm, B. B., et al. (2009). LTP impairment by fractalkine/CX3CL1 in mouse hippocampus is mediated through the activity of adenosine receptor type 3 (A3R). *J. Neuroimmunol.* 215, 36–42. doi: 10.1016/j.jneuroim.2009.07.016

Marin-Teva, J. L., Dusart, I., Colin, C., Gervais, A., van Rooijen, N., and Mallat, M. (2004). Microglia promote the death of developing Purkinje cells. *Neuron* 41, 535–547. doi: 10.1016/s0896-6273(04)00069-8

Mizutani, M., Pino, P. A., Saederup, N., Charo, I. F., Ransohoff, R. M., and Cardona, A. E. (2012). The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood. *J. Immunol.* 188, 29–36. doi: 10.4049/jimmunol.1100421

Mody, M., Cao, Y., Cui, Z., Tay, K. Y., Shyong, A., Shimizu, E., et al. (2001). Genome-wide gene expression profiles of the developing mouse hippocampus. *Proc. Natl. Acad. Sci. U S A* 98, 8862–8867. doi: 10.1073/pnas.141244998

Mosher, K. I., Andres, R. H., Fukuhara, T., Bieri, G., Hasegawa-Moriyama, M., He, Y., et al. (2012). Neural progenitor cells regulate microglia functions and activity. *Nat. Neurosci.* 15, 1485–1487. doi: 10.1038/nn.3233

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* 308, 1314–1318. doi: 10.1126/science.1110647

Nishiyori, A., Minami, M., Ohtani, Y., Takami, S., Yamamoto, J., Kawaguchi, N., et al. (1998). Localization of fractalkine and CX3CR1 mRNAs in rat brain: does fractalkine play a role in signaling from neuron to microglia? *FEBS Lett.* 429, 167–172. doi: 10.1016/s0014-5793(98)00583-3

Pan, Y., Lloyd, C., Zhou, H., Dolich, S., Deeds, J., Gonzalo, J. A., et al. (1997). Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature* 387, 611–617. doi: 10.1038/42491

Pan, X. D., Zhu, Y. G., Lin, N., Zhang, J., Ye, Q. Y., Huang, H. P., et al. (2011). Microglial phagocytosis induced by fibrillar beta-amyloid is attenuated by oligomeric beta-amyloid: implications for Alzheimer's disease. *Mol. Neurodegener.* 6:45. doi: 10.1186/1750-1326-6-45

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., et al. (2011). Synaptic pruning by microglia is necessary for normal brain development. *Science* 333, 1456–1458. doi: 10.1126/science.1202529

Parkhurst, C. N., Yang, G., Ninan, I., Savas, J. N., Yates, J. R. 3rd, Lafaille, J. J., et al. (2013). Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. *Cell* 155, 1596–1609. doi: 10.1016/j.cell.2013.11.030

Pascual, O., Ben Achour, S., Rostaing, P., Triller, A., and Bessis, A. (2012). Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. *Proc. Natl. Acad. Sci. U S A* 109, E197–E205. doi: 10.1073/pnas.1111098109

Piccinin, S., Di Angelantonio, S., Piccioni, A., Volpini, R., Cristalli, G., Fredholm, B. B., et al. (2010). CX3CL1-induced modulation at CA1 synapses reveals multiple mechanisms of EPSC modulation involving adenosine receptor subtypes. *J. Neuroimmunol.* 224, 85–92. doi: 10.1016/j.jneuroim.2010.05.012

Proudfoot, A. E. I., India, S., Hamel, D., and Handel, T. M. (2010). "Structural aspects of chemokines and their interactions with receptors and glycosaminoglycans," in *Chemokine Receptors as Drug Targets* (Weinheim, Germany: Wiley-VCH Verlag GmbH and Co. KGaA), 1–32.

Ragozzino, D., Di Angelantonio, S., Trettel, F., Bertollini, C., Maggi, L., Gross, C., et al. (2006). Chemokine fractalkine/CX3CL1 negatively modulates active glutamatergic synapses in rat hippocampal neurons. *J. Neurosci.* 26, 10488–10498. doi: 10.1523/jneurosci.3192-06.2006

Rogers, J. T., Morganti, J. M., Bachstetter, A. D., Hudson, C. E., Peters, M. M., Grimmig, B. A., et al. (2011). CX3CR1 deficiency leads to impairment of hippocampal cognitive function and synaptic plasticity. *J. Neurosci.* 31, 16241–16250. doi: 10.1523/jneurosci.3667-11.2011

Rossi, D. L., Hardiman, G., Copeland, N. G., Gilbert, D. J., Jenkins, N., Zlotnik, A., et al. (1998). Cloning and characterization of a new type of mouse chemokine. *Genomics* 47, 163–170. doi: 10.1006/geno.1997.5058

Rossi, D., and Zlotnik, A. (2000). The biology of chemokines and their receptors. *Annu. Rev. Immunol.* 18, 217–242. doi: 10.1146/annurev.immunol.18.1.217

Ruchaya, P. J., Paton, J. F., Murphy, D., and Yao, S. T. (2012). A cardiovascular role for fractalkine and its cognate receptor, CX3CR1, in the rat nucleus of the solitary tract. *Neuroscience* 209, 119–127. doi: 10.1016/j.neuroscience.2012.02.018

Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., et al. (2012). Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron* 74, 691–705. doi: 10.1016/j.neuron.2012.03.026

Schwaeble, W. J., Stover, C. M., Schall, T. J., Dairaghi, D. J., Trinder, P. K., Linington, C., et al. (1998). Neuronal expression of fractalkine in the presence and absence of inflammation. *FEBS Lett.* 439, 203–207. doi: 10.1016/s0014-5793(98)01384-2

Scianni, M., Antonilli, L., Chece, G., Cristalli, G., Di Castro, M. A., Limatola, C., et al. (2013). Fractalkine (CX3CL1) enhances hippocampal N-methyl-D-aspartate receptor (NMDAR) function via D-serine and adenosine receptor type A2 (A2AR) activity. *J. Neuroinflammation* 10:108. doi: 10.1186/1742-2094-10-108

Shigemoto-Mogami, Y., Hoshikawa, K., Goldman, J. E., Sekino, Y., and Sato, K. (2014). Microglia enhance neurogenesis and oligodendrogenesis in the early postnatal subventricular zone. *J. Neurosci.* 34, 2231–2243. doi: 10.1523/jneurosci.1619-13.2014

Sierra, A., Beccari, S., Diaz-Aparicio, I., Encinas, J. M., Comeau, S., and Tremblay, M. E. (2014). Surveillance, Phagocytosis and Inflammation: how never-resting microglia influence adult hippocampal Neurogenesis. *Neural Plast.* 2014:610343. doi: 10.1155/2014/610343

Sierra, A., Encinas, J. M., Deudero, J. J., Chancey, J. H., Enikolopov, G., Overstreet-Wadiche, L. S., et al. (2010). Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Cell Stem Cell* 7, 483–495. doi: 10.1016/j.stem.2010.08.014

Sousa, N., Almeida, O. F., and Wotjak, C. T. (2006). A hitchhiker's guide to behavioral analysis in laboratory rodents. *Genes Brain Behav.* 5(Suppl. 2), 5–24. doi: 10.1111/j.1601-183x.2006.00228.x

Stevens, B., Allen, N. J., Vazquez, L. E., Howell, G. R., Christopherson, K. S., Nouri, N., et al. (2007). The classical complement cascade mediates CNS synapse elimination. *Cell* 131, 1164–1178. doi: 10.1016/j.cell.2007.10.036

Sunnemark, D., Eltayeb, S., Nilsson, M., Wallstrom, E., Lassmann, H., Olsson, T., et al. (2005). CX3CL1 (fractalkine) and CX3CR1 expression in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis: kinetics and cellular origin. *J. Neuroinflammation* 2:17. doi: 10.1186/1742-2094-2-17

Tarozzo, G., Bortolazzi, S., Crochemore, C., Chen, S. C., Lira, A. S., Abrams, J. S., et al. (2003). Fractalkine protein localization and gene expression in mouse brain. *J. Neurosci. Res.* 73, 81–88. doi: 10.1002/jnr.10645

Tremblay, M. E. (2011). The role of microglia at synapses in the healthy CNS: novel insights from recent imaging studies. *Neuron Glia Biol.* 7, 67–76. doi: 10.1017/s1740925x12000038

Tremblay, M. E., Lowery, R. L., and Majewska, A. K. (2010). Microglial interactions with synapses are modulated by visual experience. *PLoS Biol.* 8:e1000527. doi: 10.1371/journal.pbio.1000527

Tremblay, M. E., Zettel, M. L., Ison, J. R., Allen, P. D., and Majewska, A. K. (2012). Effects of aging and sensory loss on glial cells in mouse visual and auditory cortices. *Glia* 60, 541–558. doi: 10.1002/glia.22287

Ueno, M., Fujita, Y., Tanaka, T., Nakamura, Y., Kikuta, J., Ishii, M., et al. (2013). Layer V cortical neurons require microglial support for survival during postnatal development. *Nat. Neurosci.* 16, 543–551. doi: 10.1038/nn.3358

Wake, H., Moorhouse, A. J., Jinno, S., Kohsaka, S., and Nabekura, J. (2009). Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. *J. Neurosci.* 29, 3974–3980. doi: 10.1523/jneurosci.4363-08.2009

Wakselman, S., Bechade, C., Roumier, A., Bernard, D., Triller, A., and Bessis, A. (2008). Developmental neuronal death in hippocampus requires the microglial CD11b integrin and DAP12 immunoreceptor. *J. Neurosci.* 28, 8138–8143. doi: 10.1523/jneurosci.1006-08.2008

Wolf, Y., Yona, S., Kim, K. W., and Jung, S. (2013). Microglia, seen from the CX3CR1 angle. *Front. Cell Neurosci.* 7:26. doi: 10.3389/fncel.2013.00026

Zhan, Y., Paolicelli, R. C., Sforazzini, F., Weinhard, L., Bolasco, G., Pagani, F., et al. (2014). Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior. *Nat. Neurosci.* 17, 400–406. doi: 10.1038/nn.3641

Zhang, M., Xu, G., Liu, W., Ni, Y., and Zhou, W. (2012). Role of fractalkine/CX3CR1 interaction in light-induced photoreceptor degeneration through regulating retinal microglial activation and migration. *PLoS One* 7:e35446. doi: 10.1371/journal.pone.0035446

Zlotnik, A., and Yoshie, O. (2000). Chemokines: a new classification system and their role in immunity. *Immunity* 12, 121–127. doi: 10.1016/S1074-7613(00)80165-X

Zlotnik, A., and Yoshie, O. (2012). The chemokine superfamily revisited. *Immunity* 36, 705–716. doi: 10.1016/j.immuni.2012.05.008

**Conflict of Interest Statement**: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 17 March 2014; accepted: 24 April 2014; published online: 13 May 2014.*
*Citation: Paolicelli RC, Bisht K and Tremblay M-È (2014) Fractalkine regulation of microglial physiology and consequences on the brain and behavior. Front. Cell. Neurosci.* ***8****:129. doi: 10.3389/fncel.2014.00129*
*This article was submitted to the journal Frontiers in Cellular Neuroscience.*
*Copyright © 2014 Paolicelli, Bisht and Tremblay. This is an open-access article distributed under the terms of the* Creative Commons Attribution License *(CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 31 March 2015
doi: 10.3389/fncel.2015.00111

# Defective microglial development in the hippocampus of *Cx3cr1* deficient mice

**Francesca Pagani[1*†], Rosa C. Paolicelli[2,3†], Emanuele Murana[4], Barbara Cortese[5], Silvia Di Angelantonio[1,4], Emanuele Zurolo[6], Eva Guiducci[3], Tiago A. Ferreira[3], Stefano Garofalo[4], Myriam Catalano[4,7], Giuseppina D'Alessandro[4,7], Alessandra Porzia[8], Giovanna Peruzzi[1], Fabrizio Mainiero[9], Cristina Limatola[4,7], Cornelius T. Gross[3] and Davide Ragozzino[4,7]**

*[1] Center for Life Nanoscience – Istituto Italiano di Tecnologia@Sapienza, Rome, Italy, [2] Division of Psychiatry Research, University of Zürich, Zürich, Switzerland, [3] Mouse Biology Unit, European Molecular Biology Laboratory, Monterotondo, Italy, [4] Department of Physiology and Pharmacology, Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza University of Rome, Rome, Italy, [5] Consiglio Nazionale delle Ricerche – Institute of Inorganic Methodologies and Plasmas, Department of Physics, Sapienza University of Rome, Rome, Italy, [6] Department of Neuropathology, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands, [7] Istituto di Ricovero e Cura a Carattere Scientifico Neuromed, Pozzilli, Italy, [8] Department of Molecular Medicine, Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza University of Rome, Rome, Italy, [9] Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy*

OPEN ACCESS

**Edited by:**
*Silvia Alboni, University of Modena and Reggio Emilia, Italy*

**Reviewed by:**
*Claudia Verderio, Consiglio Nazionale delle Ricerche, Italy*
*Claudia Eder, University of London, UK*
*Etienne Audinat, Paris Descartes University, France*

***Correspondence:**
*Francesca Pagani, Center for Life Nanoscience – Istituto Italiano di Tecnologia@Sapienza, Viale Regina Elena 291, 00161 Roma, Italy*
*francesca.pagani@iit.it*

*† These authors have contributed equally to this work.*

**Received:** 08 January 2015
**Accepted:** 11 March 2015
**Published:** 31 March 2015

**Citation:**
*Pagani F, Paolicelli RC, Murana E, Cortese B, Di Angelantonio S, Zurolo E, Guiducci E, Ferreira TA, Garofalo S, Catalano M, D'Alessandro G, Porzia A, Peruzzi G, Mainiero F, Limatola C, Gross CT and Ragozzino D (2015) Defective microglial development in the hippocampus of Cx3cr1 deficient mice. Front. Cell. Neurosci. 9:111. doi: 10.3389/fncel.2015.00111*

Microglial cells participate in brain development and influence neuronal loss and synaptic maturation. Fractalkine is an important neuronal chemokine whose expression increases during development and that can influence microglia function via the fractalkine receptor, CX3CR1. Mice lacking *Cx3cr1* show a variety of neuronal defects thought to be the result of deficient microglia function. Activation of CX3CR1 is important for the proper migration of microglia to sites of injury and into the brain during development. However, little is known about how fractalkine modulates microglial properties during development. Here we examined microglial morphology, response to ATP, and $K^+$ current properties in acute brain slices from *Cx3cr1* knockout mice across postnatal hippocampal development. We found that fractalkine signaling is necessary for the development of several morphological and physiological features of microglia. Specifically, we found that the occurrence of an outward rectifying $K^+$ current, typical of activated microglia, that peaked during the second and third postnatal week, was reduced in *Cx3cr1* knockout mice. Fractalkine signaling also influenced microglial morphology and ability to extend processes in response to ATP following its focal application to the slice. Our results reveal the developmental profile of several morphological and physiological properties of microglia and demonstrate that these processes are modulated by fractalkine signaling.

**Keywords: microglia, CX3CR1, fractalkine, development, rearrangement, potassium currents**

## Introduction

Microglia, traditionally known as resident immune cells in the central nervous system (CNS), play essential roles in brain circuit maturation during development, participating in the precise refinement of synaptic connections (Tremblay et al., 2010; Paolicelli et al., 2011; Schafer et al., 2012;

Zhan et al., 2014). Microglial cells derive from myeloid lineage and colonize the immature brain during development, progressively acquiring a ramified morphology, commonly associated to surveying activity (Prinz and Mildner, 2011; Walker et al., 2014). Ramified microglia lie very close to each other and have highly motile filopodia-like protrusions of variable shape, defining rarely overlapping niches of competency (Raivich, 2005). Microglial processes continuously sample the extracellular space and contact pre- and post-synaptic elements (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009; Tremblay et al., 2010). Among the functional consequences of this active and continuous surveillance of the developing brain appear to be the shaping neuronal circuits by phagocytosis (Paolicelli et al., 2011) and favoring synaptic maturation (Hoshiko et al., 2012). In damaged brain, microglial processes rapidly rearrange toward the site of injury (Nimmerjahn et al., 2005; Lee et al., 2008). Among soluble factors activating microglial cells, ATP regulates microglial processes extention and ramification in both physiological and pathological conditions (Davalos et al., 2005; Inoue et al., 2007). To reach the site of activating stimuli, microglia retract their cellular processes and rearrange them in a directional manner toward the lesion. In addition, a marked change in ionic channel expression has been observed (Boucsein et al., 2000). Indeed, in acute brain slices non-activated microglia frequently display a small linear conductance, while following nerve lesion (Boucsein et al., 2000) or status epilepticus (Avignone et al., 2008) they transiently express inward and outward rectifier potassium ($K^+$) currents (Menteyne et al., 2009; Kettenmann et al., 2011). Ion channels have been involved in many microglial functional properties (Eder, 2005), such as cell volume regulation (Schlichter et al., 1996; Eder et al., 1998), proliferation (Kotecha and Schlichter, 1999), migration (Rappert et al., 2002; Schilling et al., 2004) and cell process extension and retraction (Eder et al., 1998). However, microglia activation is no longer considered an all or none event, but rather a sequence of progressive stages (Ponomarev et al., 2007; Olah et al., 2012; Crain et al., 2013) depending on the balance between pro-inflammatory and anti-inflammatory signals in the surrounding environment (Biber et al., 2007; Lively and Schlichter, 2013).

Chemokines are key molecules in neuron-microglia communication in the developing and mature brain under both physiological and pathological conditions (de Jong et al., 2005). Fractalkine is an important neuron-microglia chemokine signal. In the CNS, fractalkine is expressed by neurons, especially in forebrain structures (Tarozzo et al., 2003), while its receptor, CX3CR1, is expressed uniquely by microglia (Harrison et al., 1998; Jung et al., 2000; Cardona et al., 2006; Mizutani et al., 2012). Recent studies showed that fractalkine-mediated microglia-neuron signaling modulates numerous physiological processes across the lifespan, including the maturation of synaptic connections (Paolicelli et al., 2011; Zhan et al., 2014), neuronal survival (Limatola et al., 2005; Lauro et al., 2008), and synaptic transmission and plasticity (Bertollini et al., 2006; Ragozzino et al., 2006; Piccinin et al., 2010; Maggi et al., 2011; Hoshiko et al., 2012). Mice lacking fractalkine signaling display deficits in hippocampal-dependent learning and memory (Rogers et al., 2011; Zhan et al., 2014) and in microglial properties in barrel cortex (Arnoux et al., 2013). Moreover, reduced microglia colonization during development has been described in hippocampus (Paolicelli et al., 2011) and somatosensory cortex (Hoshiko et al., 2012) in these mice.

In order to better understand the role of fractalkine signaling in microglia physiology and its possible impact on brain development, we studied several functional properties of microglia in acute brain slices from *Cx3cr1* knockout mice across the first postnatal weeks (PNWs). We report that the functional properties of microglia undergo dynamic changes during development and that these changes are absent or delayed in mice lacking fractalkine signaling. These data reveal the highly dynamic nature of microglial maturation during brain development and further highlight the importance of fractalkine signaling in this process.

## Materials and Methods

### Animals and Ethical Approval

For acute slice preparation, $Cx3cr1^{GFP/+}$ and $Cx3cr1^{GFP/GFP}$ mice were used (Jung et al., 2000). For microglia morphometric analysis in perfused brains, $Cx3cr1^{GFP/+}$ and $Cx3cr1^{GFP/KO}$ littermates were produced by intercrossing $Cx3cr1^{GFP/GFP}$ with $Cx3cr1^{KO/+}$ breeders (Haskell et al., 2001). This breeding strategy was required for the progeny to have a single GFP allele, regardless the Cx3cr1 genotype. Such approach was necessary for imaging microglia with similar fluorescent intensity thresholds. Procedures using laboratory animals were in accordance with the international guidelines on the ethical use of animals from the European Communities Council Directive of November 24, 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to reduce the number of animals used, in accordance with the European Communities Council Directive of September 20, 2010 (2010/63/UE).

### Slice Preparation

Acute hippocampal slices were prepared from $Cx3cr1^{+/GFP}$ and $Cx3cr1^{GFP/GFP}$ mice (Jung et al., 2000) in the first six PNWs. Animals were decapitated under halothane anesthesia, and whole brains were rapidly immersed for 10 min in chilled artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 125, KCl 2.3, $CaCl_2$ 2, $MgCl_2$ 1, $NaHPO_4$ 1, $NaHCO_3$ 26, and glucose 10 (Sigma Aldrich). The ACSF was continuously oxygenated with 95% $O_2$, 5% $CO_2$ to maintain physiological pH. Transverse 250 μm hippocampal slices were cut at 4°C with a vibratome (DSK, Kyoto, Japan), placed in a chamber containing oxygenated ACSF and allowed to recover for at least 1 h at room temperature. All recordings were performed at room temperature on slices submerged in ACSF and perfused (1 ml/min) with the same solution in the recording chamber under the microscope. For microglia morphometric analysis from perfused brains, on post-natal day 8 (P8), pups were anesthetized intraperitoneally with Avertin (Sigma-Aldrich, St Louis, MO, USA) and perfused transcardially with 4% paraformaldehyde. Brains were postfixed overnight at 4°C, and sliced afterward on a vibratome (100 μm thick sections; Leica Microsystems, Wetzlar, Germany). Upon

DAPI staining, slices were mounted on glass slides and kept for images acquisition.

## Time-Lapse Imaging in Acute Hippocampal Slices

Time-lapse fluorescence determinations were acquired at room temperature (24–25°C) using a customized digital imaging microscope. Excitation of GFP was achieved using a 1-nm-bandwidth polychromatic light selector (Till Polychrome V), equipped with a 150 W xenon lamp (Till Photonics, Germany). Fluorescence was visualized using an upright microscope (Axioskope) equipped with a 40x water-immersion objective (Achroplan CarlZeiss, USA) and a digital 12 bit CCD camera system (SensiCam, PCO AG, Germany). All the peripheral hardware control, image acquisition and image processing were achieved using customized software Till Vision v. 4.0 (Till Photonics. A glass pipette containing adenosine 5′-triphosphate magnesium salt (ATP, 3 mM; Sigma Aldrich) was placed in the stratum radiatum in the center of the recording field. Mg-ATP was pressure applied to the slices (100 ms; 5 psi) with a Picospritzer III (Parker Instrumentation). Changes in GFP fluorescence distribution were monitored by acquiring a fluorescent image every 10 s for 50 min. To quantify the speed of microglial processes rearrangement toward the pipette tip, we measured the increase of GFP fluorescence in a circular area centered on the pipette tip (10 μm radius). At each time point the fluorescence increase in the area was calculated as $\Delta F = F\text{-}F_0$, and then divided for $F_0$ ($\Delta F/F_0$, where $F_0$ is the average fluorescence before ATP puff), to normalize the difference in basal GFP fluorescence in slices from the two genotypes. Slices were used from 2 to 7 h after cutting.

## Tracking Analysis of Single Microglial Process

All images were processed using ImageJ software (Schindelin et al., 2012; Schneider et al., 2012). Images stacks were exported as .avi files to enable manual cell processes tracking on the ImageJ "Manual Tracking" plug-in (http://imagej.nih.gov/ij/plugins/track/track.html). To obtain quantitative distributions of tracks parameters, data were analyzed with ImageJ and Origin 7 (OriginLab Co.) software. Stacks were first background subtracted to optimize contrast. To obtain x–y coordinates of single processes, track positions were transferred into a new coordinate system, in which the ATP-containing pipette tip was set as origin ($x = 0$, $y = 0$). For each moving process (i), with position vector Ri(t), the change in position from one frame to the next *[*$\Delta Ri(t)$*]*, and the instantaneous velocity *[*vi(t)*]* were given by $\Delta Ri(t) = Ri(t+\Delta t) - Ri(t)$, and $vi(t) = \Delta Ri(t)/\Delta t$ respectively, where $\Delta t$ is the elapsed time among the two frames. The mean velocity of each process was calculated as $<v> = dx/dt$, expressed in μm/min, defining dx as the mean accumulated distance of each process i sampled within the time interval dt. The position of each process was converted in polar coordinates from Cartesian coordinates. The radial distance r in every pair of successive images was calculated by measuring the Euclidean distance of individual tracks considering $r(t)^2 = (x(t))^2 + (y(t))^2$. The mean velocity vector was then expressed as in polar coordinates oriented to the center, corresponding to the ATP-containing pipette tip. Displacement is defined as the vector length of the final coordinates from the origin. Directionality is an index of the straightness of the processes trajectory. Index values close to *one* indicates a straight migration trajectory toward the ATP-containing pipette; low values refer to process following a meandering path. Migration directionality was calculated as the ratio of the vector displacement of each process to their accumulated distance (process track) with the help of ImageJ software as previously described (Wu et al., 2012).

## Whole Cell Patch Clamp Recordings

Visually identified GFP-expressing microglial cells were patched in whole-cell configuration in the CA1 stratum radiatum. Micropipettes (4–5 MΩ) were usually filled with a solution containing the following composition (in mM): KCl 135, BAPTA 5, $MgCl_2$ 2, HEPES 10, and Mg-ATP 2 (pH 7.3 adjusted with KOH, osmolarity 290 mOsm; Sigma Aldrich). Voltage-clamp recordings were performed using an Axopatch 200A amplifier (Molecular Devices). Currents were filtered at 2 kHz, digitized (10 kHz) and collected using Clampex 10 (Molecular Devices); the analysis was performed off-line using Clampfit 10 (Molecular Devices). Slicing procedure might activate microglial cells especially near the surface of the slice, whereby recordings were performed on deep cells. Moreover, experiments were performed from 1 to 7 h after slicing. The current/voltage (I/V) relationship of each cell was determined applying voltage steps from −170 to +70 mV ($\Delta V = 10$ mV) for 50 ms holding the cell at −70 mV between steps. Resting membrane potential and membrane capacitance were measured at start of recording. Membrane capacitance was estimated as the total charge (i.e., the current integral, Qstep) mobilized in each cell by a 10 mV depolarizing step (Vstep): Qstep/Vstep (Supplementary Material). Outward and inward rectifier $K^+$ current amplitude were evaluated after subtraction of the leak current by a linear fit of the I/V curve between −100 and −50 mV. Cells were considered as expressing the outward rectifier $K^+$ current when the I/V relationship showed a rectification above −30 mV and the amplitude measured at 0 mV was at least 10 pA, after leak subtraction; similarly cells showing a small inward rectification below −100 mV were classified as expressing the inward rectifier $K^+$ current when subtracted current amplitude was at least 5 pA at −150 mV. Current densities, reported in **Table 1**, were obtained by normalization of current amplitude to cell capacitance. 4-aminopyridine (2 mM, Sigma Aldrich) was used as blocker of outward rectifier $K^+$ current.

**TABLE 1 | Current densities of outward ($I_K$; MP = +50 mV) and inward ($I_{Kir}$; MP = −150 mV) rectifier currents of microglia, in hippocampal slices from *Cx3cr1*$^{+/GFP}$ and *Cx3cr1*$^{GFP/GFP}$ mice at PNW 2 (*p*, *t*-test).**

| | Cx3cr1$^{+/GFP}$ (n/$n_{tot}$) | Cx3cr1$^{GFP/GFP}$ (n/$n_{tot}$) | *p* |
|---|---|---|---|
| $I_K$ (pA/pF) | 5.2 ± 0.8 (46/88) | 5.6 ± 0.8 (20/56) | 0.74 |
| $I_{Kir}$ (pA/pF) | −2.6 ± 0.2 (38/88) | −3.0 ± 0.7 (13/56) | 0.5 |

### Morphological Analysis of Microglial Cells

In slices from perfused brains, endogenous GFP signal was acquired in the hippocampus CA1 stratum radiatum, for a total of 52 serial optical sections (42 μm thickness in the *z*-axis, 129 × 129 μm in xy-axis). Representative images of those fields were obtained as a z-projection based on the maximal intensity signal, using ImageJ software. Within each acquired stack, all the cells that appear entire were subjected to three-dimensional reconstruction.

Three dimensional reconstruction of recorded microglial cells was achieved by injecting biocytin (0.4% dissolved in the internal solution) throughout the recording electrode for at least 10 min. Slices were removed from recording chamber and fixed with paraformaldehyde 4% for 20 min at room temperature and stored at 4°C. After Triton X100 permeabilization, sections were stained with primary antibody at 4°C (anti-GFP antibody, Aves Labs, Inc., Portland, Oregon; 1:800), then incubated with a secondary antibody conjugated to fluorescein (Aves Lab. 1:800), and with streptavidin conjugated to fluorophore Alexa594 (Abcam, 1:800).

Confocal microscopy analysis was performed with a TCS-SP5 (Leica) Laser Scanning System, at 40x magnification. Acquisition files were then processed with ImageJ software for two-dimensional analysis. Three dimensional reconstructions were generated with Imaris software (Bitplane, Zurich, Switzerland) and morphometric analysis of each reconstructed cell, both in acute slices and in perfused brain sections, was performed after surface and volume rendering.

### Isolation of Microglial GFP Positive Cells from Hippocampus

Two weeks old $Cx3cr1^{+/GFP}$ and $Cx3cr1^{GFP/GFP}$ mice were anesthetized and decapitated. Brains were removed, and isolated hippocampi were cut into small pieces. Single-cell suspension was achieved by mechanical dissociation and the suspension was applied to a 70 μm cell strainer. Cells were sorted based on GFP expression using a BD FACSAriaIII (BD Biosciences) equipped with a 488 nm laser and FACSDiva software 6.1.3 (BD Biosciences). Briefly, cells were first gated based on forward and side scatter area plot (FSC-A and SSC-A), and then detected in the green fluorescence channel for GFP expression. Following this gate strategy, GFP positive cells sorted were enriched to >98%, used for total RNA isolation with Single Cell RNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada), and processed for real-time PCR. The quality and yield of RNAs were verified using the Ultraspec 2000 UV/Visible (Pharmacia Biotech).

### Real-Time PCR

Reverse transcription reaction was performed in a thermocycler (MJ Mini Personal Thermal Cycler; Biorad, Milano, Italy) using IScript TM Reverse Transcription Supermix (Biorad) according to the manufacturer's protocol, under the following conditions: incubation at 25°C for 5 min, reverse transcription at 42°C for 30 min, inactivation at 85°C for 5 min. Real-time PCR (RT-PCR) was carried out in a I-Cycler IQ Multicolor RT-PCR Detection System (Biorad) using SsoFast EvaGreen Supermix (Biorad) according to the manufacturer's instructions. The RT-PCR protocol consisted of 40 cycles of denaturation at 95°C for 30 s and annealing/extension at 60°C for 30 s. For quantification analysis the comparative Threshold Cycle (Ct) method was used. The Ct values from each gene were normalized to the Ct value of GAPDH in the same RNA samples. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008) and expressed as fold changes in arbitrary values. The following pairs of primers were used: *p2y12:* 5′-CCTGTCGTCAGAGACTACAAG-3′ (F); 5′-GGATTTACTGCGGATCTGAAAG-3′ (R); *p2Y6* 5′-ATCAGCTTCCTGCCTTTCC-3′ (F); 5′-CTGTGAGCCTCTGTAAGAGAGATCG-3′ (R); *cd86:* 5′-AGAACTTACGGAAGCACCCA-3′ (F); 5′-GGCAGATATGCAGTCCCATT-3′ (R); *il-15:* 5′-CATCCATCTCGTGCTACTTGTGTT-3′ (F); 5′-CATCTATCCAGTTGGCCTCTGTTT-3′ (R); *tnf-α :* 5′-GTGGAACTGGCAGAAGAG-3′ (F); 5′-CCATAGAACTGATGAGAGG-3′ (R); *arg-1:* 5′-CTCCAAGCCAAAGTCCTTAGAG-3′ (F); 5′-AGGAGCTGTCATTAGGGACATC-3′ (R); *ym-1* 5′-CAGGTCTGGCAATTCTTCTGAA-3′ (F); 5′-GTCTTGCTCATGTGTGTAAGTGA-3′ (R); *il-1β:* 5′-GCAACTGTTCCTGAACTCAACT-3′ (F); 5′-ATCTTTTGGGGTCCGTCAACT-3′ (R); *cd206:* 5′-CAAGGAAGGTTGGCATTTGT-3′ (F); 5′-CCTTTCAGTCCTTTGCAAGC-3′ (R); *fizz-1:* 5′-CCAATCCAGCTAACTATCCCTCC-3′ (F); 5′-ACCCAGTAGCAGTCATCCCA-3′ (R); *gapdh* 5′-TCGTCCCGTAGACAAAATGG-3′ (F); 5′-TTGAGGTCAATGAAGGGGTC-3′ (R).

### Statistical Analysis

Data, analyzed offline, are presented as mean ± SEM. Statistical analyses were performed using Imaris software for quantitative morphometric analysis; Origin 7 and SigmaPlot 11 (Systat Software Inc., San Jose, CA, USA) software were used for statistical analysis of electrophysiological and imaging data. Paired and unpaired *t*-test and one-way ANOVA were used for parametrical data, as indicated; multiple comparison procedures were performed with Holm-Sidak method. We constructed I/V plots, cumulative distribution plots, and fitted data points by linear or non-linear regression analysis using Origin 7 software. Statistical significance for cumulative distributions was assessed with Kolmogorov-Smirnov test. For statistical analysis of microglial surface areas in fixed brains, we arbitrarily subdivided the cells of each genotype in two classes as smaller or larger respect to the median of each distribution. For statistical analysis of current occurrence in different PNWs and genotypes, statistical difference of proportions was obtained with *z*-test (SigmaPlot 11). For multiple comparisons, multiplicity-adjusted *p* values are indicated on the respective figures when appropriate; otherwise the *p* values were indicated by $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

## Results

### Lack of Fractalkine Signaling Impairs Microglial Expression of Voltage-Dependent $K^{+}$ Currents in the Second Postnatal Week

We analyzed the electrophysiological properties of microglial cells by whole-cell recordings in CA1 stratum radiatum of acute

hippocampal slices from *Cx3cr1*$^{+/GFP}$ and *Cx3cr1*$^{GFP/GFP}$ mice, during PNW 2. Microglial cells recorded from CX3CR1$^{+/GFP}$ slices, displayed, in different proportion, both outward and inward voltage-dependent $K^+$ currents (**Figures 1A,B**). The outward $K^+$ current ($I_K$, **Figures 1A**, right; **1B**), recorded in 46/88 (52%) cells, was blocked by 4-aminopyridine (2 mM; $n = 3$, data not shown). The inward rectifier $K^+$ current ($I_{Kir}$, **Figures 1A**, right; **1B**) was present in 43% of microglial cells. As shown in **Figure 1C** (purple bars), representing the electrophysiological profile of CX3CR1$^{+/GFP}$ microglia, about one fourth of cells displayed both currents (26%, **Figure 1A**, right), while a similar proportion was silent (30%, **Figure 1A**, left). Microglial cells lacking fractalkine signaling showed a remarkably different electrophysiological profile, with lower frequency of currents expression: $I_K$ (36%), $I_{Kir}$ (23%), both currents (5%), silent (46%; **Figure 1C**, orange bars, $p < 0.05$, Chi-square test). However, both $I_K$ and $I_{Kir}$ were detected with similar current densities in the two genotypes (**Table 1**).

These results suggest that in the developing hippocampus, a relevant proportion of microglial cells displays a pattern of voltage-dependent $K^+$ currents ($I_K$ and $I_{Kir}$) resembling that of activated microglia; the occurrence of this phenotype is reduced in mice lacking fractalkine signaling.

## Reduced Ramification of Microglia in *Cx3cr1*$^{GFP/GFP}$ Mice

To characterize microglial morphology and disclose possible differences between the two genotypes, a morphometric analysis was performed in fixed mouse brains of *Cx3cr1*$^{GFP/+}$ and *Cx3cr1*$^{GFP/KO}$ littermates at P8, both bearing a single GFP copy (**Figure 2A**). The tridimensional reconstruction of microglial cells did not reveal significant differences in cell surface area and volume between the two genotypes (mean surface area: CX3CR1$^{GFP/+}$ $1271 \pm 105\ \mu m^2$, $n = 68$; CX3CR1$^{GFP/KO}$ $1168 \pm 69\ \mu m^2$, $n = 86$; $p = 0.4$, *t*-test; mean volume: CX3CR1$^{GFP/+}$ $1026 \pm 93\ \mu m^3$, $n = 68$; CX3CR1$^{GFP/KO}$ $878 \pm 54\ \mu m^3$ $n = 86$; $p = 0.2$, *t*-test). However, a more detailed analysis on the distributions of microglial surface areas showed that a small population of CX3CR1$^{GFP/+}$ cells was characterized by a very large surface area and that this population was absent in CX3CR1$^{GFP/KO}$ brains (Figure S1). The subdivision of cells in each genotype in two classes, as smaller (small) or larger (large)

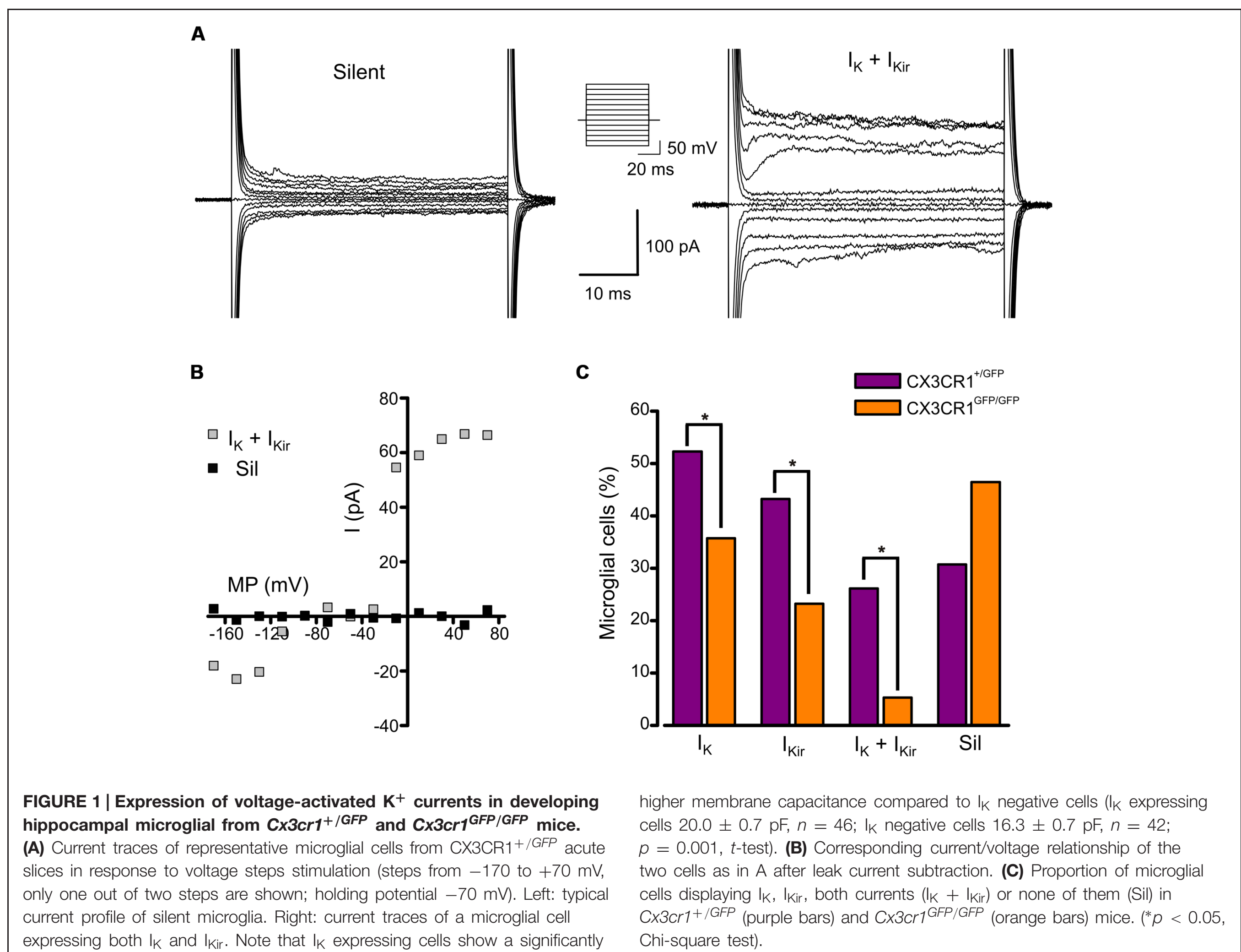



**FIGURE 1 | Expression of voltage-activated $K^+$ currents in developing hippocampal microglial from *Cx3cr1*$^{+/GFP}$ and *Cx3cr1*$^{GFP/GFP}$ mice.** **(A)** Current traces of representative microglial cells from CX3CR1$^{+/GFP}$ acute slices in response to voltage steps stimulation (steps from −170 to +70 mV, only one out of two steps are shown; holding potential −70 mV). Left: typical current profile of silent microglia. Right: current traces of a microglial cell expressing both $I_K$ and $I_{Kir}$. Note that $I_K$ expressing cells show a significantly higher membrane capacitance compared to $I_K$ negative cells ($I_K$ expressing cells $20.0 \pm 0.7$ pF, $n = 46$; $I_K$ negative cells $16.3 \pm 0.7$ pF, $n = 42$; $p = 0.001$, *t*-test). **(B)** Corresponding current/voltage relationship of the two cells as in A after leak current subtraction. **(C)** Proportion of microglial cells displaying $I_K$, $I_{Kir}$, both currents ($I_K + I_{Kir}$) or none of them (Sil) in *Cx3cr1*$^{+/GFP}$ (purple bars) and *Cx3cr1*$^{GFP/GFP}$ (orange bars) mice. (*$p < 0.05$, Chi-square test).

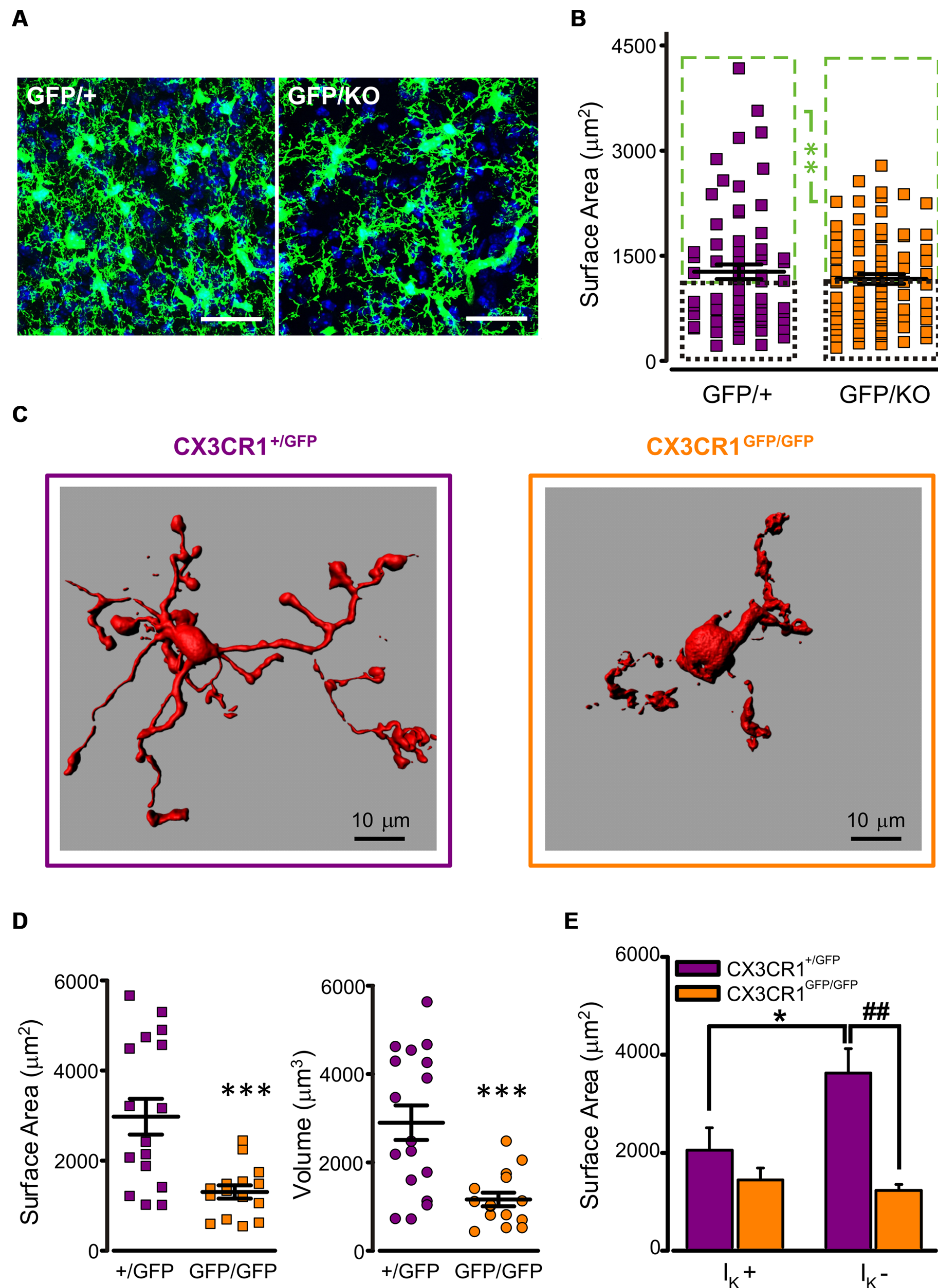



**FIGURE 2 | Reduced ramification of CX3CR1$^{GFP/GFP}$ microglia.** **(A)** Representative confocal *z*-stack projections showing microglial cells in CA1 stratum radiatum from P8 *Cx3cr1*$^{GFP/+}$ (left) and *Cx3cr1*$^{GFP/KO}$ (right) mice (blu: DAPI; bar 30 mm). **(B)** Quantitative morphometric analysis of CX3CR1$^{GFP/+}$ and CX3CR1$^{GFP/KO}$ microglial cells; notice that the difference in surface area is limited to large cells (mean surface area in large cells (green boxes): CX3CR1$^{GFP/+}$ 1924 ± 133 μm$^2$, $n = 34$; CX3CR1$^{GFP/KO}$ 1705 ± 64 μm$^2$, $n = 43$; ** referred to green boxes, $p < 0.01$, two way ANOVA, Holm-Sidak; mean surface area in small cells (black boxes): CX3CR1$^{GFP/+}$ 618 ± 32 μm$^2$, $n = 34$; CX3CR1$^{GFP/KO}$ 630 ± 38 μm$^2$, $n = 43$, $p = 0.9$, two way ANOVA, Holm-Sidak). **(C)** 3D reconstruction, by Imaris software, of representative biocytin-loaded microglial cells in CA1 stratum radiatum from CX3CR1$^{+/GFP}$ (left) and CX3CR1$^{GFP/GFP}$ (right) slices, showing reduced processes extension in CX3CR1$^{GFP/GFP}$ cells. **(D)** Quantitative morphometric analysis of cell surface area (left) and volume (right) of CX3CR1$^{+/GFP}$ (+/GFP, purple) and CX3CR1$^{GFP/GFP}$ (GFP/GFP, orange) biocytin-loaded microglia. Note that CX3CR1$^{GFP/GFP}$ cells are significantly less extended (mean surface area: CX3CR1$^{+/GFP}$ 2975 ± 398 μm$^2$, $n = 17$; CX3CR1$^{GFP/GFP}$ 1302 ± 146 μm$^2$, $n = 15$; ***$p < 0.0005$, *t*-test; mean volume: CX3CR1$^{+/GFP}$ 2899 ± 391 μm$^3$, $n = 17$; CX3CR1$^{GFP/GFP}$ 1160 ± 155 μm$^3$ $n = 15$; ***$p < 0.0005$, *t*-test). **(E)** Area correlation between genotypes (CX3CR1$^{+/GFP}$, purple bars; CX3CR1$^{GFP/GFP}$, orange bars) in cells expressing $I_K$ ($I_K$ +) and cells not expressing $I_K$ ($I_K$ –). Note that the surface area increase is restricted to CX3CR1$^{+/GFP}$ $I_K$ – cells (*$p < 0.05$ vs. $I_K$ +; ##$p < 0.01$ vs. CX3CR1$^{GFP/GFP}$, *t*-test).

respect to the median of each distribution, revealed a significant difference between large cells, with surface area significantly smaller in *Cx3cr1*$^{GFP/KO}$ mice (**Figure 2B**; $p < 0.01$, two-way ANOVA, Holm-Sidak) No difference was observed in the small cell group.

Further investigation was carried out on microglial cells from acute hippocampal slices in PNW 2. A subset of microglial cells was filled with biocytin through the recording pipette, allowing high resolution 3D reconstruction and the correlation between electrophysiological profile and morphology. As shown in the representative images in **Figure 2C**, in slices, CX3CR1$^{GFP/GFP}$ cells (right) displayed strikingly reduced processes extension compared to CX3CR1$^{+/GFP}$ cells (left). Quantitative morphometric analysis confirmed that surface area and volume of CX3CR1$^{GFP/GFP}$ microglial cells ($n = 15$) were significantly smaller with respect to those of CX3CR1$^{+/GFP}$ microglia ($n = 17$; $p < 0.0005$, *t*-test; **Figure 2D**).

Moreover, in CX3CR1$^{+/GFP}$ microglial cells, processes extension was negatively correlated with $I_K$ expression (**Figure 2E**, purple bars). Indeed, $I_K$ positive cells (7/17) showed less ramified morphology and smaller surface area, compared to $I_K$ negative cells ($n = 10/17$; $p < 0.05$, *t*-test). In addition, $I_K$ positive cells had a significant higher membrane capacitance compared to that of $I_K$ negative cells ($I_K$ positive cells: $21.7 \pm 1.4$ pF, $n = 7$; $I_K$ negative cells: $16.4 \pm 1.4$ pF, $n = 10$, $p < 0.05$, *t*-test).

These data point to a subdivision of PNW 2 microglial cells in two functional groups: one resembling *active* microglia, characterized by retracted phenotype and $I_K$ expression and another *surveying* microglia, $I_K$ negative and ramified. This correlation was not observed in CX3CR1$^{GFP/GFP}$ microglial cells (**Figure 2E**, orange bars). In fact, in brain slices from *Cx3cr1*$^{GFP/GFP}$ mice, both $I_K$ positive ($n = 8$) and negative ($n = 7$) cells displayed poorly ramified morphology. Consistently, the difference in cell surface area between the two genotypes was restricted to $I_K$ negative cells ($p < 0.01$, *t*-test; **Figure 2E**), suggesting that in *Cx3cr1*$^{GFP/GFP}$ mice surveying microglia are less ramified and pointing to a functional monitoring defect. None of the two genotypes showed a correlation between $I_{Kir}$ expression and the morphological parameters of surface area and volume (data not shown).

To determine whether the above reported phenotypical differences could rely on cellular polarization, we compared in *Cx3cr1*$^{+/GFP}$ and *Cx3cr1*$^{GFP/GFP}$ mice the microglial expression profile of specific genes related to M1/M2 activation states. RT-PCR was performed on mRNAs extracted from hippocampal GFP positive cells from the two genotypes. As reported in **Table 2**, no statistically significant differences were observed for either M1 (cd86, il-1β, il-15, tnf-α) or M2 (arg-1, cd206, fizz, ym-1) genes, indicating that the phenotypical changes associated with the lack of fractalkine signaling are not associated with polarization toward "classical" M1 or "alternative" M2 activation states.

Altogether, these data indicate that CX3CR1$^{GFP/GFP}$ microglia show functional and morphological differences respect to CX3CR1$^{+/GFP}$ microglia, which are not relying on a different M1/M2 microglial polarization in the two genotypes.

## ATP-Induced Microglial Processes Migration is Impaired in Cx3cr1 Knockout Mice

Using time-lapse imaging, we characterized the extension of microglial processes toward the focal application of ATP in hippocampal slices. To quantify the directional movement in the two genotypes, we monitored the fluorescence increase in concentric areas around the ATP-containing pipette tip before and after a short ATP puff (3 mM, 100 ms). As shown in **Figure 3A**, the increase in fluorescence measured around the pipette (10 μm radius) was reduced in *Cx3cr1*$^{GFP/GFP}$ mice (orange squares, $n = 18$ fields/5 mice) compared to *Cx3cr1*$^{+/GFP}$ mice (purple circles, $n = 18$ fields/10 mice; $p < 0.05$, unpaired *t*-test).

To determine whether the impairment in process extension was due to a reduction in expression of ATP receptors, p2y12 and p2y6 mRNAs levels were evaluated by RT-PCR in hippocampal microglial cells. As reported in **Table 2**, no expression differences were observed for either p2y12 or p2y6 transcripts, suggesting that ATP sensing was not altered in developing hippocampal microglial cells lacking *Cx3cr1*.

To understand the basis of the observed difference in processes extension between the two genotypes, we performed a tracking analysis of single microglial processes (**Figure 3B**; movies 1 and 2 in Supplementary Material). At the start of fluorescence monitoring ($t_0$), the number of detected processes was significantly higher in CX3CR1$^{+/GFP}$ than in CX3CR1$^{GFP/GFP}$ slices ($p < 0.01$, *t*-test; **Figure 3C**). Moreover, at $t_0$ the process positions in CX3CR1$^{GFP/GFP}$ slices (orange symbols, **Figure 4A**) were more distant from the ATP-containing pipette, compared to CX3CR1$^{+/GFP}$ (purple, $p < 0.05$, Kolmogorov-Smirnov test). The observed differences may reflect the reduced ramification of CX3CR1$^{GFP/GFP}$ microglia, as well as their lower cell density (Paolicelli et al., 2011). Besides, in both genotypes the number of traced processes increased during the experiments, which was significantly higher 45 min after ATP puff ($t_{45}$; **Figure 3C**). We noticed that the mean velocity of processes

**TABLE 2 | Expression of selected M1/M2 polarization markers, P2y6 and P2y12 in microglial GFP positive cells isolated from hippocampus of *Cx3cr1*$^{+/GFP}$ ($n = 4$) and *Cx3cr1*$^{GFP/GFP}$ ($n = 4$) mice in the PNW 2.**

| | CX3CR1$^{+/GFP}$ | CX3CR1$^{GFP/GFP}$ | *p* |
|---|---|---|---|
| M1 polarization markers | | | |
| cd86 | 1.00 ± 0.32 | 0.55 ± 0.16 | 0.23 |
| il-1β | 1.00 ± 0.19 | 1.06 ± 0,16 | 0.80 |
| il-15 | 1.00 ± 0.38 | 1.14 ± 0.29 | 0.83 |
| tnf-α | 1.00 ± 0.22 | 1.72 ± 0.20 | 0.06 |
| M2 polarization markers | | | |
| arg-1 | 1.00 ± 0.25 | 0.63 ± 0.37 | 0.47 |
| cd206 | 1.00 ± 0.52 | 1.03 ± 0.50 | 0.96 |
| fizz-1 | 1.00 ± 0.19 | 0.71 ± 0.12 | 0.22 |
| ym-1 | 1.00 ± 0.22 | 0.65 ± 0.11 | 0.20 |
| Purinergic receptors | | | |
| p2y6 | 1.00 ± 0.19 | 1.43 ± 0.29 | 0.26 |
| p2y12 | 1.00 ± 0.28 | 1.47 ± 0.36 | 0.49 |

*Transcript levels for CX3CR1$^{GFP/GFP}$ microglia are expressed as n-fold increase respect to CX3CR1$^{+/GFP}$ (p, t-test).*

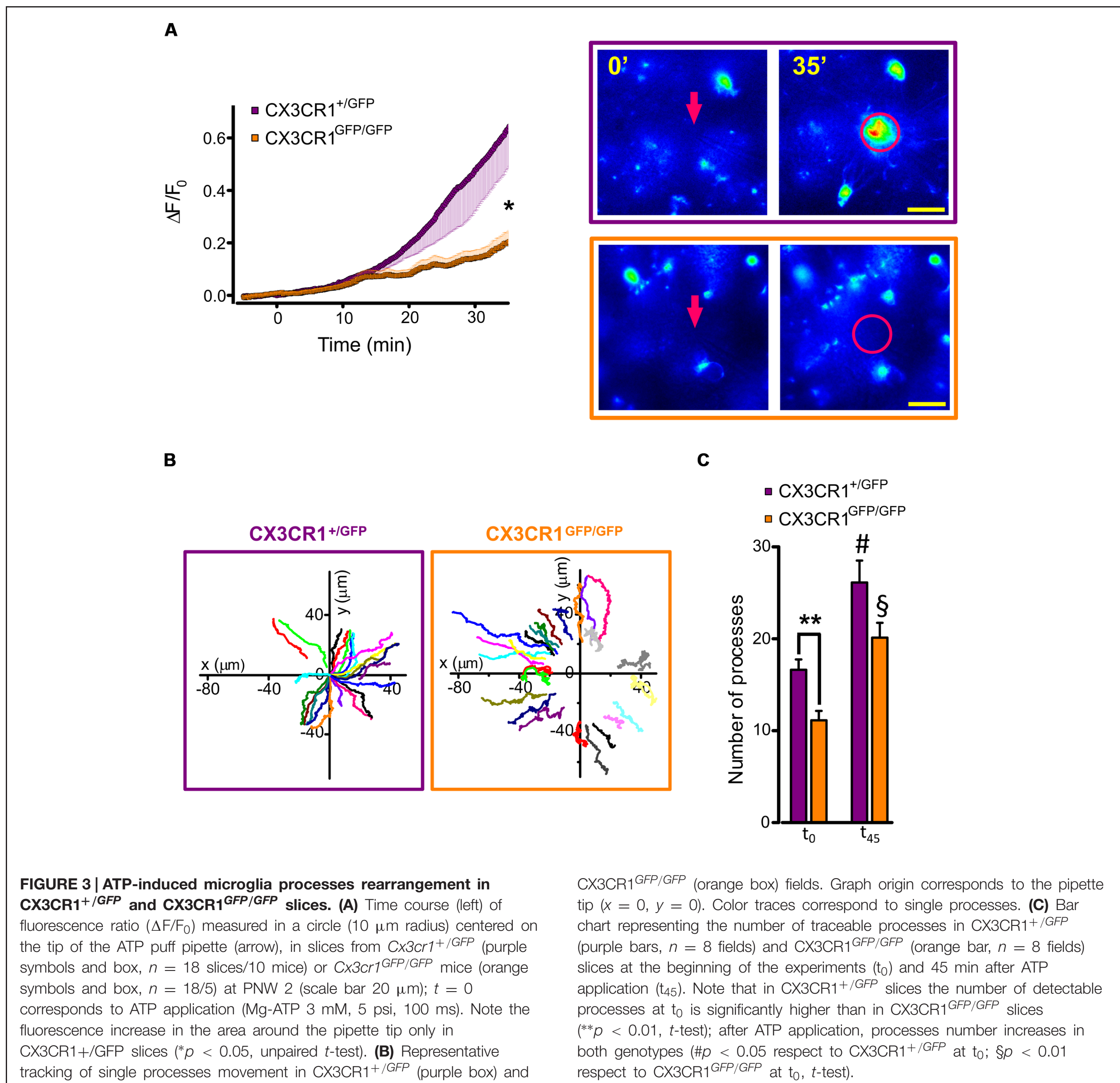



**FIGURE 3 | ATP-induced microglia processes rearrangement in CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ slices. (A)** Time course (left) of fluorescence ratio ($\Delta F/F_0$) measured in a circle (10 µm radius) centered on the tip of the ATP puff pipette (arrow), in slices from *Cx3cr1*$^{+/GFP}$ (purple symbols and box, $n$ = 18 slices/10 mice) or *Cx3cr1*$^{GFP/GFP}$ mice (orange symbols and box, $n$ = 18/5) at PNW 2 (scale bar 20 µm); $t = 0$ corresponds to ATP application (Mg-ATP 3 mM, 5 psi, 100 ms). Note the fluorescence increase in the area around the pipette tip only in CX3CR1+/GFP slices (*$p < 0.05$, unpaired $t$-test). **(B)** Representative tracking of single processes movement in CX3CR1$^{+/GFP}$ (purple box) and CX3CR1$^{GFP/GFP}$ (orange box) fields. Graph origin corresponds to the pipette tip ($x = 0$, $y = 0$). Color traces correspond to single processes. **(C)** Bar chart representing the number of traceable processes in CX3CR1$^{+/GFP}$ (purple bars, $n$ = 8 fields) and CX3CR1$^{GFP/GFP}$ (orange bar, $n$ = 8 fields) slices at the beginning of the experiments ($t_0$) and 45 min after ATP application ($t_{45}$). Note that in CX3CR1$^{+/GFP}$ slices the number of detectable processes at $t_0$ is significantly higher than in CX3CR1$^{GFP/GFP}$ slices (**$p < 0.01$, $t$-test); after ATP application, processes number increases in both genotypes (#$p < 0.05$ respect to CX3CR1$^{+/GFP}$ at $t_0$; §$p < 0.01$ respect to CX3CR1$^{GFP/GFP}$ at $t_0$, $t$-test).

elongation was similar in the two genotypes (CX3CR1$^{+/GFP}$ 2.39 ± 0.05 µm/min, $n$ = 210 processes/8 fields/4 mice; CX3CR1$^{GFP/GFP}$ 2.48 ± 0.08, $n$ = 161/8/4; $p$ = 0.36; not shown). Nevertheless, the distribution of microglial processes positions was remarkably different between the two genotypes also after ATP-induced extension, as CX3CR1$^{+/GFP}$ processes were significantly closer to the pipette tip at $t_{45}$ (**Figure 4B**; $p < 0.0001$, Kolmogorov-Smirnov test). This was likely due to the lower directionality shown by microglial processes in their elongation. As shown in **Figure 4C**, CX3CR1$^{GFP/GFP}$ processes displayed lower displacement and directionality toward ATP source.

In addition, the correlation between instantaneous velocity and position of single process (measured as radial distance from the ATP source), showed a non-linear profile of extension velocity in CX3CR1$^{+/GFP}$ cells (**Figure 4C**, left). The speed of single process movement increased when processes of CX3CR1$^{+/GFP}$ microglia were in the proximity of the ATP stimulus (**Figure 4D**). This rise in instantaneous velocity as the processes approach the site of ATP application was absent in CX3CR1$^{GFP/GFP}$ slices (**Figure 4D**), contributing to the reduced efficacy of ATP-directed processes rearrangement in the absence of fractalkine signaling.

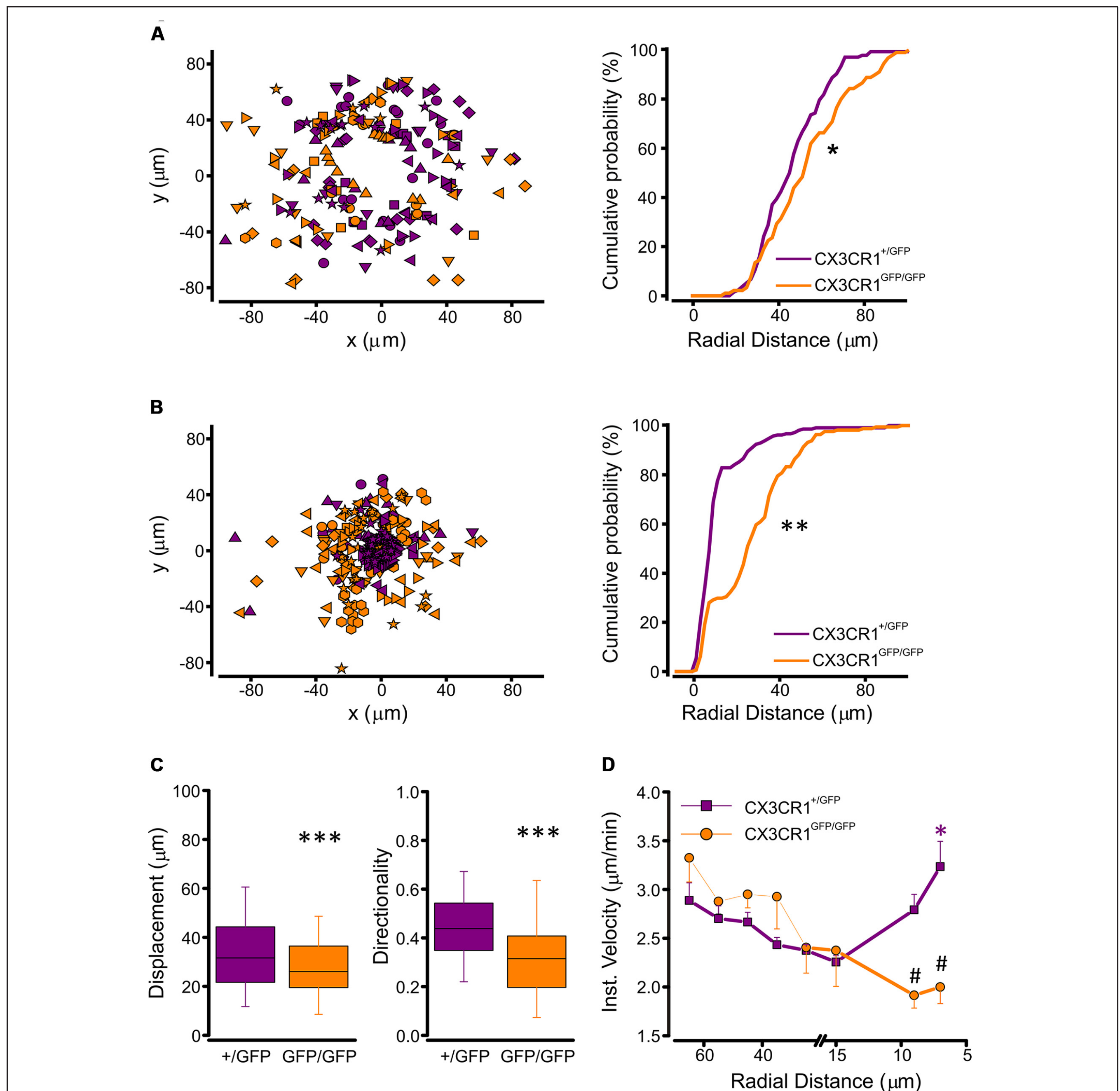



**FIGURE 4 | Tracking analysis of single processes in CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ microglia. (A)** Left, plot of spatial x-y coordinates respect to the ATP pipette tip of microglial processes in CX3CR1$^{+/GFP}$ (purple symbols, $n = 133$ processes/8 fields) and CX3CR1$^{GFP/GFP}$ hippocampal slices (orange symbols, $n = 89/8$) at start of recordings ($t_0$). Right, cumulative distributions of radial distances of microglial processes from the pipette tip in CX3CR1$^{+/GFP}$ (purple) or CX3CR1$^{GFP/GFP}$ (orange) slices, at $t_0$. Note that CX3CR1$^{GFP/GFP}$ processes are significantly more distant than CX3CR1$^{+/GFP}$ ones (*$p < 0.05$, Kolmogorov-Smirnov test). **(B)** Left, plot of spatial x-y coordinates of CX3CR1$^{+/GFP}$ (purple symbols, $n = 209/8$) and CX3CR1$^{GFP/GFP}$ (orange symbols, $n = 161/8$) microglial processes 45 min after ATP application ($t_{45}$). Right, cumulative distributions of radial distances of microglial processes in CX3CR1$^{+/GFP}$ (purple) or CX3CR1$^{GFP/GFP}$ (orange) slices, at $t_{45}$. Note that at $t_{45}$ the majority of CX3CR1$^{GFP/GFP}$ processes do not reach the ATP pipette (**$p < 0.0001$, Kolmogorov-Smirnov test). **(C)** Box charts of displacement (left) and directionality (right) of CX3CR1$^{+/GFP}$ (purple) and CX3CR1$^{GFP/GFP}$ (orange) microglial processes. In slices from Cx3cr1$^{+/GFP}$ mice displacement and directionality are significantly higher than in those from Cx3cr1$^{GFP/GFP}$ mice (***$p < 0.0001$, *t*-test), **(D)** Correlation plot showing mean instantaneous velocities vs. radial distance of CX3CR1$^{+/GFP}$ (purple) and CX3CR1$^{GFP/GFP}$ (orange) microglial processes. The velocity of CX3CR1$^{+/GFP}$ processes increase significantly 7 mm far from the ATP pipette tip (*$p < 0.001$, Kruskal-Wallis One Way ANOVA on Ranks, and $p < 0.05$ multiple comparison Dunn's method versus 15, 25, and 35 μm radial distance). Note that in the proximity of the ATP pipette, the velocity of CX3CR1$^{GFP/GFP}$ processes is significantly slower than that of CX3CR1$^{+/GFP}$ processes (#$p < 0.01$ *t*-test).

## Absence of Fractalkine Signaling Affects Developmental Profile of Microglia Functional Properties in the Hippocampus

In order to determine whether the physiological deficits we identified in *Cx3cr1* knockout mice were specific to the early postnatal period, we analyzed the morphological and functional properties of hippocampal microglia at a later developmental stage (PNW 6). Surface area quantification of biocytin-injected microglia, revealed that CX3CR1$^{+/GFP}$ microglia at PNW 6 had significantly greater process extensions relative to PNW 2 (mean surface area = 4662 ± 404 $\mu m^2$, $n = 15$, $p < 0.01$; unpaired *t*-test; Figure S2). Conversely, membrane surface area of CX3CR1$^{GFP/GFP}$ microglia at PNW 6 was not greater than at PNW 2 (mean surface area = 1816 ± 286 $\mu m^2$, $n = 16$, $p = 0.13$ PNW 6 vs. PNW 2; unpaired *t*-test) and was significantly smaller when compared to control mice at PNW 6 ($p < 0.01$, *Cx3cr1*$^{GFP/GFP}$ vs. *Cx3cr1*$^{+/GFP}$ unpaired *t*-test). Moreover, CX3CR1$^{GFP/GFP}$ microglia showed significantly lower capacitance than CX3CR1$^{+/GFP}$ cells (23.7 ± 0.7 pF, $n = 63$; 28.3 ± 0.7 pF, $n = 63$; $p < 0.001$, unpaired *t*-test; PNWs 5–6; Figure S2).

We also analyzed the expression of voltage dependent $K^+$ currents and ATP-induced process extension during PNWs 1–6. Patch clamp recordings of microglia in CX3CR1$^{+/GFP}$ slices showed that the occurrence of $I_K$ was developmentally regulated, transiently increasing in PNWs 2 and 3 ($p < 0.05$ respect to PNW 1, *z*-test; **Figure 5A**, purple). Conversely, in the absence of fractalkine signaling, no developmental increase in $I_K$ expression was observed with about one third of cells expressing $I_K$ (**Figure 5A**, orange). Consistent with this observation the proportion of cells expressing $I_K$ at PNWs 2 and 3 in CX3CR1$^{+/GFP}$ slices was significantly higher than in CX3CR1$^{GFP/GFP}$ slices ($p < 0.05$, **Figure 5A**).

We then tested whether the proportion of $I_{Kir}$ expressing microglia followed a similar temporal pattern. In CX3CR1$^{+/GFP}$ slices, the proportion of microglia displaying $I_{Kir}$ showed a developmental increase to about 90% at PNW 6 ($p < 0.001$ vs. PNWs 1, 2, and 3, *z*-test, **Figure 5B**, purple). Conversely, in CX3CR1$^{GFP/GFP}$ slices this developmental increase of $I_{Kir}$ was not observed, although a transient reduction was found at PNW 2 (**Figure 5B**, orange, $p < 0.05$, vs. PNW 1, *z*-test). Consistently the proportion of $I_{Kir}$ expressing cells in CX3CR1$^{GFP/GFP}$ slices was significantly lower when compared to CX3CR1$^{+/GFP}$ slices at PNWs 5 and 6 ($p < 0.01$, **Figure 5B**). These results show that fractalkine signaling is involved in modulating the profile of microglial voltage-dependent potassium currents during postnatal development.

Finally, we characterized changes in ATP-directed process extension across developmental time points (PNWs 1–5). Quantitative time-lapse imaging analysis revealed that in *Cx3cr1*$^{+/GFP}$ mice the speed of extension peaked at PNW 2 ($p < 0.01$, vs PNW 1 ANOVA) with a non-significant tendency to decrease in the following weeks (**Figure 5C**, purple bars). In CX3CR1$^{GFP/GFP}$ slices, a later increase of extension speed was observed, with a peak at PNW 4 ($p < 0.01$, vs. PNW 1 ANOVA; **Figure 5C**, orange bars), pointing to a delay

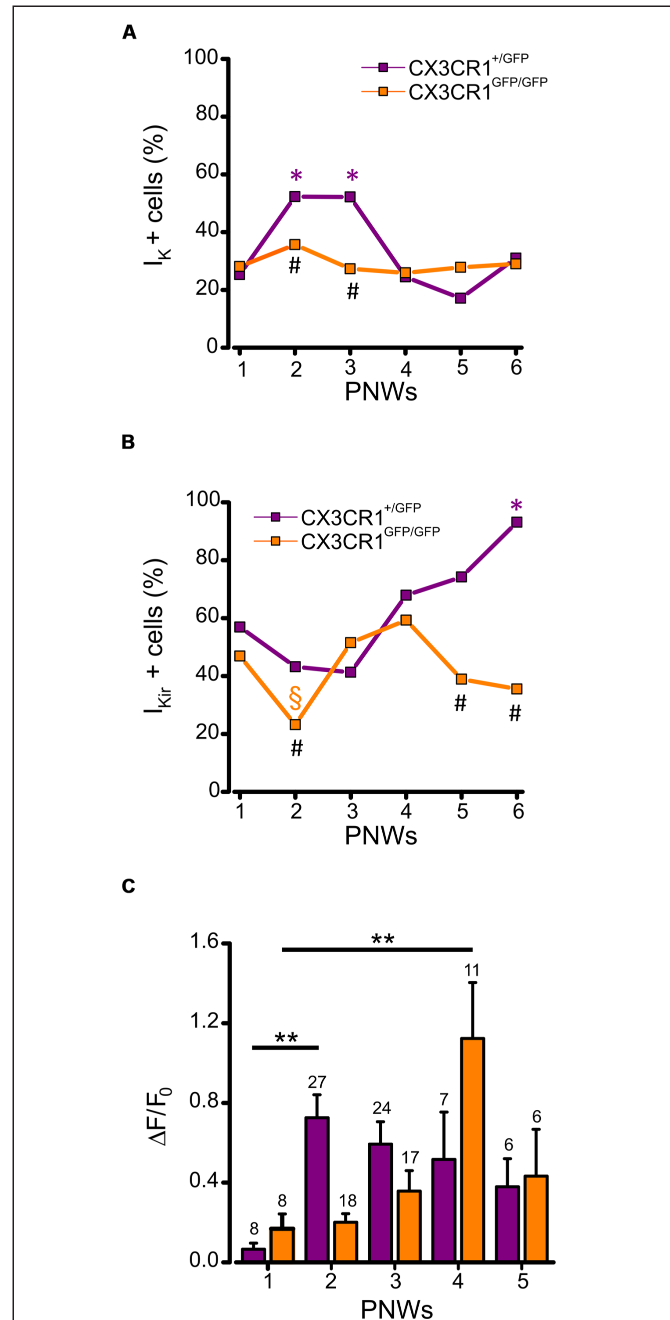



**FIGURE 5 | Developmentally regulated changes in microglial electrophysiological properties and processes rearrangement in CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ hippocampal slices. (A)** Time course displaying the occurrence of microglial cells expressing $I_K$ ($I_K$ + cells) during PNWs 1–6. $I_K$ expression transiently increases only in CX3CR1$^{+/GFP}$ microglia (purple symbols) during PNWs 2–3 (*$p < 0.05$ respect to PNW 1; #$p < 0.05$ between the two genotypes). **(B)** Time course displaying the occurrence of microglial cells expressing $I_{Kir}$ ($I_{Kir}$ + cells). $I_{Kir}$ expression transiently decreases only in CX3CR1$^{GFP/GFP}$ microglia (orange symbols) during PNW 2 (§ $p < 0.05$; * and # as in panel A). **(C)** Bar chart of mean total fluorescence ratio ($\Delta F/F_0$) measured during ATP induced processes rearrangement in slices from *Cx3cr1*$^{+/GFP}$ (purple bars) or *Cx3cr1*$^{GFP/GFP}$ mice (orange bars) during PNWs 1-5. Number of recorded fields is reported on the top of each bar. Note that the peak of microglial ability to respond to ATP stimulus is delayed in *Cx3cr1*$^{GFP/GFP}$ mice (**$p < 0.01$).

in microglial maturation. These results indicate that fractalkine signaling plays a critical role in the dynamics of maturation of microglia.

## Discussion

We report here that mice lacking *Cx3cr1* are defective in developmentally regulated changes of microglial properties, highlighting the role of fractalkine signaling in neuron-microglia interaction during brain development. Our data show that the lack of CX3CR1 strongly affects both microglial morphology and functional properties. Specifically, microglia in *Cx3cr1*$^{GFP/GFP}$ mice were characterized by a less ramified morphology, by the lack of developmental regulation of $K^+$ currents and, most remarkably, by defective tissue monitoring, as demonstrated by a reduction in ATP-directed rearrangement.

Our 3D confocal imaging reconstructions from hippocampal slices revealed reduced microglial branching in CX3CR1$^{GFP/GFP}$ slices (**Figure 2**), which was partially confirmed by analysis of perfused brains. CX3CR1 signaling has been shown to influence microglial function under both normal and pathological conditions (Limatola and Ransohoff, 2014; Paolicelli et al., 2014). For example, a transient reduction of microglial density during hippocampal development has been reported in *Cx3cr1* knockout mice (Paolicelli et al., 2011). However, previous studies examining microglia ramification did not evidentiate morphological differences (Liang et al., 2009; Hoshiko et al., 2012), suggesting that they may be the result of the acute hippocampal slices preparation and/or recording conditions, with ATP containing whole cell pipette, used in our studies. The observed difference may, thus, depend on higher susceptibility to insults of *Cx3cr1* knockout mice. Indeed, it is known that several types of brain injury, driving microglia into an activated state, induce stronger response in mice lacking *Cx3cr1* (Cardona et al., 2006). On the other hand, our reconstructions on perfused brains show that morphological differences among the two genotypes might be restricted to a subset of cells showing the largest surface area. It can't be ruled out that patch clamp recording might have been primarily performed on these cells, as most visible in the slice depth, thus highlighting CX3CR1-dependent morphological defect.

Microglia is characterized by the ability to extend processes in response to ATP, an "alarm signal" released by injured or dying cells (Davalos et al., 2005; Avignone et al., 2008). We found that ATP-directed microglial processes extension was reduced in slices from postnatal hippocampus of *Cx3cr1*$^{GFP/GFP}$ mice. This finding is consistent with a previous report showing a reduced ability of microglia to migrate and extend processes in response to focal lesions in *Cx3cr1*$^{GFP/GFP}$ mice retina (Liang et al., 2009). Our data point to a defect relying in the lower number and less ramified morphology of microglial cells, rather than in a decrease in the intrinsic motility of processes. Quantitative time-lapse imaging analysis of individual microglial processes showed (i) lower density of processes and (ii) reduced directionality toward ATP in *Cx3cr1*$^{GFP/GFP}$ mice, but (iii) similar elongation speed in the two genotypes. Despite stronger GFP expression, CX3CR1$^{GFP/GFP}$ microglial processes are less numerous and are detected at higher distances from the ATP source, compared to CX3CR1$^{+/GFP}$. Moreover, we measured a comparable mean velocity of processes elongation in the two genotypes. Another possible explanation, taking into account the reduced directionality, relies on lower ATP sensitivity of CX3CR1$^{GFP/GFP}$ microglial processes. However, it is unlikely that the observed difference in ATP induced processes extension is due to changes in the expression of purinergic receptors on CX3CR1$^{GFP/GFP}$ microglia (Arnoux et al., 2013), as RT-PCR analysis of hippocampal microglia at PNW 2 showed similar expression of p2y12 and p2y6 transcripts in the two genotypes (**Table 2**).

Furthermore, RT-PCR analysis of the two genotypes did not highlight differences in polarization toward the least (M1) or the most (M2) migratory activation states (Lively and Schlichter, 2013), suggesting that CX3CR1-dependent functions at this stage of development do not involve changes in microglial polarization. It should be considered, however, that our bulk RT-PCR analysis may not have been sufficiently sensitive to detect small variations in gene expression or changes that occurred in a small fraction of cells. Finally, the lack of velocity increase in the close proximity of the ATP pipette tip observed in CX3CR1$^{GFP/GFP}$ processes may depend on longer time needed by knockout processes to reach the ATP-containing pipette. In alternative, it could be a direct effect of CX3CR1 deficiency, as fractalkine signaling is known to promote microglial migration (Maciejewski-Lenoir et al., 1999) and rapidly change in microglia morphology (Liang et al., 2009).

## Conclusion

We can speculate that the observed difference in ATP-induced microglia rearrangement is due to defective branching and directional elongation of microglial processes, pointing to a reduced monitoring capacity of CX3CR1$^{GFP/GFP}$ microglia in the developing hippocampus.

The functional impairment of CX3CR1$^{GFP/GFP}$ microglia is further highlighted by the altered electrophysiological profile observed during postnatal development. Indeed, developing hippocampal microglia display two voltage dependent $K^+$ currents: an outward rectifier current, resembling the delayed rectifier $K^+$ current described in cultured rat microglia ($I_K$) and an inward rectifier $K^+$ current ($I_{Kir}$; Nörenberg et al., 1994). Although microglial cells in brain slices are generally considered silent (Boucsein et al., 2000), similar currents have been described in postnatal hippocampus (Schilling and Eder, 2007) and barrel cortex (Arnoux et al., 2013). We made the novel observation that the frequency of occurrence of $K^+$ currents in hippocampal microglia changed during postnatal development: $I_K$ were more frequent at PNWs 2–3, while $I_{Kir}$ were more frequent at PNW 6. Remarkably, the presence of $I_K$ is associated to a retracted morphology and reduced cell surface area resembling that of active microglia, following nerve lesion (Boucsein et al., 2000), epileptic state (Avignone et al., 2008), or LPS treatment (Nörenberg et al., 1994). These data suggest that microglia may have a transient propensity to be activated during hippocampal maturation, as the presence of $I_K$ is a typical feature of activated microglia in both slices and dissociated cell cultures,

likely associated with the expression of $Kv_{1.3}$ channels (Menteyne et al., 2009; Moussaud et al., 2009). However, RT-PCR analysis does not highlight a clear polarization toward the classical (M1) or alternative (M2) activation states, pointing to a localized phenomenon. In addition, the level of $I_K$ functional expression in developing microglia is lower, compared to typically active microglia in pathological contexts (Avignone et al., 2008; Menteyne et al., 2009), suggesting that this regulation may be part of physiological changes, due to varying environmental challenges during development (Eggen et al., 2013). Indeed, developmental regulation of $I_K$ has been reported in the barrel cortex (Arnoux et al., 2013). Such phenotypical changes, constituting a sort of developmental activation of microglia (Dalmau et al., 1998), could be induced by local neuronal signals, including cell death or synaptic elimination during developmental circuit refinement (Perry et al., 1985) and participate to the fine tuning of hippocampal synaptic connections (Paolicelli and Gross, 2011).

Strikingly, the occurrence of $I_K$ in the hippocampus was not developmentally regulated in mice lacking fractalkine signaling (**Figure 5C**). In this respect, our results differ from a previous report in the barrel cortex of *Cx3cr1*$^{GFP/GFP}$ mice, pointing to a delay in microglial maturation, associated with a delay in microglial migration into the barrels (Arnoux et al., 2013). We can speculate that lack of any developmental increase in $I_K$ expression in the hippocampus is associated with a loss of environmental influence on microglia maturation in a precise time window.

The expression of $I_{Kir}$ also showed a dynamic developmental profile in control animals (apparently opposite to $I_K$ profile), that was absent in *Cx3cr1*$^{GFP/GFP}$ mice. This current, typical of dissociated microglia cell cultures (Kettenmann et al., 1990), has been proposed to represent a marker of early microglial activation (Boucsein et al., 2000; Kettenmann et al., 2011). Functional expression of $I_{Kir}$ has been reported in the hippocampus (Schilling and Eder, 2007) and corpus callosum microglia during early postnatal development (Brockhaus et al., 1993), increasing in adult and aging mice (Schilling and Eder, 2014). Under our conditions, $I_{Kir}$ was found in almost all microglia in control animals at PNWs 5 and 6, and was not associated to specific morphological features. Although its function is still unknown, it can be speculated that $I_{Kir}$ frequency is maximal when microglia shows the greatest ramification, thus being maximally capable of sensing tissue signals. The reduced frequency of $I_{Kir}$ in *Cx3cr1*$^{GFP/GFP}$ mice could reflect a reduced environmental sensitivity of these cells.

In conclusion, our data support the idea that CX3CR1 deficient microglia is unable to respond properly to specialized environmental signals during normal development (Paolicelli et al., 2014). We speculate that the observed deficit in microglia maturation implies reduced tissue monitoring, suggesting that a developmental microglial defect, partially corrected in the adult, could lead to the delay in synaptic maturation and defective synaptic connectivity reported in these mice (Paolicelli et al., 2011; Hoshiko et al., 2012; Zhan et al., 2014).

## Acknowledgments

The work was supported by grants from PRIN 2009 (to DR, CL, and SA), and AIRC (to CL.) EM. was supported by the PhD program in Neurophysiology, Sapienza University, Rome. RP, EG, TF, and CG. were supported by funds from EMBL and received invaluable support from the EMBL Microscopy Facility under the direction of Giulia Bolasco. Author contributions: FP designed carried out and analyzed all patch clamp experiments, RP designed carried out and analyzed all morphometric experiments; BC designed and performed tracking analysis; imaging experiments were designed, carried out, and analyzed by FP, EM, and EZ; confocal images acquisition was performed by EG. and TF; SG, MC, GA, AP, GP, FM, and CL designed and performed cell sorting and RT-PCR analysis; DR and SA wrote the manuscript with help of FP; DR. conceived the project with help of CG.

## Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2015.00111/abstract

## References

Arnoux, I., Hoshiko, M., Mandavy, L., Avignone, E., Yamamoto, N., and Audinat, E. (2013). Adaptive phenotype of microglial cells during the normal postnatal development of the somatosensory "Barrel" cortex. *Glia* 61, 1582–1594. doi: 10.1002/glia.22503

Avignone, E., Ulmann, L., Levavasseur, F., Rassendren, F., and Audinat, E. (2008). Status epilepticus induces a particular microglial activation state characterized by enhanced purinergic signaling. *J. Neurosci.* 28, 9133–9144. doi: 10.1523/JNEUROSCI.1820-08.2008

Bertollini, C., Ragozzino, D., Gross, C., Limatola, C., and Eusebi, F. (2006). Fractalkine/CX3CL1 depresses central synaptic transmission in mouse hippocampal slices. *Neuropharmacology* 51, 816–821. doi: 10.1016/j.neuropharm.2006.05.027

Biber, K., Neumann, H., Inoue, K., and Boddeke, H. W. (2007). Neuronal "On" and "Off" signals control microglia. *Trends Neurosci.* 30, 596–602. doi: 10.1016/j.tins.2007.08.007

Boucsein, C., Kettenmann, H., and Nolte, C. (2000). Electrophysiological properties of microglial cells in normal and pathological rat brain slices. *Eur. J. Neurosci.*12, 2049–2058. doi: 10.1046/j.1460-9568.2000.00100.x

Brockhaus, J., Ilschner, S., Banati, R. B., and Kettenmann, H. (1993). Membrane properties of ameboid microglial cells in the corpus callosum slice from early postnatal mice. *J. Neurosci.* 13, 4412–4421.

Cardona, A. E., Pioro, E. P., Sasse, M. E., Kostenko, V., Cardona, S. M., Dijkstra, I. M., et al. (2006). Control of microglial neurotoxicity by the fractalkine receptor. *Nat. Neurosci.* 9, 917–924. doi: 10.1038/nn1715

Crain, J. M., Nikodemova, M., and Watters, J. J. (2013). Microglia express distinct M1 and M2 phenotypic markers in the postnatal and adult central nervous system in male and female mice. *J. Neurosci. Res.* 91, 1143–1151. doi: 10.1002/jnr. 23242

Dalmau, I., Finsen, B., Zimmer, J., González, B., and Castellano, B. (1998). Development of microglia in the postnatal rat hippocampus. *Hippocampus* 8, 458–474. doi: 10.1002/(SICI)1098-1063(1998)8:5<458::AID-HIPO6>3.0.CO;2-N

Davalos, D., Grutzendler, J., Yang, G., Kim, J. V., Zuo, Y., Jung, S., et al. (2005). ATP mediates rapid microglial response to local brain injury in vivo. *Nat. Neurosci.* 8, 752–758. doi: 10.1038/nn1472

de Jong, E. K., Dijkstra, I. M., Hensens, M., Brouwer, N., van Amerongen, M., Liem, R. S., et al. (2005). Vesicle-mediated transport and release of CCL21 in endangered neurons: a possible explanation for microglia activation remote from a primary lesion. *J. Neurosci.* 25, 7548–7557. doi: 10.1523/JNEUROSCI.1019-05.2005

Eder, C. (2005). Regulation of microglial behavior by ion channel activity. *J. Neurosci. Res.* 81, 314–321. doi: 10.1002/jnr.20476

Eder, C., Klee, R., and Heinemann, U. (1998). Involvement of stretch-activated Cl-channels in ramification of murine microglia. *J. Neurosci.* 18, 7127–7137.

Eggen, B. J., Raj, D., Hanisch, U. K., and Boddeke, H. W. (2013). Microglial phenotype and adaptation. *J. Neuroimmune Pharmacol.* 8, 807–823. doi: 10.1007/s11481-013-9490-4

Harrison, J. K., Jiang, Y., Chen, S., Xia, Y., Maciejewski, D., McNamara, R. K., et al. (1998). Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10896–10901. doi: 10.1073/pnas.95.18.10896

Haskell, C. A., Hancock, W. W., Salant, D. J., Gao, W., Csizmadia, V., Peters, W., et al. (2001). Targeted deletion of CX(3)CR1 reveals a role for fractalkine in cardiac allograft rejection. *J. Clin. Invest.* 108, 679–688. doi: 10.1172/JCI12976

Hoshiko, M., Arnoux, I., Avignone, E., Yamamoto, N., and Audinat, E. (2012). Deficiency of the microglial receptor CX3CR1 impairs postnatal functional development of thalamocortical synapses in the barrel cortex. *J. Neurosci.* 32, 15106–15111. doi: 10.1523/JNEUROSCI.1167-12.2012

Inoue, K., Koizumi, S., and Tsuda, M. (2007). The role of nucleotides in the neuron–glia communication responsible for the brain functions. *J. Neurochem.* 102, 1447–1458. doi: 10.1111/j.1471-4159.2007.04824.x

Jung, S., Aliberti, J., Graemmel, P., Sunshine, M. J., Kreutzberg, G. W., Sher, A., et al. (2000). Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol. Cell. Biol.* 20, 4106–4114. doi: 10.1128/MCB.20.11.4106-4114.2000

Kettenmann, H., Hanisch, U. K., Noda, M., and Verkhratsky, A. (2011). Physiology of microglia. *Physiol. Rev.* 91, 461–553. doi: 10.1152/physrev.00011.2010

Kettenmann, H., Hoppe, D., Gottmann, K., Banati, R., and Kreutzberg, G. (1990). Cultured microglial cells have a distinct pattern of membrane channels different from peritoneal macrophages. *J. Neurosci. Res.* 26, 278–287. doi: 10.1002/jnr.490260303

Kotecha, S. A., and Schlichter, L. C. (1999). A Kv1.5 to Kv1.3 switch in endogenous hippocampal microglia and a role in proliferation. *J. Neurosci*. 19, 10680–10693.

Lauro, C., Di Angelantonio, S., Cipriani, R., Sobrero, F., Antonilli, L., Brusadin, V., et al. (2008). Activity of adenosine receptors type 1 is required for CX3CL1-mediated neuroprotection and neuromodulation in hippocampal neurons. *J. Immunol.* 180, 7590–7596. doi: 10.4049/jimmunol.180.11.7590

Lee, J. E., Liang, K. J., Fariss, R. N., and Wong, W. T. (2008). Ex vivo dynamic imaging of retinal microglia using time-lapse confocal microscopy. *Invest. Ophthalmol. Vis. Sci.* 49, 4169–4176. doi: 10.1167/iovs.08-2076

Liang, K. J., Lee, J. E., Wang, Y. D., Ma, W., Fontainhas, A. M., Fariss, R. N., et al. (2009). Regulation of dynamic behavior of retinal microglia by CX3CR1 signaling. *Invest. Ophthalmol. Vis. Sci.* 50, 4444–4451. doi: 10.1167/iovs.08-3357

Limatola, C., Lauro, C., Catalano, M., Ciotti, M. T., Bertollini, C., Di Angelantonio, S., et al. (2005). Chemokine CX3CL1 protects rat hippocampal neurons against glutamate-mediated excitotoxicity. *J. Neuroimmunol.* 166, 19–28. doi: 10.1016/j.jneuroim.2005.03.023

Limatola, C., and Ransohoff, R. M. (2014). Modulating neurotoxicity through CX3CL1/CX3CR1 signaling. *Front. Cell. Neurosci.* 8:229. doi: 10.3389/fncel.2014.00229

Lively, S., and Schlichter, L. C. (2013). The microglial activation state regulates migration and roles of matrix-dissolving enzymes for invasion. *J. Neuroinflammation.* 10:75. doi: 10.1186/1742-2094-10-75

Maciejewski-Lenoir, D., Chen, S., Feng, L., Maki, R., and Bacon, K. B. (1999). Characterization of fractalkine in rat brain cells: migratory and activation signals for CX3CR-1-expressing microglia. *J. Immunol.* 163, 1628–1635.

Maggi, L., Scianni, M., Branchi, I., D'Andrea, I., Lauro, C., and Limatola C. (2011). CX(3)CR1 deficiency alters hippocampal-dependent plasticity phenomena blunting the effects of enriched environment. *Front. Cell. Neurosci.* 5:22. doi: 10.3389/fncel.2011.00022

Menteyne, A., Levavasseur, F., Audinat, E., and Avignone, E. (2009). Predominant functional expression of Kv1.3 by activated microglia of the hippocampus after status epilepticus. *PLoS ONE* 4:e6770. doi: 10.1371/journal.pone.0006770

Mizutani, M., Pino, P. A., Saederup, N., Charo, I. F., Ransohoff, R. M., and Cardona, A. E. (2012). The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood. *J. Immunol.* 188, 29–36. doi: 10.4049/jimmunol.1100421

Moussaud, S., Lamodière, E., Savage, C., and Draheim, H. J. (2009). Characterisation of $K^+$ currents in the C8-B4 microglial cell line and their regulation by microglia activating stimuli. *Cell. Physiol. Biochem.* 24, 141–152. doi: 10.1159/000233240

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* 308, 1314–1318. doi: 10.1126/science.1110647

Nörenberg, W., Gebicke-Haerter, P. J., and Illes, P. (1994). Voltage-dependent potassium channels in activated rat microglia. *J. Physiol.* 475, 15–32. doi: 10.1113/jphysiol.1994.sp020046

Olah, M., Amor, S., Brouwer, N., Vinet, J., Eggen, B., Biber, K., et al. (2012). Identification of a microglia phenotype supportive of remyelination. *Glia* 60, 306–321. doi: 10.1002/glia.21266

Paolicelli, R. C., Bisht, K., and Tremblay, M. E. (2014). Fractalkine regulation of microglial physiology and consequences on the brain and behavior. *Front. Cell. Neurosci.* 8:129. doi: 10.3389/fncel.2014.00129

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., et al. (2011). Synaptic pruning by microglia is necessary for normal brain development. *Science* 333, 1456–1458. doi: 10.1126/science.1202529

Paolicelli, R. C., and Gross, C. T. (2011). Microglia in development: linking brain wiring to brain environment. *Neuron Glia Biol*. 7, 77–83. doi: 10.1017/S1740925X12000105

Perry, V. H., Hume, D. A., and Gordon, S. (1985). Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. *Neuroscience* 15, 313–326. doi: 10.1016/0306-4522(85)90215-5

Piccinin, S., Di Angelantonio, S., Piccioni, A., Volpini, R., Cristalli, G., Fredholm, B. B., et al. (2010). CX3CL1-induced modulation at CA1 synapses reveals multiple mechanisms of EPSC modulation involving adenosine receptor subtypes. *J. Neuroimmunol.* 224, 85–92. doi: 10.1016/j.jneuroim.2010.05.012

Ponomarev, E. D., Maresz, K., Tan, Y., and Dittel, B. N. (2007). CNS-derived interleukin-4 is essential for the regulation of autoimmune inflammation and induces a state of alternative activation in microglial cells. *J. Neurosci.* 27, 10714–10721. doi: 10.1523/JNEUROSCI.1922-07.2007

Prinz, M., and Mildner, A. (2011). Microglia in the CNS: immigrants from another world. *Glia* 59, 177–187. doi: 10.1002/glia.21104

Ragozzino, D., Di Angelantonio, S., Trettel, F., Bertollini, C., Maggi, L., Gross, C., et al. (2006). Chemokine fractalkine/CX3CL1 negatively modulates active glutamatergic synapses in rat hippocampal neurons. *J. Neurosci.* 26, 10488–10498. doi: 10.1523/JNEUROSCI.3192-06.2006

Raivich, G. (2005). Like cops on the beat: the active role of resting microglia. *Trends Neurosci*. 28, 571–573. doi: 10.1016/j.tins.2005.09.001

Rappert, A., Biber, K., Nolte, C., Lipp, M., Schubel, A., Lu, B., et al. (2002). Secondary lymphoid tissue chemokine (CCL21) activates CXCR3 to trigger a Cl-current and chemotaxis in murine microglia. *J. Immunol.* 168, 3221–3226. doi: 10.4049/jimmunol.168.7.3221

Rogers, J. T., Morganti, J. M., Bachstetter, A. D., Hudson, C. E., Peters, M. M., Grimmig, B. A., et al. (2011). CX3CR1 deficiency leads to impairment of hippocampal cognitive function and synaptic plasticity. *J. Neurosci.* 31, 16241–16250. doi: 10.1523/JNEUROSCI.3667-11.2011

Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., et al. (2012). Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron* 74, 691–705. doi: 10.1016/j.neuron.2012.03.026

Schilling, T., and Eder, C. (2007). Ion channel expression in resting and activated microglia of hippocampal slices from juvenile mice. *Brain Res.* 1186, 21–28. doi: 10.1016/j.brainres.2007.10.027

Schilling, T., and Eder, C. (2014). Microglial $K^+$ channel expression in young adult and aged mice. *Glia* 63, 664–672. doi: 10.1002/glia.22776

Schilling, T., Stock, C., Schwab, A., and Eder, C. (2004). Functional importance of $Ca2^+$-activated $K^+$ channels for lysophosphatidic acid-induced microglial migration. *Eur. J. Neurosci.* 19, 1469–1474. doi: 10.1111/j.1460-9568.2004.03265.x

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019

Schlichter, L. C., Sakellaropoulos, G., Ballyk, B., Pennefather, P. S., and Phipps, D. J. (1996). Properties of $K^+$ and Cl–channels and their involvement in proliferation of rat microglial cells. *Glia* 17, 225–236. doi: 10.1002/(SICI)1098-1136(199607)17:3<225::AID-GLIA5>3.0.CO;2-#

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T)method. *Nat. Protoc*. 3, 1101–1108. doi: 10.1038/nprot.2008.73

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675. doi: 10.1038/nmeth.2089

Tarozzo, G., Bortolazzi, S., Crochemore, C., Chen, S. C., Lira, A. S., Abrams, J. S., et al. (2003). Fractalkine protein localization and gene expression in mouse brain. *J. Neurosci. Res.* 73, 81–88. doi: 10.1002/jnr.10645

Tremblay, M. È., Lowery, R. L., and Majewska, A. K. (2010). Microglial interactions with synapses are modulated by visual experience. *PLoS Biol.* 8:e1000527. doi: 10.1371/journal.pbio.1000527

Wake, H., Moorhouse, A. J., Jinno, S., Kohsaka, S., and Nabekura, J. (2009). Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. *J. Neurosci.* 29, 3974–3980. doi: 10.1523/JNEUROSCI.4363-08.2009

Walker, F. R., Beynon, S. B., Jones, K. A., Zhao, Z., Kongsui, R., Cairns, M., et al. (2014). Dynamic structural remodelling of microglia in health, and disease: a review of the models, the signals, and the mechanisms. *Brain Behav. Immun*. 37, 1–14. doi: 10.1016/j.bbi.2013.12.010

Wu, C., Asokan, S. B., Berginski, M. E., Haynes, E. M., Sharpless, N. E., Griffith, J. D., et al. (2012). Arp2/3 is critical for lamellipodia and response to extracellular matrix cues but is dispensable for chemotaxis. *Cell* 148, 973–987. doi: 10.1016/j.cell.2011.12.034

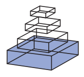
Zhan, Y., Paolicelli, R. C., Sforazzini, F., Weinhard, L., Bolasco, G., Pagani, F., et al. (2014). Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior. *Nat. Neurosci*. 17, 400–406. doi: 10.1038/nn.3641

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Copyright © 2015 Pagani, Paolicelli, Murana, Cortese, Di Angelantonio, Zurolo, Guiducci, Ferreira, Garofalo, Catalano, D'Alessandro, Porzia, Peruzzi, Mainiero, Limatola, Gross and Ragozzino. This is an open-access article distributed under the terms of the* Creative Commons Attribution License (CC BY)*. The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 12 August 2014
doi: 10.3389/fncel.2014.00233



# $CX_3CL1$ is up-regulated in the rat hippocampus during memory-associated synaptic plasticity

**Graham K. Sheridan[1,2], Anita Wdowicz[1], Mark Pickering[3], Orla Watters[4], Paul Halley[1], Niamh C. O'Sullivan[4], Claire Mooney[1], David J. O'Connell[4], John J. O'Connor[4] and Keith J. Murphy[1]***

[1] Neurotherapeutics Research Group, UCD School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Dublin, Ireland
[2] Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, UK
[3] School of Medicine and Medical Science, Health Sciences Centre, University College Dublin, Dublin, Ireland
[4] UCD School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Dublin, Ireland

***Edited by:***
*Laura Maggi, Sapienza University of Rome, Italy*

***Reviewed by:***
*Astrid E. Cardona, The University of Texas at San Antonio, USA*
*Davide Ragozzino, Università Sapienza, Italy*

***Correspondence:***
*Keith J. Murphy, Neurotherapeutics Research Group, UCD School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland*
*e-mail: keith.murphy@ucd.ie*

Several cytokines and chemokines are now known to play normal physiological roles in the brain where they act as key regulators of communication between neurons, glia, and microglia. In particular, cytokines and chemokines can affect cardinal cellular and molecular processes of hippocampal-dependent long-term memory consolidation including synaptic plasticity, synaptic scaling and neurogenesis. The chemokine, $CX_3CL1$ (fractalkine), has been shown to modulate synaptic transmission and long-term potentiation (LTP) in the CA1 pyramidal cell layer of the hippocampus. Here, we confirm widespread expression of $CX_3CL1$ on mature neurons in the adult rat hippocampus. We report an up-regulation in $CX_3CL1$ protein expression in the CA1, CA3 and dentate gyrus (DG) of the rat hippocampus 2 h after spatial learning in the water maze task. Moreover, the same temporal increase in $CX_3CL1$ was evident following LTP-inducing theta-burst stimulation in the DG. At physiologically relevant concentrations, $CX_3CL1$ inhibited LTP maintenance in the DG. This attenuation in dentate LTP was lost in the presence of $GABA_A$ receptor/chloride channel antagonism. $CX_3CL1$ also had opposing actions on glutamate-mediated rise in intracellular calcium in hippocampal organotypic slice cultures in the presence and absence of $GABA_A$ receptor/chloride channel blockade. Using primary dissociated hippocampal cultures, we established that $CX_3CL1$ reduces glutamate-mediated intracellular calcium rises in both neurons and glia in a dose dependent manner. In conclusion, $CX_3CL1$ is up-regulated in the hippocampus during a brief temporal window following spatial learning the purpose of which may be to regulate glutamate-mediated neurotransmission tone. Our data supports a possible role for this chemokine in the protective plasticity process of synaptic scaling.

**Keywords: calcium imaging, chemokine signaling, fractalkine, learning and memory, LTP, water maze**

## INTRODUCTION

While higher inflammatory and pathophysiological levels of cytokines are implicated in a range of neuropsychiatric and neurodegeneration diseases, it is now equally evident that, within the central nervous system (CNS), cytokines, including the chemoattractant cytokines (chemokines), mediate physiological signaling functions far beyond and independent of their traditional roles in inflammation and disease (Hopkins and Rothwell, 1995; Rothwell and Hopkins, 1995; Reichenberg et al., 2001; Pollmächer et al., 2002; Wilson et al., 2002; Adler and Rogers, 2005; Adler et al., 2006; McAfoose and Baune, 2009; Hoshiko et al., 2012; Williamson and Bilbo, 2013). In particular, pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor alpha (TNF-α) have all been implicated in cardinal cellular and molecular processes of long-term hippocampal-dependent memory consolidation including synaptic plasticity, synaptic scaling and neurogenesis (Malenka and Bear, 2004; Bruel-Jungerman et al., 2007a,b; Turrigiano, 2007; Baier et al., 2009; McAfoose and Baune, 2009; Bachstetter et al., 2011; Ben Menachem-Zidon et al., 2011; Yirmiya and Goshen, 2011; del Rey et al., 2013; Gemma and Bachstetter, 2013). For example, at physiological levels, IL-1 promotes long-term potentiation (LTP), a widely employed electrophysiological model of memory-associated synaptic plasticity, whereas IL-6 appears to exert inhibitory influences on excessive excitation during LTP maintenance (Li et al., 1997; Coogan et al., 1999; Ross et al., 2003; Balschun et al., 2004). Interestingly, production of both IL-1 and IL-6 is increased following LTP induction, further supporting a role for cytokines in modulating memory-associated synaptic plasticity and network-protective synaptic scaling (Schneider et al., 1998; Jankowsky et al., 2000). Our aim in the current study was to assess the effects of the chemokine, $CX_3CL1$, also known as neurotactin or fractalkine, on hippocampal-dependent synaptic plasticity processes such as spatial memory and LTP. $CX_3CL1$ is highly expressed on hippocampal neurons in the post-natal and adult rat and declines in old age which has been linked to cognitive decline in rodents (Lyons et al., 2009). In this study, we investigated if $CX_3CL1$ plays a normal physiological role in hippocampal-dependent synaptic plasticity.

Several chemokines are widely expressed throughout the CNS during development and throughout life where they have been shown to play diverse roles in cell migration and differentiation

(Lu et al., 2002), regulation of cellular communication in the adult brain (Tran and Miller, 2003) and neuroprotection (Araujo and Cotman, 1993; Meucci et al., 1998; Robinson et al., 1998; Bruno et al., 2000; Limatola et al., 2000; Hatori et al., 2002; Deiva et al., 2004; Krathwohl and Kaiser, 2004; Limatola et al., 2005; Catalano et al., 2013; Shepherd et al., 2013). $CX_3CL1$ is the only member of the chemokine δ subfamily (Rostene et al., 2007). Most other chemokines bind several G protein-coupled receptors to mediate their activities and so $CX_3CL1$ is unusual in that it appears to bind only one receptor, the $G_i$ protein-coupled receptor, $CX_3CR1$ (Allen et al., 2007). The full-length molecule is also larger than most other chemokines, containing approximately 373 amino acid residues compared to the more common 70–80 amino acid size. The 95 kDa full-length protein is a type I transmembrane protein consisting of a 76-amino acid *N*-terminal chemokine domain, a 241-amino acid glycosylated mucin-like stalk, an 18-amino acid transmembrane region and an intracellular *C*-terminal domain. The approximately 70 kDa soluble *N*-terminal chemokine domain can be released from the full-length protein via the action of several metalloproteinases such as cathepsin S, ADAM10, and ADAM17 (TACE: TNF-α-converting enzyme) in both the periphery and CNS (Garton et al., 2001; Hundhausen et al., 2003; Clark et al., 2007; Cook et al., 2010; Jones et al., 2013). Unlike most chemokines, $CX_3CL1$ is constitutively expressed in the CNS with particularly high levels in hippocampal neurons (Harrison et al., 1998). $CX_3CR1$, the only known receptor for $CX_3CL1$, is expressed predominantly on microglia in the mouse CNS (Cardona et al., 2006). The cell type expression pattern of $CX_3CR1$ in the CNS remains controversial, however, since several studies report $CX_3CR1$ expression on neurons *in vitro* as well as in brain regions including the hippocampus, Raphe nucleus, nucleus of the solitary tract (NTS) and paraventricular nucleus (PVN) of the hypothalamus in rats (Meucci et al., 1998, 2000; Maciejewski-Lenoir et al., 1999; Hatori et al., 2002; Hughes et al., 2002; Tarozzo et al., 2003; Verge et al., 2004; Limatola et al., 2005; Zhuang et al., 2007; Heinisch and Kirby, 2009; Ruchaya et al., 2012, 2014).

The high basal level of $CX_3CL1$ mRNA and protein expression in the hippocampus is suggestive of a physiological, non-inflammatory function. Indeed, there is mounting evidence which implicates both $CX_3CL1$ and its receptor, $CX_3CR1$, in synaptic plasticity and neuromodulation (Bertollini et al., 2006; Ragozzino et al., 2006; Piccinin et al., 2010; Maggi et al., 2011; Rogers et al., 2011; Roseti et al., 2013; Scianni et al., 2013). For example, $CX_3CL1$ has been shown to reduce spontaneous glutamate release and post-synaptic glutamate currents (Meucci et al., 1998; Limatola et al., 2005). The latter effect has been linked to increased intracellular calcium and dephosphorylation of the GluR1 AMPA receptor subunit (Ragozzino et al., 2006). These synaptic effects are consistent with a direct action of $CX_3CL1$ on neurons most likely exerted through the $CX_3CR1$ receptor, which is reportedly expressed on the dendrites of hippocampal neurons (Meucci et al., 2000; Limatola et al., 2005). Overall, previous studies indicate a predominantly inhibitory role for $CX_3CL1$, perhaps as a component of neuroprotective synaptic scaling mechanisms necessary for hippocampal memory-associated synaptic plasticity processes (Bertollini et al., 2006; Turrigiano, 2008; Piccinin et al., 2010). Consistent with this hypothesis, ADAM17-mediated increase in soluble $CX_3CL1$ is observed in multiple settings of glutamatergic neurotransmission where the chemokine is suggested to perform a neuroprotective function (Chapman et al., 2000; Tsou et al., 2001; Erichsen et al., 2003; Limatola et al., 2005; Ragozzino et al., 2006; Lauro et al., 2010; Pabon et al., 2011). At levels reached during inflammatory conditions, $CX_3CL1$ signaling has previously been associated with activation of pro-survival and anti-apoptotic pathways through phosphorylation of molecules such as Akt, as well as activation of MAP kinases such as p-38 and Erk1/2 (p44/42; Maciejewski-Lenoir et al., 1999; Meucci et al., 2000; Cambien et al., 2001; Deiva et al., 2004; Klosowska et al., 2009; Lyons et al., 2009).

In the present study, we investigated if $CX_3CL1$ expression is actively regulated in the hippocampus during a normal spatial learning event and also after the induction of LTP. We demonstrate the ability of physiological levels of $CX_3CL1$ to inhibit the maintenance of LTP and the importance of dentate gyrus (DG) GABAergic neurotransmission to facilitating this attenuation of hippocampal synaptic plasticity. Finally, we provide evidence that the effects of $CX_3CL1$ on synaptic plasticity may relate to suppression of glutamate-mediated calcium influx, particularly in hippocampal neurons.

## MATERIALS AND METHODS

### ANIMAL MAINTENANCE AND BEHAVIORAL ASSESSMENT

Postnatal day 80 male Wistar rats (330–380 g) were used for behavioral studies and were obtained from the Biomedical Facility at University College Dublin, Ireland. All experimental procedures were approved by the Animal Research Ethics Committee of the Biomedical Facility at UCD and were carried out by individuals who held the appropriate license issued by the Minister for Health and Children. Animals were housed in groups of four and given *ad libitum* access to food and water. The experimental room was kept on a 12 h light/dark cycle at 22 ± 2°C. The behavior of each animal was assessed in an open field apparatus (620 mm long, 620 mm wide, and 150 mm high) both 48 and 24 h prior to commencement of training. The base of the open field box was demarcated into an 8 × 8 grid. The animals' locomotion, rearing, and grooming behavior was monitored over a 5 min period and deemed normal prior to water maze training (data not shown). Their weights were also recorded immediately following the open field. Behavioral assessment was conducted in a quiet room under low-level red light illumination.

### WATER MAZE TRAINING

On postnatal day 80, animals were trained in the Morris water maze spatial learning task. Briefly, the water maze apparatus consists of a large circular pool (150 cm diameter, 80 cm deep) and a hidden platform (11 cm diameter). Both were constructed from black polyvinyl plastic, offering no intramaze visual cues that may help guide escape behavior. The platform was submerged 1.5 cm below the water surface (temperature 26 ± 1°C) and positioned 30 cm from the edge of the maze wall. The platform remained in the same position throughout the training session. The experimental room contained several extra-maze visual cues. The rat was lowered into the water facing the wall of the maze (30 cm high) at one of three locations which were alternated with each

trial. Trials lasted a maximum of 90 s and the length of time taken for the rat to find the hidden platform was recorded. Rats failing to find the platform within the 90 s were placed on it for 10 s and allowed orient themselves. The training session consisted of five trials with an inter-trial interval of 300 s. Each trained animal was assigned a corresponding passive control animal that spent the same lengths of time swimming in the pool, minus the platform. After training, the rats were dried-off and placed back into their home cages. They were then killed by cervical dislocation at specific time-points post-training, i.e. 1, 2, or 3 h after commencement of the third trial. Brains used for immunofluorescent labeling procedures were quickly dissected out, covered in optimal cutting temperature (OCT compound; Agar Scientific) and snap frozen in *N*-hexane cooled to –80°C with $CO_2$.

### IMMUNOFLUORESCENT LABELING OF HIPPOCAMPAL $CX_3CL1$

Coronal cryosections of whole brain were taken at –3.3 mm with respect to Bregma in order to examine the dorsal hippocampus (Paxinos and Watson, 2005). The 12 μm sections were adhered to glass slides coated with poly-L-lysine. Sections were fixed in 70% ethanol for 25 min and then washed in phosphate-buffered saline (PBS). Sections were then incubated for 18 h at room temperature in primary antibody solution. The primary antibodies used were: (1) AF537 (R&D Systems; 1:250 dilution), a goat IgG polyclonal antibody that labels recombinant rat $CX_3CL1$ and; (2) MAB377 (Millipore; 1:500 dilution), a mouse IgG monoclonal antibody that detects the neuronal marker NeuN. The primary antibody solution consisted of 1% bovine serum albumin (BSA) and 1% normal rabbit serum in PBS. Following two 10 min washes in PBS, sections were incubated for 3 h with a rabbit anti-goat IgG secondary antibody conjugated to FITC (Sigma; 1:1000 dilution) which detected the $CX_3CL1$ antibody. The NeuN primary antibody was detected by a rabbit anti-mouse IgG TRITC-labeled secondary antibody (Sigma; 1:1000 dilution). Where applicable, nuclei were visualized using either propidium iodide or Hoechst 33258 (Invitrogen). Sections were mounted in Citifluor glycerol PBS solution (Agar Scientific), cover-slipped and stored in darkness at 4°C until imaged. To minimize any potential confounder effects from the immunohistochemical technique, trained sections were prepared, stained and imaged at the same time as their relevant passive control.

### ACUTE HIPPOCAMPAL SLICE PREPARATION AND fEPSP RECORDING

Post-natal day 21–25 male Wistar rats (60–100 g) were obtained from the Biomedical Facility, University College Dublin, Ireland. All experimental procedures were approved by the Animal Research Ethics Committee of the Biomedical Facility at UCD. Animals were anesthetized using isoflurane (Abbott Laboratories Ireland Ltd.) and decapitated by guillotine. The brain was rapidly removed and placed into ice-cold artificial cerebro-spinal fluid (aCSF) bubbled with 95% $O_2$ and 5% $CO_2$ (aCSF composition: 120 mM NaCl, 26 mM $NaHCO_3$, 1.25 mM $NaH_2PO_4$, 2.5 mM KCl, 2 mM $MgSO_4 \cdot 7H_20$, 2 mM $CaCl_2$ and 10 mM D-glucose). This high magnesium aCSF facilitates slice viability and recovery through greater NMDA receptor blockade. Transverse hippocampal slices (400 μm) were cut from both hemispheres using a vibroslice (Campden Instruments). They were then transferred to a submerged incubation chamber containing bubbled, room temperature aCSF and allowed to recover for 90 min. Following this recovery period, slices were transferred to a recording chamber perfused with aCSF at a flow rate of 4–5 ml/min at 31 ± 0.5°C. The composition of aCSF used for recording was a modified version of that used during slice recovery, i.e., the $MgSO_4 \cdot 7H_2O$ content was reduced to 1.3 mM to decrease NMDA receptor blockade and facilitate LTP induction. Extracellular field excitatory postsynaptic potentials (fEPSPs) were elicited by stimulation of the medial perforant path of the DG by a monopolar glass electrode at a frequency of 0.05 Hz. Responses were recorded using a glass electrode placed in the middle third of the molecular layer and stimulus strength was adjusted to give a response 35% of maximal. The effect of 500 pM $rCX_3CL1$ (recombinant rat $CX_3CL1$, Peprotech EC) on LTP in the DG was investigated both in the presence and absence of 100 μM of the $GABA_A$ receptor/chloride channel inhibitor, picrotoxin (Sigma–Aldrich). Stable baseline recordings were made for at least 20 min prior to application of drugs. LTP was induced by theta-burst stimulation (TBS) consisting of eight trains (40 ms duration) of eight pulses at 200 Hz with 2 s intervals between trains and at a stimulus strength corresponding to 70% of maximal. Following TBS, the stimulus voltage was returned to that of baseline levels and fEPSPs were recorded every 20 s for a further 60 min.

### FREEZING OF HIPPOCAMPAL SLICES FOR IMMUNOSTAINING

Slices were coated in OCT (Agar UK) and snap-frozen in *n*-hexane cooled to –80°C with compressed $CO_2$. Hippocampal slices were cryosectioned into 12 μm sections that were adhered to glass slides and immunofluorescently stained for $CX_3CL1$ (as above). Nuclei were counterstained with propidium iodide.

### PREPARATION OF ORGANOTYPIC HIPPOCAMPAL SLICE CULTURES

Organotypic hippocampal cultures were prepared according to Stoppini et al. (1991). Briefly, post-natal day 7 male Wistar rats were decapitated without anaesthetic and their brains quickly dissected out and placed into ice-cold Earle's balanced salt solution (EBSS) for 1 min. Both hippocampi were removed and cut into 400 μm slices using a McIlwain tissue chopper. Slices were separated and arranged onto organotypic inserts (three per insert, Millicell PICMORG50). The inserts were housed in standard six-well cell culture plates which were kept in an incubator at 35°C and 5% $CO_2$ in air. The slices were grown using an interface method with 1 ml organotypic medium supplying the under-surface of the slice. The organotypic medium consisted of 50% minimum essential medium (MEM, Gibco), 25% EBSS (Gibco), 25% heat-inactivated horse serum (Sigma) and supplemented with 2 mM glutamine, 28 mM D-glucose, 100 U/ml penicillin/streptomycin, and 25 mM HEPES. The first medium change was conducted 24 h following slice preparation with subsequent medium changes occurring every 2 days. Slices were maintained for 21 days *in vitro* (DIV) prior to experimentation.

### CONFOCAL MICROSCOPY

All confocal images used for quantitative analysis of immunofluorescence (12-bit; 1024 × 1024 pixels) were captured using a 40×/0.8 W water-dipping lens (Zeiss Achroplan). Images of

hippocampal sections from water maze-trained animals were captured from three defined regions of the hippocampus, i.e., CA1, CA3, and the apex of the DG. Images taken from acute slice preparations were captured from the upper (unstimulated) and lower (TBS-stimulated) blades of the DG. The specific areas of the hippocampal neuronal circuit captured were kept consistent between sections. Three sections from each rat brain and acute hippocampal slice were used for analysis.

### IMAGE ANALYSIS

Image analysis was conducted using EBImage; a package for the R programming environment (Pau et al., 2010). Analysis of the combined nuclear and surrounding cell soma expression of $CX_3CL1$ fluorescence was calculated for every cell in each image. Briefly, taking the DG confocal image in **Figure S1** as a typical example, the red, green and blue channels were first separated for each image and every pixel within the images ($1024 \times 1024$) was assigned an intensity value between 0 and 1. Using the blue channel as a nuclear reference, size and fluorescence intensity thresholds were set in order to select only those pixels likely to represent Hoechst-labeled nuclei. The nuclei were then 'dilated' using morphological kernel expansion. This step allowed the designation of a soma region surrounding each nucleus. A distance map was then generated for the image which calculates the distance of each foreground pixel (white) to the nearest background pixel (black). The watershed segmentation algorithm is then employed which accurately separates clusters of nuclei that are very close together, or touching, into individual cells. Minimum distance between objects and minimum radius criteria are written into the analysis scripts which further refines object separation. $CX_3CL1$ immunofluorescence is calculated for every cell as the average pixel fluorescence intensity.

### LIVE-CELL CALCIUM IMAGING IN ORGANOTYPIC SLICES AND PRIMARY HIPPOCAMPAL CULTURES

At 21 days *in vitro*, organotypic hippocampal cultures were prepared for calcium imaging experiments by transferring inserts to room-temperature BSS (buffered salt solution) composed of 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgSO_4$, 5.5 mM D-glucose, and 20 mM HEPES, pH 7.3. The insert membranes were cut using a scalpel and individual slices were transferred to 35 mm Petri dishes containing 2 ml of 3 μM fluo-4 AM calcium indicator (Invitrogen) in BSS and allowed incubate in the dark for 30 min. Similarly, cover-slips containing mixed neuronal-glial cultures were individually transferred to standard six-well plates, containing 3 μM fluo-4 AM in BSS, for 20 min.

Time-series calcium imaging experiments in organotypic slices were conducted in the CA1 pyramidal cell layer of the hippocampus using an upright LSM V Pascal confocal microscope (Zeiss). The field of view on the 10× water immersion lens was halved allowing us to monitor calcium responses at a rate of two frames per second. We measured baseline calcium levels in the first 20 s (i.e. average of 40 frames). After 20 s a solution of glutamate (30 μM) in BSS was washed onto the slice and filled the slice chamber. This glutamate solution remained in the chamber for 105 s. The calcium response of each cell over the 125 s time-course was calculated using EBImage software. The concentrations of $rCX_3CL1$ (500 pM for 15 min) and picrotoxin (100 μM for 15 min) used for live-cell calcium imaging experiments in organotypic slices was the same as those used in acute slice electrophysiology experiments.

For live-cell calcium imaging experiments in primary mixed neuron-glial cultures, cells were loaded with 3 μM fluo-4 AM calcium indicator (Invitrogen) in BSS as above, and were then transferred to a custom-built imaging chamber containing fresh BSS. The imaging chamber allowed for wash-in/out of $CX_3CL1$ and glutamate solutions (Pickering et al., 2008). Experiments were conducted at room temperature using an upright LSM V Pascal confocal microscope and a 10×/0.3 W Ph1 water-dipping lens so as to capture several hundred cells per experiment. Time-series confocal images were captured at frame-rate of 1 Hz. Cells were pre-treated for 15 min with either 500 pM or 2 nM $rCX_3CL1$. Baseline calcium levels for every cell were monitored for 20 s prior to a 30 μM glutamate exposure. This glutamate solution remained in the chamber for 90 s before being washed out with fresh BSS solution and frames were captured for a further 40 s. The calcium response of each cell was calculated every second for 150 s using EBImage software.

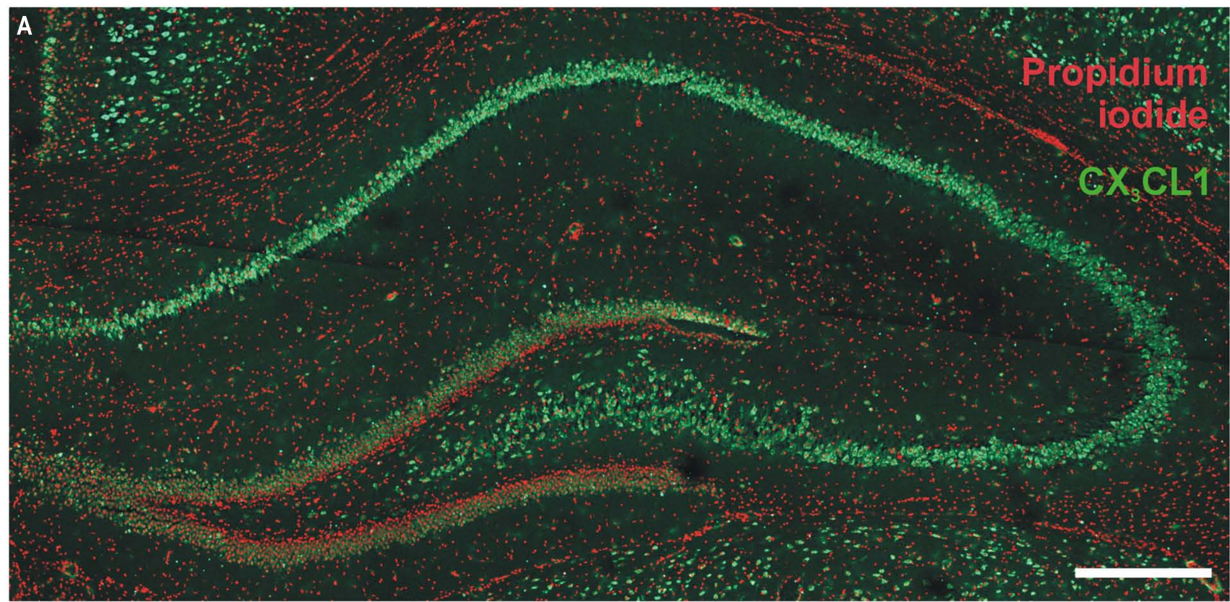
### STATISTICAL ANALYSIS

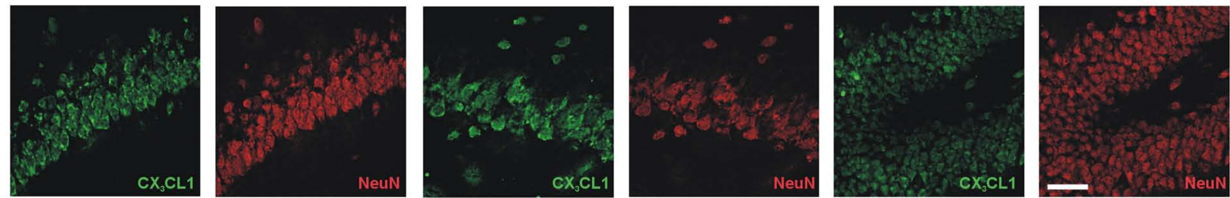
All raw data was imported into GraphPad Prism 6 software where statistical analyses were performed. Kruskal–Wallis non-parametric analysis of variance (ANOVA) tests were performed in conjunction with Dunn's multiple comparisons *post hoc* analyses in order to determine statistical significance ($p < 0.01$) for: (1) $CX_3CL1$ regulation post-spatial learning in rats; (2) $CX_3CL1$ regulation post-theta burst stimulation in acute hippocampal slices and; (3) glutamate-mediated calcium responses in primary mixed neuron-glial cell cultures. One-way ANOVA tests were performed in conjunction with Bonferroni *post hoc* analyses in order to determine statistical significance ($p < 0.05$) for: (1) the reduction in swim-times during water maze training; and (2) the effect of $rCX_3CL1$ on LTP and paired pulse ratio. Mann–Whitney $U$ tests were performed in order to determine statistical significance ($p < 0.001$) for the effects of $rCX_3CL1$ and picrotoxin on glutamate-mediated calcium responses in organotypic hippocampal slice cultures. The relationship between NeuN expression and $CX_3CL1$ expression in the rat hippocampus was analyzed by Pearson correlation and linear regression.

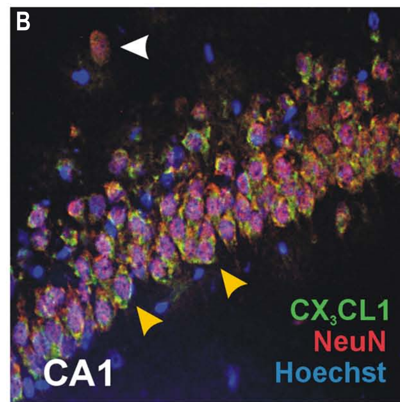
## RESULTS

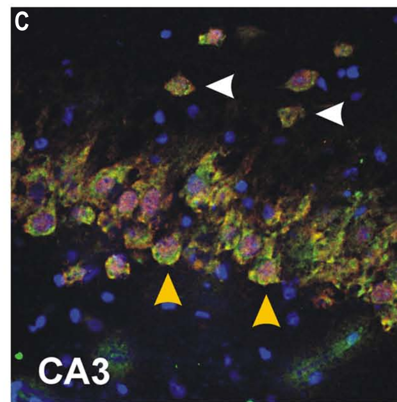
### $CX_3CL1$ IS HIGHLY EXPRESSED BY NEURONAL CELL TYPES IN THE ADULT RAT HIPPOCAMPUS

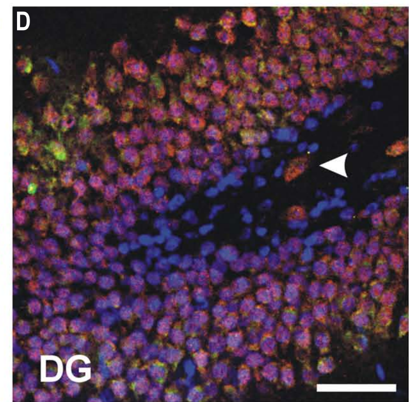
We characterized the expression and distribution of $CX_3CL1$ in the rat dorsal hippocampus using immunofluorescence. $CX_3CL1$ expression was predominately restricted to the glutamatergic pyramidal and granule neurons of the hippocampus (**Figure 1A**). At higher magnification, it is clear that $CX_3CL1$ expression co-localizes with the neuronal marker NeuN in CA1 and CA3 pyramidal neurons (**Figures 1B,C**; yellow arrowheads), dentate granule cells (**Figure 1D**) and presumptive interneurons outside the primary cell layers in all three hippocampal subfields (**Figures 1B–D**; white arrowheads). In all cases, the chemokine appears to be



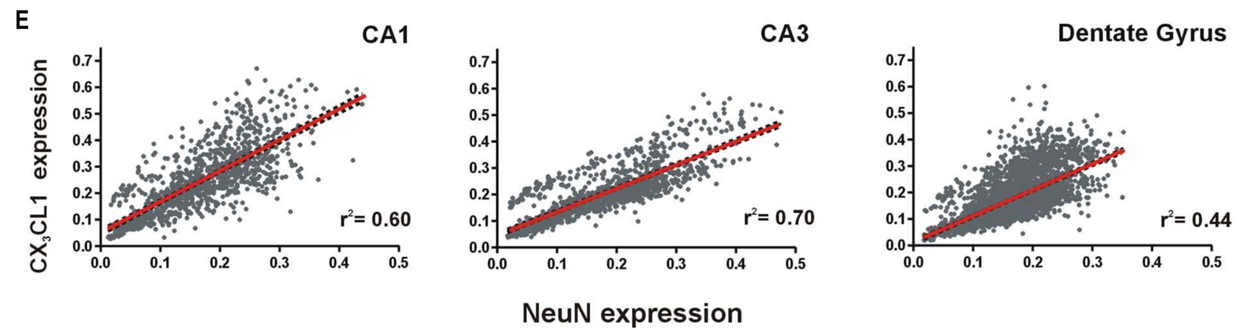




**FIGURE 1 | Expression of $CX_3CL1$ in the CA1, CA3 and dentate gyrus (DG) of the rat hippocampus. (A)** Basal levels of $CX_3CL1$ expression in the rat dorsal hippocampus. Green: $CX_3CL1$; Red: propidium iodide-stained nuclei. Scale bar = 200 μm. **(B–D)** CA1, CA3 and DG regions of the hippocampus, respectively, showing $CX_3CL1$ (green), NeuN (red) and Hoechst (blue) expression. White arrowheads: interneurons; yellow arrowheads: CA pyramidal neurons. **(E)** Relationship between $CX_3CL1$ and NeuN expression. Each dot represents a single cell. Linear regression analyses (red lines) appear on each plot with corresponding $r^2$ values indicated. Linear regression $p < 0.05$ for CA1, CA3 and DG regions of the hippocampus.

predominantly expressed on the plasma membrane of the cell soma. We assigned expression intensity values for NeuN and $CX_3CL1$ to each cell in the image dataset (see **Figure S1** for image analysis method) and then analyzed the relationship between expression of the mature neuronal marker, NeuN, and $CX_3CL1$ by Pearson correlation in each sub-region of the hippocampus. A high degree of correlation was evident between $CX_3CL1$ and NeuN expression in all three regions of the hippocampus (**Figure 1E**). The CA3 region showed the strongest linear regression coefficients of determination for correlation between $CX_3CL1$ and NeuN expression ($r^2 = 0.70$, $p < 0.05$). These data confirm previous reports that mature NeuN + hippocampal neurons in the adult rat hippocampus express high levels of $CX_3CL1$ under naïve resting conditions.

#### $CX_3CL1$ IS UP-REGULATED 2 h POST-SPATIAL LEARNING IN THE RAT HIPPOCAMPUS

Rats were trained in a five-trial water maze session ($n = 12$ in total; $n = 4$ per time point) and the latencies to find the platform were recorded. Latency-to-platform times decreased significantly (one-way ANOVA, $p < 0.001$) over the five trials of the training session indicating that the animals acquired the task (**Figure 2A**). We quantified $CX_3CL1$ expression in the hippocampus of these rats using immunofluorescence. Total $CX_3CL1$ expression was very similar in trained and passive animals 1 h following training in all hippocampal regions (**Figures 2B–D**). At the 2 h time-point, $CX_3CL1$ expression was significantly higher in trained animals compared to passive controls in all three hippocampal regions analyzed (Kruskal–Wallis ANOVA, Dunn's multiple comparisons *post hoc* test, $p < 0.001$). At the 3 h time-point in the DG, $CX_3CL1$ expression in trained animals again matched that of passive controls, highlighting the temporal specificity and transient nature of the 2 h up-regulation in dentate granule cells of rats that learned the spatial task. In the CA3, however, there was a significant, training-specific down-regulation in $CX_3CL1$ expression whereas levels of $CX_3CL1$ remained elevated in the CA1 region of trained animals compared to passive control counterparts at the 3 h time-point (Kruskal–Wallis ANOVA, Dunn's multiple comparisons *post hoc* test, $p < 0.01$). It should be noted that passive control animals exhibited an up-regulation in $CX_3CL1$ at the 2 h post-swim time-point indicating that, in addition to the learning-specific regulations described above, this chemokine is also responsive to the general stressors associated with the paradigm.

### $CX_3CL1$ IS UP-REGULATED 2 h POST-THETA BURST STIMULATION IN DENTATE GRANULE NEURONS

After measuring a learning-specific up-regulation in $CX_3CL1$ in pyramidal and dentate granule neurons 2 h post-water maze-training, we next asked whether $CX_3CL1$ is up-regulated after LTP-inducing TBS in acute hippocampal slices. TBS was delivered to the medial perforant path of the lower DG blade, as illustrated in **Figure 3A**. LTP was induced and recorded for 2 h, after which slices were snap-frozen. $CX_3CL1$ expression on stimulated dentate granule cells was compared to that on neurons located in the upper unstimulated blade (US) of the DG (**Figure 3A**). LTP-inducing TBS resulted in an up-regulation in $CX_3CL1$ expression after 2 h on the stimulated DG blade compared to the upper unstimulated DG blade (**Figure 3B**). No such difference between upper and lower blades was observed in time-matched control slices which received no stimulation.

#### EFFECTS OF $CX_3CL1$ ON LTP IN THE DENTATE GYRUS DEPENDS ON INHIBITORY TONE AND $GABA_A$ RECEPTORS

We investigated a possible functional role for $CX_3CL1$ up-regulation in modulating synaptic transmission during memory-associated synaptic plasticity. Previous studies have shown that $CX_3CL1$ inhibits LTP and mimics long-term depression (LTD) at the CA3–CA1 synapses in acute hippocampal slices (Bertollini et al., 2006; Ragozzino et al., 2006; Maggi et al., 2009). Given that we observed memory-associated increases in $CX_3CL1$ levels in CA1, CA3, and DG regions of the hippocampus following spatial learning, we next asked what effect $CX_3CL1$ exerts on LTP in the dentate granule cell synaptic field. Pre-treatment with $CX_3CL1$ (500 pM) significantly reduced induction and completely prevented maintenance of LTP in the DG (**Figure 4A**).

Consistent with previous work on pyramidal hippocampal neurons (Ragozzino et al., 2006), the paired-pulse ratio of DG neuron responses evoked by two successive stimuli (50 ms apart) was unaffected by $CX_3CL1$ treatment either at baseline or following TBS (**Figure 4B**). While not ruling out a presynaptic contribution, these data support a post-synaptic action for $CX_3CL1$-mediated inhibition of fEPSP amplitude in the DG following TBS.

Recent evidence has shown that $CX_3CL1$ reduces the activity of serotonergic neurons in the Raphe nucleus through enhanced $GABA_A$ receptor-mediated inhibition (Heinisch and Kirby, 2009). We next investigated if $CX_3CL1$-mediated control of glutamatergic neuroplasticity in the hippocampal DG requires GABAergic inhibitory transmission. LTP was induced in the presence of $GABA_A$ receptor/chloride channel blocker picrotoxin (100 $\mu$M) using the same TBS protocol as in **Figure 4A**. As expected, the degree of potentiation of fEPSP amplitude was substantially greater in the presence of $GABA_A$ receptor blockade (117 $\pm$ 5% versus 200 $\pm$ 3% of baseline average in first 10 min post-TBS in the absence and presence of picrotoxin, respectively; **Figures 4A,C**; Arima-Yoshida et al., 2011). Interestingly, $CX_3CL1$ did not prevent LTP in the presence of picrotoxin (**Figure 4C**). In fact, when picrotoxin was present, the magnitude of LTP was enhanced by $CX_3CL1$ during the initial 20 min following the TBS ($p < 0.05$). $CX_3CL1$ again had no effect on paired-pulse depression in the presence of picrotoxin (**Figure 4D**) suggesting a post-synaptic action for $CX_3CL1$-mediated short-term enhancement of fEPSP amplitude following TBS.

#### EFFECTS OF $CX_3CL1$ ON GLUTAMATE-INDUCED CALCIUM RESPONSES IN THE HIPPOCAMPUS REQUIRES $GABA_A$ RECEPTOR ACTIVATION

Long-term potentiation of excitatory synaptic transmission in the hippocampus is heavily dependent on post-synaptic intracellular calcium rise (Bliss and Collingridge, 1993). We next assessed if $CX_3CL1$ exerts differential effects on glutamate-induced intracellular calcium rise in the presence and absence of $GABA_A$ receptor blockade. Live-cell calcium imaging was performed in organotypic hippocampal slice cultures pre-treated with $CX_3CL1$ (500 pM) and/or picrotoxin (100 $\mu$M) for 15 min. Slices were

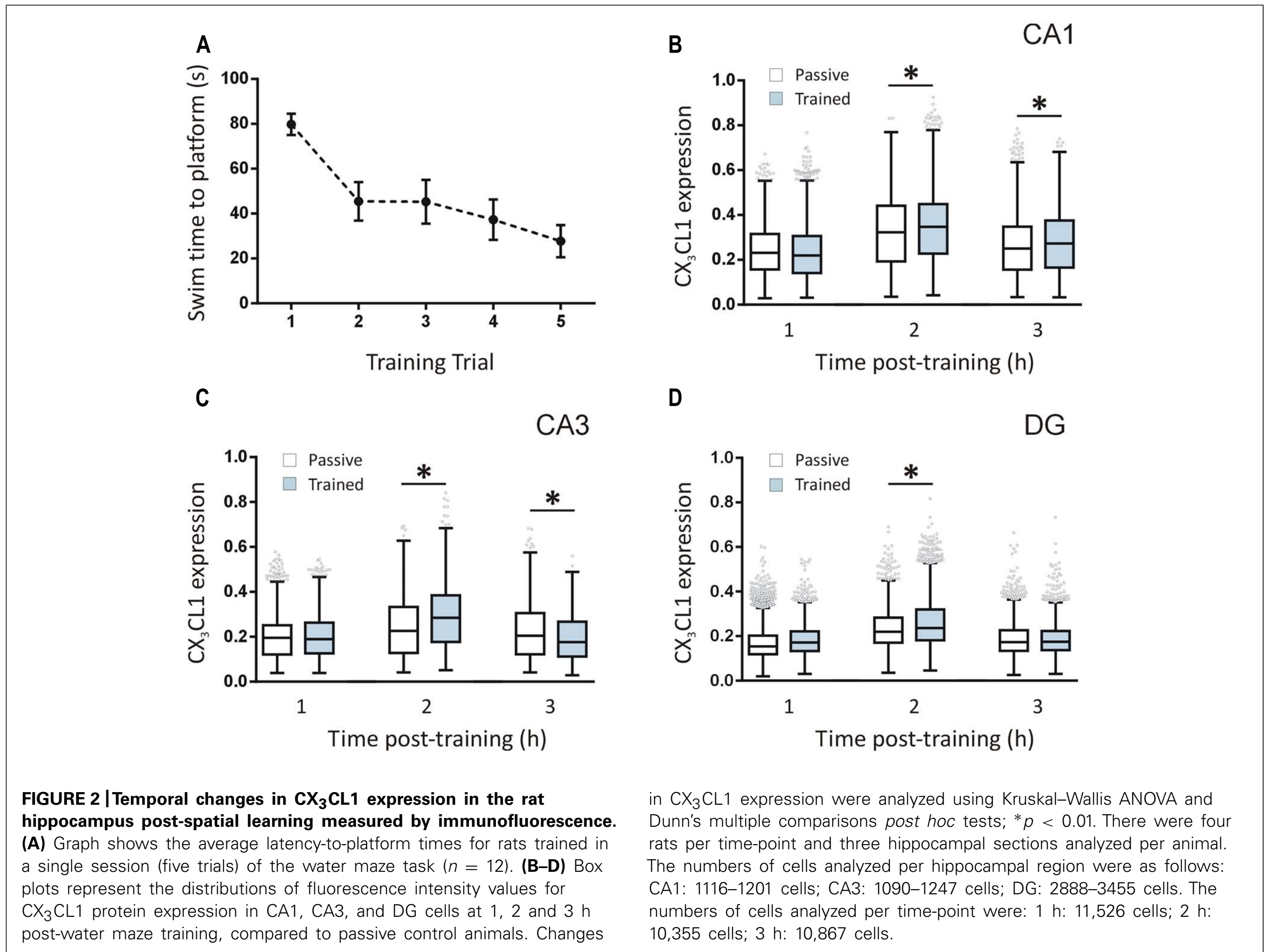



**FIGURE 2 | Temporal changes in $CX_3CL1$ expression in the rat hippocampus post-spatial learning measured by immunofluorescence.** **(A)** Graph shows the average latency-to-platform times for rats trained in a single session (five trials) of the water maze task ($n = 12$). **(B–D)** Box plots represent the distributions of fluorescence intensity values for $CX_3CL1$ protein expression in CA1, CA3, and DG cells at 1, 2 and 3 h post-water maze training, compared to passive control animals. Changes in $CX_3CL1$ expression were analyzed using Kruskal–Wallis ANOVA and Dunn's multiple comparisons *post hoc* tests; $^*p < 0.01$. There were four rats per time-point and three hippocampal sections analyzed per animal. The numbers of cells analyzed per hippocampal region were as follows: CA1: 1116–1201 cells; CA3: 1090–1247 cells; DG: 2888–3455 cells. The numbers of cells analyzed per time-point were: 1 h: 11,526 cells; 2 h: 10,355 cells; 3 h: 10,867 cells.

then exposed to glutamate (30 μM) and intracellular calcium was monitored for 1 min 45 s in the CA1 pyramidal cell layer of the hippocampus. Pre-treatment with $CX_3CL1$ prior to glutamate application resulted in an attenuated intracellular calcium rise in cells in the CA1 region of organotypic slice cultures (**Figure 4E**). While the peak amplitude of the calcium response in $CX_3CL1$-treated slices was not different than in controls (**Figure S2A**), the total calcium entry in the 60 s post-glutamate exposure was reduced, as calculated by the area under the curve (AUC) in **Figure 4E**. In contrast, in the presence of $GABA_A$ receptor/chloride channel blockade, $CX_3CL1$ enhanced glutamate-induced calcium influx as measured by AUC (**Figure 4F**) or peak intracellular calcium (**Figure S2B**). These findings are consistent with the opposing actions of $CX_3CL1$ on LTP studies in the presence and absence of picrotoxin. They suggest that neuronal responses to $CX_3CL1$ can vary depending on the balance between excitation and inhibition in the hippocampal network. Therefore, during periods of enhanced excitatory activity in the hippocampus, $CX_3CL1$ may act as a neuroprotective dampener of excessive glutamatergic neurotransmission and this action appears to be dependent on GABA-mediated inhibition.

### $CX_3CL1$ INHIBITS GLUTAMATE-INDUCED CALCIUM DYNAMICS IN BOTH NEURONS AND GLIAL CELL TYPES

In order to assess if physiological concentrations of $CX_3CL1$ exert equivalent effects on glutamate-induced calcium responses in both neurons and non-neuronal cell populations, we used hippocampal mixed cell culture preparation. Primary hippocampal cells were pre-treated with $CX_3CL1$ (500 pM or 2 nM) for 15 min prior to glutamate (30 μM) challenge. Importantly, we have shown previously that using this mixed hippocampal cell culture preparation, we can discriminate between neurons and non-neuronal cells based on the shapes of their respective glutamate-mediated calcium response curves (Pickering et al., 2008). This allowed us to simultaneously evaluate the effects of different concentrations of $CX_3CL1$ on intracellular calcium dynamics in neurons and non-neuronal cells following glutamate exposure (**Figures 5A–C**). This method of distinguishing between neurons and non-neuron cell types was shown to be as accurate as traditional methods of immunocytochemistry staining for neuronal and glial cell markers (NeuN and GFAP, respectively; Pickering et al., 2008). Neurons were identified by their substantial and prolonged increase of intracellular calcium following glutamate administration while non-neurons exhibited a sharp rise and fall back to plateaux

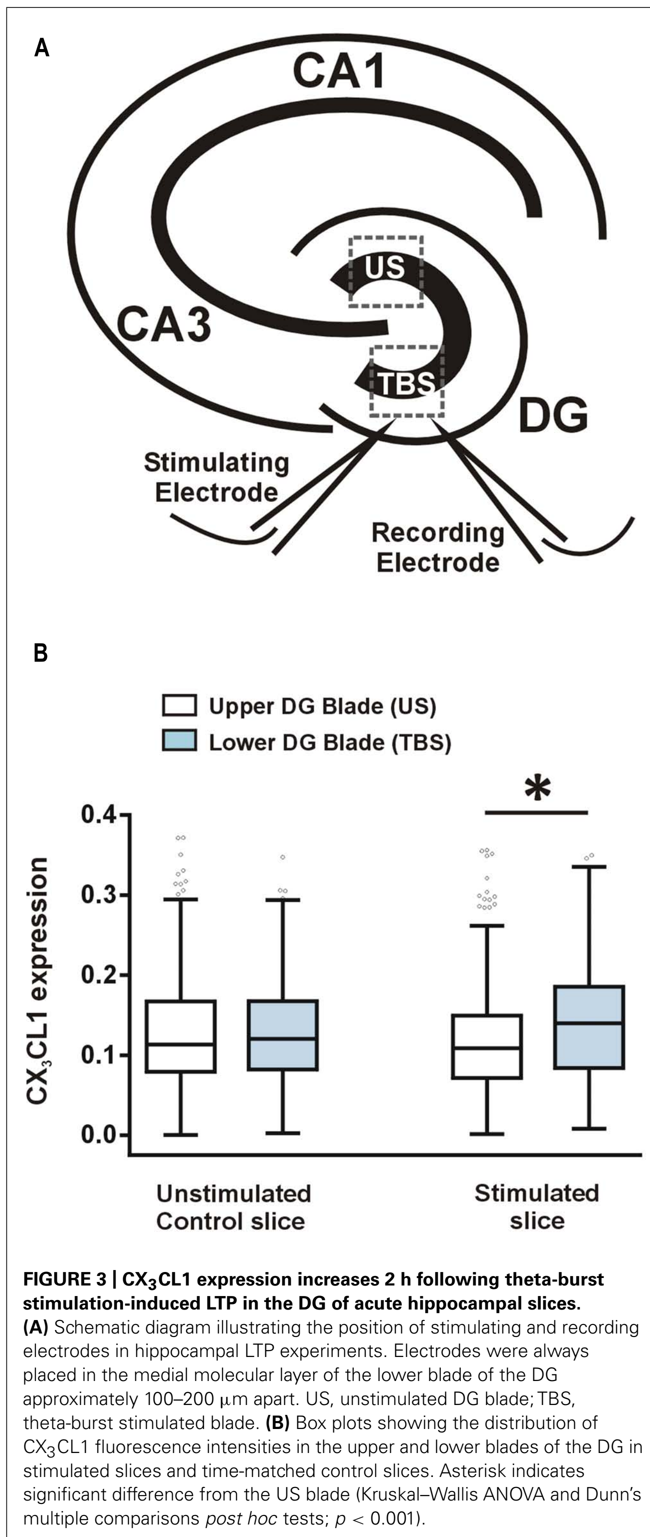



**FIGURE 3 | $CX_3CL1$ expression increases 2 h following theta-burst stimulation-induced LTP in the DG of acute hippocampal slices.** **(A)** Schematic diagram illustrating the position of stimulating and recording electrodes in hippocampal LTP experiments. Electrodes were always placed in the medial molecular layer of the lower blade of the DG approximately 100–200 μm apart. US, unstimulated DG blade; TBS, theta-burst stimulated blade. **(B)** Box plots showing the distribution of $CX_3CL1$ fluorescence intensities in the upper and lower blades of the DG in stimulated slices and time-matched control slices. Asterisk indicates significant difference from the US blade (Kruskal–Wallis ANOVA and Dunn's multiple comparisons *post hoc* tests; $p < 0.001$).

(**Figures 5D,E**, respectively). In non-neuronal cells, both low (500 pM) and higher (2 nM) physiologically relevant concentrations of $CX_3CL1$ attenuated glutamate-induced calcium influx, in a dose-dependent manner (Kruskal–Wallis ANOVA, Dunn's multiple comparisons *post hoc* test, $p < 0.05$; **Figures 5E,G**). In neurons, however, the lower concentration of $CX_3CL1$ (500 pM) had little or no effect on glutamate-induced calcium influx (**Figures 5D,F**; and **Figure S2C**). Pre-treatment of hippocampal cultures with a higher concentration (2 nM) of $CX_3CL1$ caused a substantial attenuation of glutamate-mediated calcium responses in neurons (Kruskal–Wallis ANOVA, Dunn's multiple comparisons *post hoc* test, $p < 0.001$; **Figures 5D,F**), in addition to causing further dose-dependent attenuations in calcium influx in non-neuronal cell types (**Figures 5E,G**; and **Figure S2D**). The differential responses of $CX_3CL1$ on neurons and non-neurons at the lower concentration (500 pM) may relate to variations in absolute levels of $CX_3CR1$ receptor expression on distinct cell populations in the hippocampus.

## DISCUSSION

$CX_3CL1$ is among an increasing number of cytokines and chemokines implicated in both normal functions and pathophysiological conditions of the brain (White and Greaves, 2012; Mattison et al., 2013; Sheridan and Murphy, 2013; Wu et al., 2013; Briones et al., 2014). In the present study, we identified a transient up-regulation of $CX_3CL1$ production in the hippocampus 2 h following spatial learning or induction of LTP. Importantly, these up-regulations were specific to either the memory encoding process or the TBS since the up-regulations measured were compared directly to swim-matched passive controls or unstimulated dentate granule cells, respectively. In addition, we did observe a 2 h up-regulation in $CX_3CL1$ in the passive control group of animals, indicating that this chemokine may also be responsive to stress and anxiogenic environmental conditions. However, the significant training-specific up-regulations in $CX_3CL1$, over and above those measured in passive control animals at the 2 h timepoint, implicate $CX_3CL1$ in memory-related synaptic plasticity in the hippocampus. The enhanced glutamate neurotransmission and resultant increase in hippocampal neuronal activity associated with both spatial memory formation and LTP are the most likely drivers of the production of $CX_3CL1$ in our studies. $CX_3CL1$ has been reportedly up-regulated in several settings of augmented glutamatergic transmission where the chemokine is clearly protective against excitotoxic cell death (Tong et al., 2000; Tarozzo et al., 2002; Limatola et al., 2005; Cipriani et al., 2011; Briones et al., 2014). Moreover, both $CX_3CL1$ and its receptor, $CX_3CR1$, have been shown to be up-regulated in hippocampal neurons after pilocarpine-induced status epilepticus (Yeo et al., 2011), a condition characterized by excessive glutamatergic excitation. Interestingly, 3 h post-learning, we observed opposing regulations in $CX_3CL1$ in the CA1 and CA3 pyramidal cell layers. This may relate to distinct plasticity mechanisms and/or distinct functions of these hippocampal subregions during spatial memory tasks (Hunsaker and Kesner, 2008; Rolls, 2010). Overall, the abundance and expression pattern of $CX_3CL1$ make the chemokine ideally suited for sensing hippocampal glutamate tone. Our data supports a possible role for $CX_3CL1$ in homeostatic mechanisms of synaptic scaling during memory-associated synaptic plasticity.

Previous studies have reported that $CX_3CL1$ inhibits LTP and induces LTD-like effects in the CA1 region of the hippocampus (Bertollini et al., 2006; Ragozzino et al., 2006; Maggi et al.,

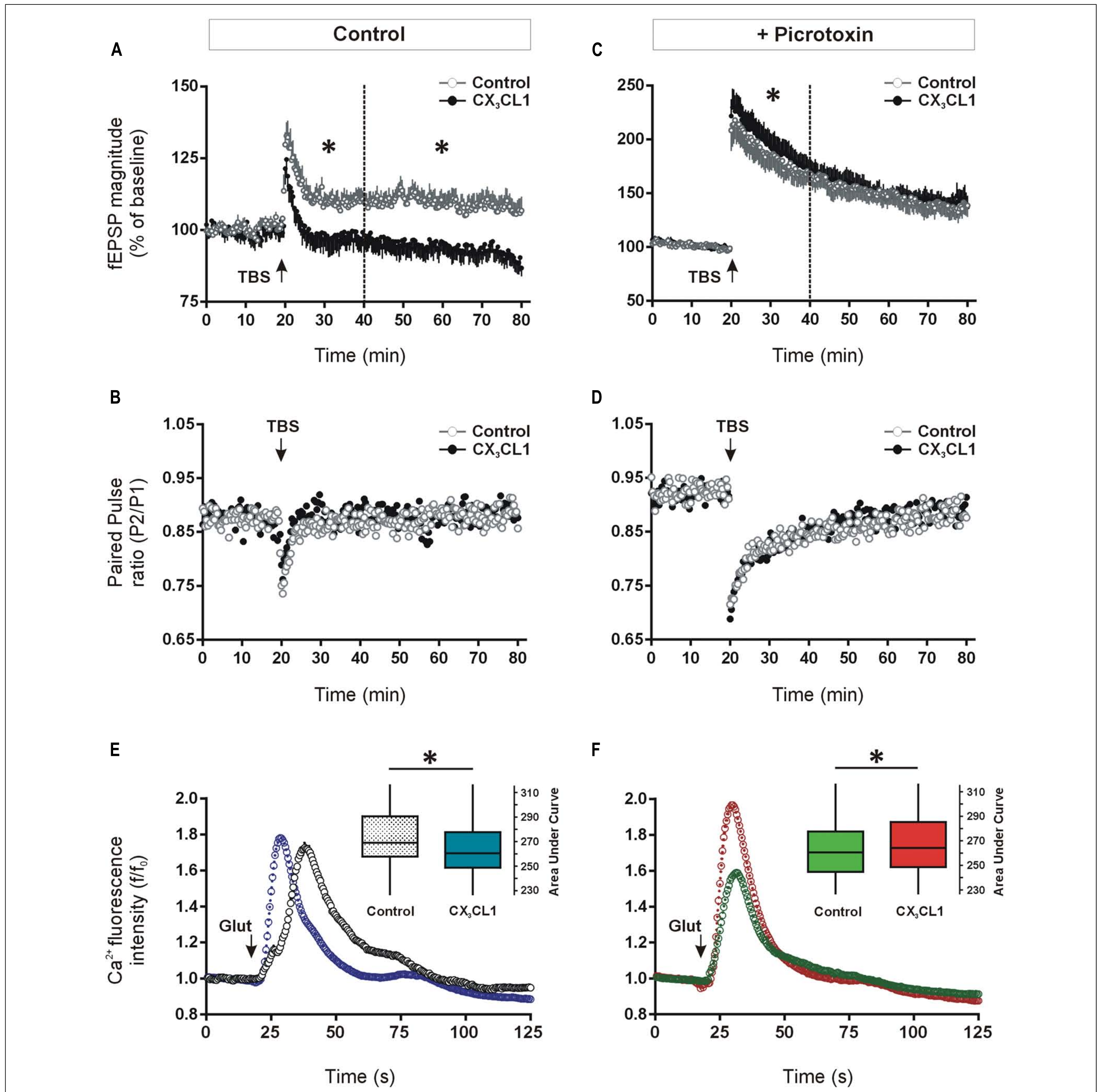



**FIGURE 4 | Differential effects of $CX_3CL1$ on long-term potentiation (LTP) in the DG and glutamate-induced intracellular calcium rise in the CA1, in the presence and absence of $GABA_A$/chloride receptor blockade. (A)** The effect of the chemokine domain of $CX_3CL1$ on LTP in acute hippocampal slices as measured by augmented field EPSP magnitude in the DG following theta-burst stimulation (TBS: 8 × 8 × 200 Hz). $CX_3CL1$ inhibited dentate LTP in both the early and late phases post-TBS (i.e., 20–40 min and 40–80 min, respectively; one-way ANOVA; $p < 0.05$, indicated by an asterisk; $n = 8$ slices per group). $CX_3CL1$ (500 pM) was present for the duration of the time period shown. **(B)** Shows the paired-pulse ratio between the first and second stimulations (50 ms interval) in the LTP experiment in **A**. **(C)** Shows the effect of $CX_3CL1$ on dentate LTP in acute hippocampal slices as measured by augmented field EPSP magnitude following TBS in the presence of picrotoxin (100 μM). $CX_3CL1$ (500 pM) and picrotoxin were present for the duration of the time period shown. $CX_3CL1$ enhanced early LTP (one-way ANOVA; $p < 0.05$, indicated by an asterisk; $n = 8$ slices per group) while having no effect on late LTP. **(D)** Shows the paired-pulse ratio between the first and second stimulations (50 ms interval) in the LTP experiment in **C**. **(E)** Shows the effect of $CX_3CL1$ on glutamate-induced calcium influx in the CA1 region of organotypic hippocampal slices cultured for 21 DIV. Pre-treatment of slice cultures with $CX_3CL1$ (500 pM) for 15 min prior to glutamate exposure reduced calcium influx in the CA1 region (Mann–Whitney *U* test; $p < 0.001$). Box plot inset shows the area under the curve (AUC) for the whole experimental time-course. $CX_3CL1$ (500 pM) was present for the duration of the time period shown. **(F)** The effect of $CX_3CL1$ on glutamate-induced calcium influx in the CA1 region of organotypic hippocampal slices in the presence of picrotoxin. Pre-treatment of slice cultures with $CX_3CL1$ (500 pM) and picrotoxin (100 μM) versus picrotoxin alone (control) for 15 min prior to glutamate exposure enhanced calcium influx in the CA1 region (Mann–Whitney *U* test; $p < 0.001$). Box plot inset shows the area under the curve (AUC) for the whole experimental time-course. $CX_3CL1$ (500 pM) and/or picrotoxin (100 μM) were present for the duration of the time period shown.

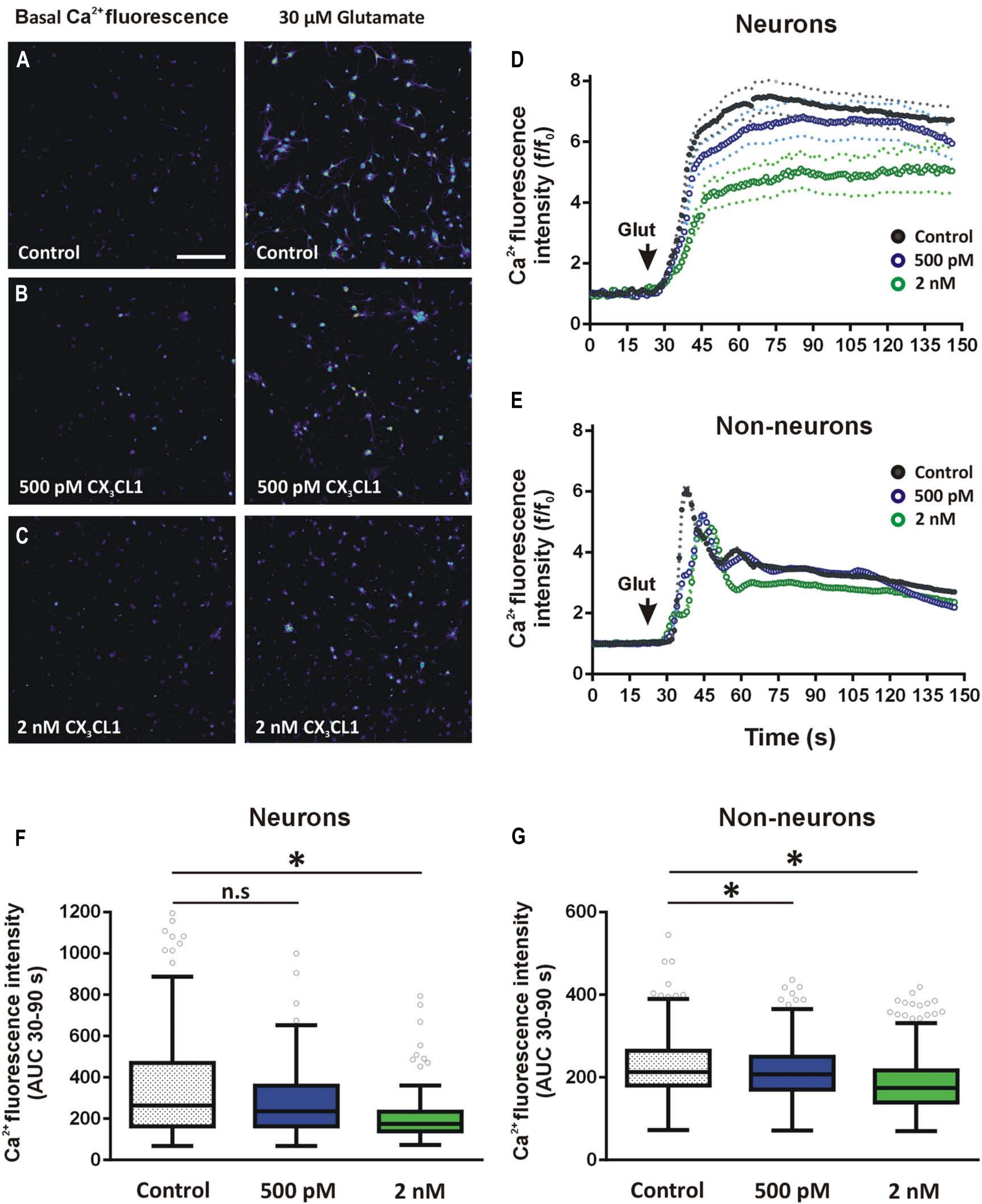



**FIGURE 5 | Effect of CX$_3$CL1 on glutamate-induced calcium dynamics in neurons and glia. (A–C)** Representative images of intracellular calcium $[Ca^{2+}]_i$ levels in mixed neuron-glial primary hippocampal cultures before and after 30 μM glutamate exposure. Control cells **(A)** were untreated prior to glutamate exposure. Treated cells were exposed to either **(B)** 500 pM or **(C)** 2 nM CX$_3$CL1 for 15 min. Scale bar = 200 μm. **(D,E)** Shows the time-course of the $[Ca^{2+}]_i$ response to glutamate in neurons **(D)** and non-neuronal cells **(E)**. Cells were treated for 15 min with either 500 pM (blue circles) or 2 nM (green circles) CX$_3$CL1 for 15 min prior to 30 μM glutamate exposure. Untreated control time-course is represented by black circles. Relative changes in $[Ca^{2+}]_i$ were calculated for each cell at each time point as $f/f_0$, where $f$ is the $[Ca^{2+}]_i$ fluorescence in each frame and $f_0$ is the average baseline fluorescence per cell, calculated 20 s prior to glutamate addition. Primary hippocampal cell cultures were divided into neurons (138, 171, and 101 cells for control, 500 pM and 2 nM CX$_3$CL1-treated cells, respectively) and non-neuronal (432, 726, and 604 cells for control, 500 pM and 2 nM CX$_3$CL1-treated cells, respectively) cell populations based on their $[Ca^{2+}]_i$ response to 30 μM glutamate (Pickering et al., 2008). **(F,G)** Quantification of the calcium imaging time-courses in **D** and **E**. 500 pM CX$_3$CL1 attenuates glutamate-induced $[Ca^{2+}]_i$ increases in non-neuronal cell types (Kruskal–Wallis ANOVA and Dunn's multiple comparisons *post hoc* tests; $*p < 0.05$), but has no significant effect on $[Ca^{2+}]_i$ response in neurons. Pre-treatment of hippocampal cell cultures for 15 min with 2 nM CX$_3$CL1, however, significantly attenuates glutamate-induced $[Ca^{2+}]_i$ influx in both non-neuronal cells and in neurons (Kruskal–Wallis ANOVA and Dunn's multiple comparisons *post hoc* tests; $*p < 0.001$). The results represent combined data from five to six individual cover-slips per treatment group and from two separate cell culturing days.

2009). We found a matching inhibitory action of $CX_3CL1$ on LTP in the DG. These observations are in good agreement with $CX_3CL1$-mediated inhibition of glutamatergic synaptic activity of hippocampal neurons (Ragozzino et al., 2006). $CX_3CL1$-mediated regulation of glutamate transmission has been shown to be due to its post-synaptic effects on neurons and involves the dephosphorylation of the GluR1 AMPA receptor subunit on serine 845, a mechanism reminiscent of LTD (Ragozzino et al., 2006). LTD can play vital roles in the context of memory-associated synaptic plasticity including synaptic scaling and enhanced signal-to-noise ratio mechanisms.

The $GABA_A$ receptor/chloride channel blocker, picrotoxin, used in the current studies would decrease GABAergic inhibitory transmission promoting depolarization in some cells with corresponding increases in intracellular calcium (Antonucci et al., 2012). Thus, the current work suggests $GABA_A$ receptor/chloride channel activity within the hippocampal neuronal network must be intact for $CX_3CL1$ to attenuate glutamatergic neurotransmission or LTP. This situation is remarkably similar to that described for $CX_3CL1$ inhibition of serotonergic neurons of the Raphe nucleus (Heinisch and Kirby, 2009). While this effect may relate to basal inhibitory tone, $CX_3CL1$ could actually be enhancing $GABA_A$ receptor function. For example, $CX_3CL1$ enhances phosphorylation and activation of Akt in neurons and this serine/threonine kinase has been associated with phosphorylation of the $GABA_A$ beta2 receptor subunit, a modification that enhances activity of the receptor (Meucci et al., 2000; Wang et al., 2003). Moreover, several signaling systems can enhance GABA-mediated inhibition through promotion of rapid insertion of $GABA_A$ receptors into the post-synaptic plasma membrane (Wan et al., 1997; Nusser et al., 1998; Mizoguchi et al., 2003; Jovanovic et al., 2004).

When discussing potential mechanisms by which $CX_3CL1$ influences neurons we must to mindful of the inconsistencies in the literature with regard to the expression of the $CX_3CR1$ receptor by neurons (Meucci et al., 1998, 2000; Maciejewski-Lenoir et al., 1999; Jung et al., 2000; Hatori et al., 2002; Hughes et al., 2002; Tarozzo et al., 2003; Deiva et al., 2004; Verge et al., 2004; Limatola et al., 2005; Cardona et al., 2006; Zhuang et al., 2007; Heinisch and Kirby, 2009; Ruchaya et al., 2012, 2014). The consensus from work with the $CX_3CR1^{-/-}$-GFP knock-in mouse (Cardona et al., 2006) suggests that $CX_3CR1$ expression is restricted to microglial cells in the CNS under naïve conditions *in vivo*. Studies of $CX_3CR1$ expression in rats, however, have found evidence of receptor expression on neuronal cell types in various brain regions (Heinisch and Kirby, 2009; Ruchaya et al., 2012, 2014). Here, we report rapid modulatory effects of $CX_3CL1$ on neuronal events in hippocampal tissue. Our data does not conclusively support a direct action of $CX_3CL1$ on neurons and we cannot discount the possibility that the effects we see on LTP and calcium influx in neuronal cell types happen as a consequence of $CX_3CL1$-mediated activation of $CX_3CR1$ solely on microglial cell types. If this is true, however, the ability of microglia to rapidly regulate multiple hippocampal memory-associated synaptic plasticity processes may be much more extensive than traditionally thought. The evidence from rats that $CX_3CR1$ is expressed on hippocampal neuron allows the possibility that, as is the case for $CX_3CL1$-mediated regulation of serotonergic neurons of the dorsal Raphe, glutamatergic neuron-derived $CX_3CL1$ may act in an autocrine/paracrine fashion in the hippocampus during periods of synaptic plasticity to regulate glutamate-mediated neurotransmission tone.

While previous studies have shown that $CX_3CL1$ causes an increase in intracellular calcium in neurons and other cell types (Oh et al., 2002; Deiva et al., 2004; Ragozzino et al., 2006) this effect of $CX_3CL1$ becomes apparent at concentrations of 25 nM and higher (Oh et al., 2002; Deiva et al., 2004), well above the levels we investigated here and we observed no such increase in intracellular calcium. At a concentration of 2 nM, in both neurons and non-neurons, $CX_3CL1$ pre-incubation suppressed glutamate-mediated rises in intracellular calcium levels. These data are in good agreement with previous findings of a protective effect of inflammatory levels of $CX_3CL1$ against glutamate excitotoxicity and glutamate NMDA receptor activation, in particular (Meucci et al., 1998; Deiva et al., 2004; Limatola et al., 2005), although, unlike the current work, the latter effect was linked to increased intracellular calcium (Ragozzino et al., 2006). Calcium oscillations in both neuronal and non-neuronal cells are important for cellular growth, migration and synaptic structural refinement (Katz and Shatz, 1996; Komuro and Rakic, 1998; Spitzer et al., 2000). Thus, the dampening of such signaling by $CX_3CL1$ is suggestive of a role in stability of network connectivity and activity. Overall, the current data along with information in the published literature reveal that a role for $CX_3CL1$ in control of glutamate-mediated excitatory neurotransmission during excitotoxic events can be extended to situations of synaptic plasticity required for normal functions such as memory formation.

An increasing number of chemokines exhibit extensive regulation across a range of situations where neuronal plasticity is involved; including memory-associated functional plasticity, protective plasticity in the setting of ischemia and maladaptive plasticity such as that underpinning neuropathic pain (Adler et al., 2006; Rostene et al., 2007; McAfoose and Baune, 2009; Old and Malcangio, 2012). Specifically, the role for $CX_3CL1$ in control of hippocampus and Raphe activity suggests that infection-, inflammation-, and/or chronic disease-associated increases in the chemokine could contribute to reduced hippocampal and Raphe output, alterations that could, in turn, precipitate depressed mood and heightened anxiety among other disorders of brain function (Meltzer, 1990; Bast, 2011; Small et al., 2011). The extensive expression of $CX_3CL1$ on neurons in the adult hippocampus and $CX_3CL1$ up-regulation post-spatial learning supports a direct role for $CX_3CL1$ in memory-associated synaptic plasticity. To better understand the role of $CX_3CL1$ up-regulation following learning it will be important to assess the consequences of blocking such chemokine signaling on learning and memory function.

## ACKNOWLEDGMENTS

This work was supported by a research cluster award from Science Foundation Ireland (03/IN3/B403C and 07/IN.1/B1322). Anita Wdowicz is part of the MolCellBiol Programme, funded under the Programme for Research in Third-Level Institutions and co-funded under the European Regional Development Fund.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2014.00233/abstract

**Figure S1 | Image analysis automation using EBImage software. (A)** 40× magnification of the apex of the hippocampal dentate gyrus. Green: $CX_3CL1$; Red: NeuN and Blue: Hoechst. Scale bar = 50 μm. **(B)** Hoechst channel alone. Stains cell nuclei. **(C)** NeuN channel alone. Labels neuronal cell bodies. **(D)** $CX_3CL1$ channel alone. Labels $CX_3CL1$ protein on dentate granule cells. **(E)** The blue fluorescence intensity was thresholded in order to select only Hoechst-stained nuclei (white). **(F)** Using a morphological kernel expansion algorithm, nuclei were dilated to select a cell soma region surrounding each nucleus. **(G)** A distance map was generated which calculates the distance each foreground (white) pixel is from the nearest background (black) pixel. **(H)** The watershed segmentation algorithm was then employed in order to separate all cell bodies from one another. The varying shades of gray in the image denote separated cells.

**Figure S2 | Effect of $CX_3CL1$ on the peak glutamate-induced calcium response in organotypic slice cultures and mixed hippocampal cell cultures. (A)** Shows the effect of $CX_3CL1$ on glutamate-induced calcium influx in the CA1 region of organotypic hippocampal slices cultured for 21 DIV. Pre-treatment of slice cultures with $CX_3CL1$ (500 pM) for 15 min prior to glutamate (30 μM) exposure had no effect on the peak $Ca^{2+}$ fluorescence intensity achieved by each cell (Mann–Whitney *U* test; $p < 0.001$). **(B)** Shows the effect of $CX_3CL1$ on glutamate-induced calcium influx in the CA1 region of organotypic hippocampal slices in the presence of picrotoxin. Pre-treatment of slice cultures with $CX_3CL1$ (500 pM) and picrotoxin (100 μM) versus picrotoxin alone (control) for 15 min prior to glutamate (30 μM) exposure enhanced the peak $Ca^{2+}$ fluorescence intensity achieved by each cell (Mann–Whitney *U* test; $p < 0.001$). **(C)** Shows the effect of $CX_3CL1$ (500 pM and 2 nM) on glutamate-induced calcium influx in neuronal cell types within mixed hippocampal cell cultures. Pre-treatment of primary hippocampal cell cultures with the lower dose of $CX_3CL1$ (500 pM) for 15 min prior to glutamate (30 μM) exposure had no effect on the peak $Ca^{2+}$ fluorescence intensity achieved by each neuronal cell (Kruskal–Wallis ANOVA and Dunn's multiple comparisons *post hoc* tests; $p < 0.001$). The higher dose of $CX_3CL1$ (2 nM), however, attenuated the peak $Ca^{2+}$ fluorescence intensity achieved by neurons in response to glutamate application. **(D)** Shows the effect of $CX_3CL1$ (500 pM and 2 nM) on glutamate-induced calcium influx in non-neuronal cell types within mixed hippocampal cell cultures. Pre-treatment of primary hippocampal cell cultures with the lower dose of $CX_3CL1$ (500 pM) for 15 min prior to glutamate (30 μM) exposure had no effect on the peak $Ca^{2+}$ fluorescence intensity achieved by each non-neuronal cell (Kruskal–Wallis ANOVA and Dunn's multiple comparisons *post hoc* tests; $p < 0.001$). The higher dose of $CX_3CL1$ (2 nM), however, attenuated the peak $Ca^{2+}$ fluorescence intensity achieved by each non-neuronal cell in response to glutamate application.

## REFERENCES


Adler, M. W., Geller, E. B., Chen, X., and Rogers, T. J. (2006). Viewing chemokines as a third major system of communication in the brain. *AAPS J.* 7, E865–E870. doi: 10.1208/aapsj070484

Adler, M. W., and Rogers, T. J. (2005). Are chemokines the third major system in the brain? *J. Leukoc. Biol.* 78, 1204–1209. doi: 10.1189/jlb.0405222

Allen, S. J., Crown, S. E., and Handel, T. M. (2007). Chemokine: receptor structure, interactions, and antagonism. *Annu. Rev. Immunol.* 25, 787–820. doi: 10.1146/annurev.immunol.24.021605.090529

Antonucci, F., Alpár, A., Kacza, J., Caleo, M., Verderio, C., Giani, A., et al. (2012). Cracking down on inhibition: selective removal of GABAergic interneurons from hippocampal networks. *J. Neurosci.* 32, 1989–2001. doi: 10.1523/JNEUROSCI.2720-11.2012

Araujo, D. M., and Cotman, C. W. (1993). Trophic effects of interleukin-4, -7, and -8 on hippocampal neuronal cultures: potential involvement of glial-derived factors. *Brain Res.* 600, 49–55. doi: 10.1016/0006-8993(93)90400-H

Arima-Yoshida, F., Watabe, A. M., and Manabe, T. (2011). The mechanisms of the strong inhibitory modulation of long-term potentiation in the rat dentate gyrus. *Eur. J. Neurosci.* 33, 1637–1646. doi: 10.1111/j.1460-9568.2011.07657.x

Bachstetter, A. D., Morganti, J. M., Jernberg, J., Schlunk, A., Mitchell, S. H., Brewster, K. W., et al. (2011). Fractalkine and $CX_3CR1$ regulate hippocampal neurogenesis in adult and aged rats. *Neurobiol. Aging* 32, 2030–2044. doi: 10.1016/j.neurobiolaging.2009.11.022

Baier, P. C., May, U., Scheller, J., Rose-John, S., and Schiffelholz, T. (2009). Impaired hippocampus-dependent and -independent learning in IL-6 deficient mice. *Behav. Brain Res.* 200, 192–196. doi: 10.1016/j.bbr.2009.01.013

Balschun, D., Wetzel, W., Del Rey, A., Pitossi, F., Schneider, H., Zuschratter, W., et al. (2004). Interleukin-6: a cytokine to forget. *FASEB J.* 18, 1788–1790. doi: 10.1096/fj.04-1625fje

Bast, T. (2011). The hippocampal learning-behavior translation and the functional significance of hippocampal dysfunction in schizophrenia. *Curr. Opin. Neurobiol.* 21, 492–501. doi: 10.1016/j.conb.2011.01.003

Ben Menachem-Zidon, O., Avital, A., Ben-Menahem, Y., Goshen, I., Kreisel, T., Shmueli, E. M., et al. (2011). Astrocytes support hippocampal-dependent memory and long-term potentiation via interleukin-1 signaling. *Brain Behav. Immun.* 25, 1008–1016. doi: 10.1016/j.bbi.2010.11.007

Bertollini, C., Ragozzino, D., Gross, C., Limatola, C., and Eusebi, F. (2006). Fractalkine/$CX_3CL1$ depresses central synaptic transmission in mouse hippocampal slices. *Neuropharmacology* 51, 816–821. doi: 10.1016/j.neuropharm.2006.05.027

Bliss, T. V. P., and Collingridge, G. L. (1993). A synaptic model of memory: long term potentiation and the hippocampus. *Nature* 361, 31–39. doi: 10.1038/361031a0

Briones, T. L., Woods, J., and Wadowska, M. (2014). Chronic neuroinflammation and cognitive impairment following transient global cerebral ischemia: role of fractalkine/$CX_3CR1$ signaling. *J. Neuroinflammation* 11:13. doi: 10.1186/1742-2094-11-13

Bruel-Jungerman, E., Davis, S., and Laroche, S. (2007a). Brain plasticity mechanisms and memory: a party of four. *Neuroscientist* 13, 492–505. doi: 10.1177/1073858407302725

Bruel-Jungerman, E., Rampon, C., and Laroche, S. (2007b). Adult hippocampal neurogenesis, synaptic plasticity and memory: facts and hypotheses. *Rev. Neurosci.* 18, 93–114. doi: 10.1515/REVNEURO.2007.18.2.93

Bruno, V., Copani, A., Besong, G., Scoto, G., and Nicoletti, F. (2000). Neuroprotective activity of chemokines against *N*-methyl-D-aspartate or beta-amyloid-induced toxicity in culture. *Eur. J. Pharmacol.* 399, 117–121. doi: 10.1016/S0014-2999(00)00367-8

Cambien, B., Pomeranz, M., Schmid-Antomarchi, H., Millet, M. A., Breittmayer, V., Rossi, B., et al. (2001). Signal transduction pathways involved in soluble fractalkine-induced monocytic cell adhesion. *Blood* 97, 2031–2037. doi: 10.1182/blood.V97.7.2031

Cardona, A. E., Pioro, E. P., Sasse, M. E., Kostenko, V., Cardona, S. M., Dijkstra, I. M., et al. (2006). Control of microglial neurotoxicity by the fractalkine receptor. *Nat. Neurosci.* 9, 917–924. doi: 10.1038/nn1715

Catalano, M., Lauro, C., Cipriani, R., Chece, G., Ponzetta, A., Di Angelantonio, S., et al. (2013). $CX_3CL1$ protects neurons against excitotoxicity enhancing GLT-1 activity on astrocytes. *J. Neuroimmunol.* 263, 75–82. doi: 10.1016/j.jneuroim.2013.07.020

Chapman, G. A., Moores, K., Harrison, D., Campbell, C. A., Stewart, B. R., and Strijbos, P. J. (2000). Fractalkine cleavage from neuronal membranes represents an acute event in the inflammatory response to excitotoxic brain damage. *J. Neurosci.* 20:RC87.

Cipriani, R., Villa, P., Chece, G., Lauro, C., Paladini, A., Micotti, E., et al. (2011). $CX_3CL1$ is neuroprotective in permanent focal cerebral ischemia in rodents. *J. Neurosci.* 31, 16327–16335. doi: 10.1523/JNEUROSCI.3611-11.2011

Clark, A. K., Yip, P. K., Grist, J., Gentry, C., Staniland, A. A., Marchand, F., et al. (2007). Inhibition of spinal microglial cathepsin S for the reversal of neuropathic pain. *Proc. Natl. Acad. Sci. U.S.A.* 104, 10655–10660. doi: 10.1073/pnas.0610811104

Coogan, A. N., O'Neill, L. A., and O'Connor, J. J. (1999). The P38 mitogen-activated protein kinase inhibitor SB203580 antagonizes the inhibitory effects of interleukin-1β on long-term potentiation in the rat dentate gyrus *in vitro*. *Neuroscience* 93, 57–69. doi: 10.1016/S0306-4522(99)00100-1

Cook, A., Hippensteel, R., Shimizu, S., Nicolai, J., Fatatis, A., and Meucci, O. (2010). Interactions between chemokines: regulation of fractalkine/$CX_3CL1$ homeostasis by SDF/CXCL12 in cortical neurons. *J. Biol. Chem.* 285, 10563–10571. doi: 10.1074/jbc.M109.035477

Deiva, K., Geeraerts, T., Salim, H., Leclerc, P., Héry, C., Hugel, B., et al. (2004). Fractalkine reduces *N*-methyl-D-aspartate-induced calcium flux and apoptosis

in human neurons through extracellular signal-regulated kinase activation. *Eur. J. Neurosci.* 20, 3222–3232. doi: 10.1111/j.1460-9568.2004.03800.x

del Rey, A., Balschun, D., Wetzel, W., Randolf, A., and Besedovsky, H. O. (2013). A cytokine network involving brain-borne IL-1β, IL-1ra, IL-18, IL-6, and TNFα operates during long-term potentiation and learning. *Brain Behav. Immun.* 33, 15–23. doi: 10.1016/j.bbi.2013.05.011

Erichsen, D., Lopez, A. L., Peng, H., Niemann, D., Williams, C., Bauer, M., et al. (2003). Neuronal injury regulates fractalkine: relevance for HIV-1 associated dementia. *J. Neuroimmunol.* 138, 144–155. doi: 10.1016/S0165-5728(03)00117-6

Garton, K. J., Gough, P. J., Blobel, C. P., Murphy, G., Greaves, D. R., Dempsey, P. J., et al. (2001). Tumor necrosis factor-α-converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine ($CX_3CL1$). *J. Biol. Chem.* 276, 37993–38001. doi: 10.1074/jbc.M106434200

Gemma, C., and Bachstetter, A. D. (2013). The role of microglia in adult hippocampal neurogenesis. *Front. Cell. Neurosci.* 7:229. doi: 10.3389/fncel.2013.00229

Harrison, J. K., Jiang, Y., Chen, S., Xia, Y., Maciejewski, D., McNamara, R. K., et al. (1998). Role for neuronally derived fractalkine in mediating interactions between neurons and $CX_3CR1$-expressing microglia. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10896–10901. doi: 10.1073/pnas.95.18.10896

Hatori, K., Nagai, A., Heisel, R., Ryu, J. K., and Kim, S. U. (2002). Fractalkine and fractalkine receptors in human neurons and glial cells. *J. Neurosci. Res.* 69, 418–426. doi: 10.1002/jnr.10304

Heinisch, S., and Kirby, L. G. (2009). Fractalkine/$CX_3CL1$ enhances GABA synaptic activity at serotonin neurons in the rat dorsal raphe nucleus. *Neuroscience* 164, 1210–1223. doi: 10.1016/j.neuroscience.2009.08.075

Hopkins, S. J., and Rothwell, N. J. (1995). Cytokines and the nervous system. I. Expression and recognition. *Trends Neurosci.* 18, 83–88. doi: 10.1016/0166-2236(95)80029-2

Hoshiko, M., Arnoux, I., Avignone, E., Yamamoto, N., and Audinat, E. (2012). Deficiency of the microglial receptor $CX_3CR1$ impairs postnatal functional development of thalamocortical synapses in the barrel cortex. *J. Neurosci.* 32, 15106–15111. doi: 10.1523/JNEUROSCI.1167-12.2012

Hughes, P. M., Botham, M. S., Frentzel, S., Mir, A., and Perry, V. H. (2002). Expression of fractalkine ($CX_3CL1$) and its receptor, $CX_3CR1$, during acute and chronic inflammation in the rodent CNS. *Glia* 37, 314–327. doi: 10.1002/glia.10037

Hundhausen, C., Misztela, D., Berkhout, T. A., Broadway, N., Saftig, P., Reiss, K., et al. (2003). The disintegrin-like metalloproteinase ADAM10 is involved in constitutive cleavage of $CX_3CL1$ (fractalkine) and regulates $CX_3CL1$-mediated cell-cell adhesion. *Blood* 102, 1186–1195. doi: 10.1182/blood-2002-12-3775

Hunsaker, M. R., and Kesner, R. P. (2008). Evaluating the differential roles of the dorsal dentate gyrus, dorsal CA3, and dorsal CA1 during a temporal ordering for spatial locations task. *Hippocampus* 18, 955–964. doi: 10.1002/hipo.20455

Jankowsky, J. L., Derrick, B. E., and Patterson, P. H. (2000). Cytokine responses to LTP induction in the rat hippocampus: a comparison of *in vitro* and *in vivo* techniques. *Learn. Mem.* 7, 400–412. doi: 10.1101/lm.32600

Jones, B. A., Riegsecker, S., Rahman, A., Beamer, M., Aboualaiwi, W., Khuder, S. A., et al. (2013). Role of ADAM-17, p38 MAPK, cathepsins, and the proteasome pathway in the synthesis and shedding of fractalkine/$CX_3CL1$ in rheumatoid arthritis. *Arthritis Rheum.* 65, 2814–2825. doi: 10.1002/art.38095

Jovanovic, J. N., Thomas, P., Kittler, J. T., Smart, T. G., and Moss, S. J. (2004). Brain-derived neurotrophic factor modulates fast synaptic inhibition by regulating $GABA_A$ receptor phosphorylation, activity, and cell-surface stability. *J. Neurosci.* 24, 522–530. doi: 10.1523/JNEUROSCI.3606-03.2004

Jung, S., Aliberti, J., Graemmel, P., Sunshine, M. J., Kreutzberg, G. W., Sher, A., et al. (2000). Analysis of fractalkine receptor $CX_3CR1$ function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol. Cell. Biol.* 20, 4106–4114. doi: 10.1128/MCB.20.11.4106-4114.2000

Katz, L. C., and Shatz, C. J. (1996). Synaptic activity and the construction of cortical circuits. *Science* 274, 1133–1138. doi: 10.1126/science.274.5290.1133

Klosowska, K., Volin, M. V., Huynh, N., Chong, K. K., Halloran, M. M., and Woods, J. M. (2009). Fractalkine functions as a chemoattractant for osteoarthritis synovial fibroblasts and stimulates phosphorylation of mitogen-activated protein kinases and Akt. *Clin. Exp. Immunol.* 156, 312–319. doi: 10.1111/j.1365-2249.2009.03903.x

Komuro, H., and Rakic, O. (1998). Orchestration of neuronal migration by activity of ion channels, neurotransmitter receptors, and intracellular $Ca^{2+}$ fluctuations. *J. Neurobiol.* 37, 110–130. doi: 10.1002/(SICI)1097-4695(199810)37:13.3.CO;2-Q

Krathwohl, M. D., and Kaiser, J. L. (2004). Chemokines promote quiescence and survival of human neural progenitor cells. *Stem Cells* 22, 109–118. doi: 10.1634/stemcells.22-1-109

Lauro, C., Cipriani, R., Catalano, M., Trettel, F., Chece, G., Brusadin, V., et al. (2010). Adenosine A1 receptors and microglial cells mediate $CX_3CL1$-induced protection of hippocampal neurons against Glu-induced death. *Neuropsychopharmacology* 35, 1550–1559. doi: 10.1038/npp.2010.26

Li, A. J., Katafuchi, T., Oda, S., Hori, T., and Oomura, Y. (1997). Interleukin-6 inhibits long-term potentiation in rat hippocampal slices. *Brain Res.* 748, 30–38. doi: 10.1016/S0006-308993(96)01283-8

Limatola, C., Ciotti, M. T., Mercanti, D., Vacca, F., Ragozzino, D., Giovannelli, A., et al. (2000). The chemokine growth-related gene product beta protects rat cerebellar granule cells from apoptotic cell death through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors. *Proc. Natl. Acad. Sci. U.S.A.* 97, 6197–6201. doi: 10.1073/pnas.090105997

Limatola, C., Lauro, C., Catalano, M., Ciotti, M. T., Bertollini, C., Di Angelantonio, S., et al. (2005). Chemokine $CX_3CL1$ protects rat hippocampal neurons against glutamate-mediated excitotoxicity. *J. Neuroimmunol.* 166, 19–28. doi: 10.1016/j.jneuroim.2005.03.023

Lu, M., Grove, E. A., and Miller, R. J. (2002). Abnormal development of the hippocampal dentate gyrus in mice lacking the CXCR4 chemokine receptor. *Proc. Natl. Acad. Sci. U.S.A.* 99, 7090–7095. doi: 10.1073/pnas.092013799

Lyons, A., Lynch, M. A, Downer, J. E., Hanley, R., O'Sullivan, B. J, Smith, A., et al. (2009). Fractalkine-induced activation of the phosphatidylinositol-3 kinase pathway attenuates microglial activation in vivo and in vitro. *J. Neurochem.* 110, 1547–1556. doi: 10.1111/j.1471-4159.2009.06253

Maciejewski-Lenoir, D., Chen, S., Feng, L., Maki, R., and Bacon, K. B. (1999). Characterization of fractalkine in rat brain cells: migratory and activation signals for $CX_3CR1$-expressing microglia. *J. Immunol.* 163, 1628–1635.

Maggi, L., Scianni, M., Branchi, I., D'Andrea, I., Lauro, C., and Limatola, C. (2011). $CX_3CR1$ deficiency alters hippocampal-dependent plasticity phenomena blunting the effects of enriched environment. *Front. Cell. Neurosci.* 5:1–10. doi: 10.3389/fncel.2011.00022

Maggi, L., Trettel, F., Scianni, M., Bertollini, C., Eusebi, F., Fredholm, B. B., et al. (2009). LTP impairment by fractalkine/$CX_3CL1$ in mouse hippocampus is mediated through the activity of adenosine receptor type 3 (A3R). *J. Neuroimmunol.* 215, 36–42. doi: 10.1016/j.jneuroim.2009.07.016

Malenka, R. C., and Bear, M. F. (2004). LTP and LTD: an embarrassment of riches. *Neuron* 44, 5–21. doi: 10.1016/j.neuron.2004.09.012

Mattison, H. A., Nie, H., Gao, H., Zhou, H., Hong, J. S., and Zhang, J. (2013). Suppressed pro-inflammatory response of microglia in $CX_3CR1$ knockout mice. *J. Neuroimmunol.* 257, 110–115. doi: 10.1016/j.jneuroim.2013.02.008

McAfoose, J., and Baune, B. T. (2009). Evidence for a cytokine model of cognitive function. *Neurosci. Biobehav. Rev.* 33, 355–366. doi: 10.1016/j.neubiorev.2008.10.005

Meltzer, H. Y. (1990). Role of serotonin in depression. *Ann. N. Y. Acad. Sci.* 600, 486–499. doi: 10.1111/j.1749-6632.1990.tb16904.x

Meucci, O., Fatatis, A., Simen, A. A., Bushell, T. J., Gray, P. W., and Miller, R. J. (1998). Chemokines regulate hippocampal neuronal signaling and gp120 neurotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* 95, 14500–14505. doi: 10.1073/pnas.95.24.14500

Meucci, O., Fatatis, A., Simen, A. A., and Miller, R. J. (2000). Expression of $CX_3CR1$ chemokine receptors on neurons and their role in neuronal survival. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8075–8080. doi: 10.1073/pnas.090017497

Mizoguchi, Y., Kanematsu, T., Hirata, M., and Nabekura, J. (2003). A rapid increase in the total number of cell surface functional $GABA_A$ receptors induced by brain-derived neurotrophic factor in rat visual cortex. *J. Biol. Chem.* 278, 44097–44102. doi: 10.1074/jbc.M305872200

Nusser, Z., Hajos, N., Somogyi, P., and Mody, I. (1998). Increased number of synaptic $GABA_A$ receptors underlies potentiation at hippocampal inhibitory synapses. *Nature* 395, 172–177. doi: 10.1038/25999

Oh, S. B., Endoh, T., Simen, A. A., Ren, D., and Miller, R. J. (2002). Regulation of calcium currents by chemokines and their receptors. *J. Neuroimmunol.* 123, 66–75. doi: 10.1016/S0165-5728(01)00485-4

Old, E. A., and Malcangio, M. (2012). Chemokine mediated neuron-glia communication and aberrant signalling in neuropathic pain states. *Curr. Opin. Pharmacol.* 12, 67–73. doi: 10.1016/j.coph.2011.10.015

Pabon, M. M., Bachstetter, A. D., Hudson, C. E., Gemma, C., and Bickford, P. C. (2011). $CX_3CL1$ reduces neurotoxicity and microglial activation in a rat model of Parkinson's disease. *J. Neuroinflammation* 8:9. doi: 10.1186/1742-2094-8-9

Pau, G., Fuchs, F., Sklyar, O., Boutros, M., and Huber, W. (2010). EBImage – an R package for image processing with applications to cellular phenotypes. *Bioinformatics* 26, 979–981. doi: 10.1093/bioinformatics/btq046

Paxinos, G., and Watson, C. (2005) *The Rat Brain in Stereotaxic Coordinates*, 5th Edn. San Diego, CA: Elsevier Academic Press.

Piccinin, S., Di Angelantonio, S., Piccioni, A., Volpini, R., Cristalli, G., Fredholm, B. B., et al. (2010). $CX_3CL1$-induced modulation at CA1 synapses reveals multiple mechanisms of EPSC modulation involving adenosine receptor subtypes. *J. Neuroimmunol.* 27, 85–92. doi: 10.1016/j.jneuroim.2010.05.012

Pickering, M., Pickering, B. W., Murphy, K. J., and O'Connor, J. J. (2008). Discrimination of cell types in mixed cortical culture using calcium imaging: a comparison to immunocytochemical labeling. *J. Neurosci. Methods* 173, 27–33. doi: 10.1016/j.jneumeth.2008.05.014

Pollmächer, T., Haack, M., Schuld, A., Reichenberg, A., and Yirmiya, R. (2002). Low levels of circulating inflammatory cytokines – do they affect human brain functions? *Brain Behav. Immun.* 16, 525–532. doi: 10.1016/S0889-1591(02)00004-1

Ragozzino, D., di Angelantonio, S., Trettel, F., Bertollini, C., Maggi, L., Gross, C., et al. (2006). Chemokine fractalkine/$CX_3CL1$ negatively modulates active glutamatergic synapses in rat hippocampal neurons. *J. Neurosci.* 26, 10488–10498. doi: 10.1523/JNEUROSCI.3192-06.2006

Reichenberg, A., Yirmiya, R., Schuld, A., Kraus, T., Haack, M., Morag, A., et al. (2001). Cytokine-associated emotional and cognitive disturbances in humans. *Arch. Gen. Psychiatry* 58, 445–452. doi: 10.1001/archpsyc.58.5.445

Robinson, S., Tani, M., Strieter, R. M., Ransohoff, R. M., and Miller, R. H. (1998). The chemokine growth-regulated oncogene-alpha promotes spinal cord oligodendrocyte precursor proliferation. *J. Neurosci.* 18, 10457–10463.

Rogers, J. T., Morganti, J. M., Bachstetter, A. D., Hudson, C. E., Peters, M. M., Grimmig, B. A., et al. (2011). $CX_3CR1$ deficiency leads to impairment of hippocampal cognitive function and synaptic plasticity. *J. Neurosci.* 31, 16241–16250. doi: 10.1523/JNEUROSCI.3667-11.2011

Rolls, E. T. (2010). A computational theory of episodic memory formation in the hippocampus. *Behav. Brain Res.* 215, 180–196. doi: 10.1016/j.bbr.2010.03.027

Roseti, C., Fucile, S., Lauro, C., Martinello, K., Bertollini, C., Esposito, V., et al. (2013). Fractalkine/$CX_3CL1$ modulates $GABA_A$ currents in human temporal lobe epilepsy. *Epilepsia* 54, 1834–1844. doi: 10.1111/epi.12354

Ross, F. M., Allan, S. M., Rothwell, N. J., and Verkhratsky, A. (2003). A dual role for interleukin-1 in LTP in mouse hippocampal slices. *J. Neuroimmunol.* 144, 61–67. doi: 10.1016/j.jneuroim.2003.08.030

Rostene, W., Kitabgi, P., and Parsadaniantz, S. M. (2007). Chemokines: a new class of neuromodulator? *Nat. Rev. Neurosci.* 8, 895–903. doi: 10.1038/nrn2255

Rothwell, N. J., and Hopkins, S. J. (1995). Cytokines and the nervous system II: actions and mechanisms of action. *Trends Neurosci.* 8, 130–136. doi: 10.1016/0166-2236(95)93890-A

Ruchaya, P. J., Antunes, V. R., Paton, J. F. R., Murphy, D., and Yao, S. T. (2014). The cardiovascular actions of fractalkine/$CX_3CL1$ in the hypothalamic paraventricular nucleus are attenuated in rats with heart failure. *Exp. Physiol.* 99, 111–122. doi: 10.1113/expphysiol.2013.075432

Ruchaya, P. J., Paton, J. F., Murphy, D., and Yao, S. T. (2012). A cardiovascular role for fractalkine and its cognate receptor, $CX_3CR1$, in the rat nucleus of the solitary tract. *Neuroscience* 209, 119–127. doi: 10.1016/j.neuroscience.2012.02.018

Schneider, H., Pitossi, F., Balschun, D., Wagner, A., del Rey, A., and Besedovsky, H. O. (1998). A neuromodulatory role of interleukin-1beta in the hippocampus. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7778–7783. doi: 10.1073/pnas.95.13.7778

Scianni, M., Antonilli, L., Chece, G., Cristalli, G., Di Castro, M. A., Limatola, C., et al. (2013). Fractalkine ($CX_3CL1$) enhances hippocampal *N*-methyl-D-aspartate receptor (NMDAR) function via D-serine and adenosine receptor type A2 (A2AR) activity. *J. Neuroinflammation* 10, 108. doi: 10.1186/1742-2094-10–108

Shepherd, A. J., Loo, L., and Mohapatra, D. P. (2013). Chemokine co-receptor CCR5/CXCR4-dependent modulation of Kv2.1 channel confers acute neuroprotection to HIV-1 glycoprotein gp120 exposure. *PLoS ONE* 8:e76698. doi: 10.1371/journal.pone.0076698

Sheridan, G. K., and Murphy, K. J. (2013). Neuron-glia crosstalk in health and disease: fractalkine and $CX_3CR1$ take centre stage. *Open Biol.* 3:130181. doi: 10.1098/rsob.130181

Small, S. A., Schobel, S. A., Buxton, R. B., Witter, M. P., and Barnes, C. A. (2011). A pathophysiological framework of hippocampal dysfunction in ageing and disease. *Nat. Rev. Neurosci.* 12, 585–601. doi: 10.1038/nrn3085

Spitzer, N. C., Lautermilch, N. J., Smith, R. D., and Gomez, T. M. (2000). Coding of neuronal differentiation by calcium transients. *Bioessays* 22, 811–817. doi: 10.1002/1521-1878(200009)22:9<811::AID-BIES6>3.0.CO;2-G

Stoppini, L., Buchs, P. A., and Muller, D. (1991). A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Methods* 37, 173–182. doi: 10.1016/0165-0270(91)90128-M

Tarozzo, G., Bortolazzi, S., Crochemore, C., Chen, S. C., Lira, A. S., Abrams, J. S., et al. (2003). Fractalkine protein localization and gene expression in mouse brain. *J. Neurosci. Res.* 73, 81–88. doi: 10.1002/jnr.10645

Tarozzo, G., Campanella, M., Ghiani, M., Bulfone, A., and Beltramo, M. (2002). Expression of fractalkine and its receptor, $CX_3CR1$, in response to ischaemia-reperfusion brain injury in the rat. *Eur. J. Neurosci.* 15, 1663–1668. doi: 10.1046/j.1460-9568.2002.02007.x

Tong, N., Perry, S. W., Zhang, Q., James, H. J., Guo, H., Brooks, A., et al. (2000). Neuronal fractalkine expression in HIV-1 encephalitis: roles for macrophage recruitment and neuroprotection in the central nervous system. *J. Immunol.* 164, 1333–1339. doi: 10.4049/jimmunol.164.3.1333

Tran, P. B., and Miller, R. J. (2003). Chemokine receptors: signposts to brain development and disease. *Nat. Rev. Neurosci.* 4, 444–455. doi: 10.1038/nrn1116

Tsou, C. L., Haskell, C. A., and Charo, I. F. (2001). Tumor necrosis factor-alpha-converting enzyme mediates the inducible cleavage of fractalkine. *J. Biol. Chem.* 276, 44622–44626. doi: 10.1074/jbc.M107327200

Turrigiano, G. (2007). Homeostatic signaling: the positive side of negative feedback. *Curr. Opin. Neurobiol.* 17, 318–324. doi: 10.1016/j.conb.2007.04.004

Turrigiano, G. G. (2008). The self-tuning neuron: synaptic scaling of excitatory synapses. *Cell* 135, 422–435. doi: 10.1016/j.cell.2008.10.008

Verge, G. M., Milligan, E. D., Maier, S. F., Watkins, L. R., Naeve, G. S., and Foster, A. C. (2004). Fractalkine ($CX_3CL1$) and fractalkine receptor ($CX_3CR1$) distribution in spinal cord and dorsal root ganglia under basal and neuropathic pain conditions. *Eur. J. Neurosci.* 20, 1150–1160. doi: 10.1111/j.1460-9568.2004.03593.x

Wan, Q., Xiong, Z. G., Man, H. Y., Ackerley, C. A., Braunton, J., Lu, W. Y., et al. (1997). Recruitment of functional $GABA_A$ receptors to postsynaptic domains by insulin. *Nature* 388, 686–690. doi: 10.1038/41792

Wang, Q., Liu, L., Pei, L., Ju, W., Ahmadian, G., Lu, J., et al. (2003). Control of synaptic strength, a novel function of Akt. *Neuron* 38, 915–928. doi: 10.1016/S0896-6273(03)00356-8

White, G. E., and Greaves, D. R. (2012). Fractalkine: a survivor's guide: chemokines as anti-apoptotic mediators. *Arterioscler. Thromb. Vasc. Biol.* 32, 589–594. doi: 10.1161/ATVBAHA.111.237412

Williamson, L. L., and Bilbo, S. D. (2013). Chemokines and the hippocampus: a new perspective on hippocampal plasticity and vulnerability. *Brain Behav. Immun.* 30, 186–194. doi: 10.1016/j.bbi.2013.01.077

Wilson, C. J., Finch, C. E., and Cohen, H. J. (2002). Cytokines and cognition – the case for a head-to-toe inflammatory paradigm. *J. Am. Geriatr. Soc.* 50, 2041–2056. doi: 10.1046/j.1532-5415.2002.50619.x

Wu, J., Bie, B., Yang, H., Xu, J. J., Brown, D. L., and Naguibemail, M. (2013). Suppression of central chemokine fractalkine receptor signaling alleviates amyloid-induced memory deficiency. *Neurobiol. Aging* 34, 2843–2852. doi: 10.1016/j.neurobiolaging.2013.06.003

Yeo, S. I., Kim, J. E., Ryu, H. J., Seo, C. H., Lee, B. C., Choi, I. G., et al. (2011). The roles of fractalkine/$CX_3CR1$ system in neuronal death following pilocarpine-induced status epilepticus. *J. Neuroimmunol.* 234, 93–102. doi: 10.1016/j.jneuroim.2011.03.005

Yirmiya, R., and Goshen, I. (2011). Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain Behav. Immun.* 25, 181–213. doi: 10.1016/j.bbi.2010.10.015

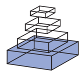
Zhuang, Z. Y., Kawasaki, Y., Tan, P. H., Wen, Y. R., Huang, J., and Ji, R. R. (2007). Role of the $CX_3CR1$/p38 MAPK pathway in spinal microglia for the development of neuropathic pain following nerve injury-induced cleavage of fractalkine. *Brain Behav. Immun.* 21, 642–651. doi: 10.1016/j.bbi.2006.11.003

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 11 April 2014; accepted: 25 July 2014; published online: 12 August 2014.*
*Citation: Sheridan GK, Wdowicz A, Pickering M, Watters O, Halley P, O'Sullivan NC, Mooney C, O'Connell DJ, O'Connor JJ and Murphy KJ (2014) $CX_3CL1$ is up-regulated in the rat hippocampus during memory-associated synaptic plasticity. Front. Cell. Neurosci.* ***8****:233. doi: 10.3389/fncel.2014.00233*
*This article was submitted to the journal Frontiers in Cellular Neuroscience.*
*Copyright © 2014 Sheridan, Wdowicz, Pickering, Watters, Halley, O'Sullivan, Mooney, O'Connell, O'Connor and Murphy. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 20 November 2014
doi: 10.3389/fncel.2014.00391



# IFN-γ differentially modulates memory-related processes under basal and chronic stressor conditions

**Darcy Litteljohn[1]*, Eric Nelson[2] and Shawn Hayley[1]***

[1] Department of Neuroscience, Carleton University, Ottawa, ON, Canada
[2] Faculty of Medicine, University of Toronto, Toronto, ON, Canada

**Edited by:**
Laura Maggi, Sapienza-Università di Roma, Italy

**Reviewed by:**
Maria Vittoria Podda, Università Cattolica del Sacro Cuore, Italy
Agnieszka Basta-Kaim, Institute of Pharmacology – Polish Academy of Sciences, Poland

***Correspondence:**
Darcy Litteljohn and Shawn Hayley, Department of Neuroscience, Carleton University, 1125 Colonel By Drive, Ottawa, ON K1S 5B6, Canada
e-mail: dlitteljohn@gmail.com; Shawn.Hayley@carleton.ca

Cytokines are inflammatory messengers that orchestrate the brain's response to immunological challenges, as well as possibly even toxic and psychological insults. We previously reported that genetic ablation of the pro-inflammatory cytokine, interferon-gamma (IFN-γ), attenuated some of the corticosteroid, cytokine, and limbic dopaminergic variations induced by 6 weeks of exposure to an unpredictable psychologically relevant stressor. Presently, we sought to determine whether a lack of IFN-γ would likewise modify the impact of chronic stress on hippocampus-dependent memory function and related neurotransmitter and neurotrophin signaling systems. As predicted, chronic stress impaired spatial recognition memory (Y-maze task) in the wild-type animals. In contrast, though the IFN-γ knockouts (KOs) showed memory disturbances in the basal state, under conditions of chronic stress these mice actually exhibited facilitated memory performance. Paralleling these findings, while overall the KOs displayed altered noradrenergic and/or serotonergic activity in the hippocampus and locus coeruleus, norepinephrine utilization in both of these memory-related brain regions was selectively increased among the chronically stressed KOs. However, contrary to our expectations, neither IFN-γ deletion nor chronic stressor exposure significantly affected nucleus accumbens dopaminergic neurotransmission or hippocampal brain-derived neurotrophic factor protein expression. These findings add to a growing body of evidence implicating cytokines in the often differential regulation of neurobehavioral processes in health and disease. Whereas in the basal state IFN-γ appears to be involved in sustaining memory function and the activity of related brain monoamine systems, in the face of ongoing psychologically relevant stress the cytokine may, in fact, act to restrict potentially adaptive central noradrenergic and spatial memory responses.

**Keywords: depression, memory, hippocampus, cytokine, knockout mouse, monoamine, BDNF**

## INTRODUCTION

It is widely accepted that the likelihood of developing depression, anxiety, and other psychological disorders is greatly influenced by exposure to stressors, particularly those of a chronic, unpredictable, and/or psychosocial nature (Hill et al., 2012). The prevailing view over many years has been that stressor-induced alterations of brain monoamine activity were largely responsible for the emotional and cognitive symptoms seen to predominate in these conditions (Schildkraut, 1965). While evidence continues to implicate monoaminergic neurotransmitter processes (Fava, 2003; Hamon and Blier, 2013), deficits in trophic growth factors such as brain-derived neurotrophic factor (BDNF), and even structural brain changes (e.g., impaired neurogenesis) have emerged as important players too in this regard (Pittenger and Duman, 2008; Calabrese et al., 2009; Mahar et al., 2014).

It's become increasingly clear that cytokines and other elements of the inflammatory immune system contribute importantly to depression and other stress-related psychological disturbances (Miller et al., 2009; Anisman and Hayley, 2012). For instance, numerous studies have reported that pro-inflammatory cytokines, most notably interleukin-1-beta (IL-1β), IL-6, interferon-alpha (IFN-α), and tumor necrosis factor-alpha (TNF-α), are altered in major depression and stressor-based animal models (Dowlati et al., 2010; Liu et al., 2012; Dahl et al., 2014). Moreover, administration of these cytokines to rodents induced behavioral, hormonal, monoamine, and neuroplastic changes that are reminiscent of at least some depressive-like clinical changes (Myint et al., 2007; Anisman et al., 2008; Kaster et al., 2012; Sukoff Rizzo et al., 2012). The fact that anti-inflammatory and anti-cytokine treatments (e.g., minocycline, curcumin, cytokine-specific antagonists) were reported to lessen the neural and behavioral impact of stressor exposure further supports a link between cytokines and stressor pathology (Koo and Duman, 2008; Hinwood et al., 2012; Jiang et al., 2013; Krügel et al., 2013).

Interferon-gamma (IFN-γ), which is a crucial mediator of both innate and adaptive immune responses, is another cytokine that has recently been posited to play a role in stressor-related psychological pathology. Several studies have reported elevated circulating levels of IFN-γ among depressed patients (Simon et al., 2008; Gabbay et al., 2009; Dahl et al., 2014; Schmidt et al., 2014), and many of the most commonly used antidepressants were found to antagonize IFN-γ signaling (Maes

et al., 1999; Kubera et al., 2001; Brustolim et al., 2006). Moreover, variation in the IFN-γ gene was recently reported to modify both depression risk (in the context of IFN-α treatment; Oxenkrug et al., 2011) and antidepressant medication effectiveness (Myint et al., 2013). Consistent with these findings, Kwant and Sakic (2004) reported that mice infected with IFN-γ adenovector displayed persistent anhedonic-like symptoms, and O'Connor et al. (2009a,b) showed that IFN-γ is a major driver of the indoleamine 2,3-dioxygenase (IDO)-enhancing and depressive-like behavioral effects of the immune-activating agents, lipopolysaccharide (LPS) and Bacillus Calmette–Guerin. Yet, compared with many of the other cytokines that have been linked to depressive illness, far fewer studies have actually set out to specifically test the influence of endogenous IFN-γ in ecologically inspired chronic stressor animal models; this is especially true in regards to the cognitive aspects of depressive-like pathology.

As one of the most potent activators of microglial cells and key regulator of the anti-viral response (Chesler and Reiss, 2002), IFN-γ is likely to be especially important for conditions in which infection overlaps with stressor exposure or in genetically vulnerable individuals (Litteljohn et al., 2010). In this regard, we previously found that IFN-γ-deficient mice had attenuated hormonal, cytokine and brain regional dopaminergic responses to chronic stress, despite showing several conspicuous behavioral and physiological differences in the basal state (i.e., increased anxiety-like behavior, elevated circulating corticosterone levels and central amygdala monoamine utilization; Litteljohn et al., 2010). This complex pattern of effects led us to theorize that IFN-γ contributes to a range of affective and perhaps cognitive processes, albeit probably in very different ways and to markedly different ends under basal and chronic stress conditions. Working under this theoretical framework, in the present investigation we sought to assess the largely unexplored role of IFN-γ in the spatial memory, psychomotor, and hippocampal BDNF and monoamine changes that are often evident following protracted exposure to psychologically relevant stressors.

## MATERIALS AND METHODS

### EXPERIMENTAL ANIMALS

Establishment of the IFN-γ –/– knockout (KO) mouse, which develops normally and is healthy in the absence of pathogenic challenge, has been described previously (Dalton et al., 1993). IFN-γ KO and wild-type (WT; IFN-γ +/+) mice raised on a C57BL/6J genetic background were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and interbred for several generations. At 6–7 weeks of age, KO and WT littermate controls from our breeding colony were singly housed in standard polycarbonate cages (27 cm × 21 cm × 14 cm). Animals were maintained on a 24 h light/dark cycle with lights on at 08:00. A diet of Ralston Purina (St. Louis, MO, USA) mouse chow and water was provided *ad libitum*, and room temperature was maintained at ~21°C. All experimental procedures were approved by the Carleton University Committee for Animal Care and complied with the guidelines set out by the Canadian Council for the Use and Care of Animals in Research. The animals were between 10 and 12 weeks of age upon commencement of the study.

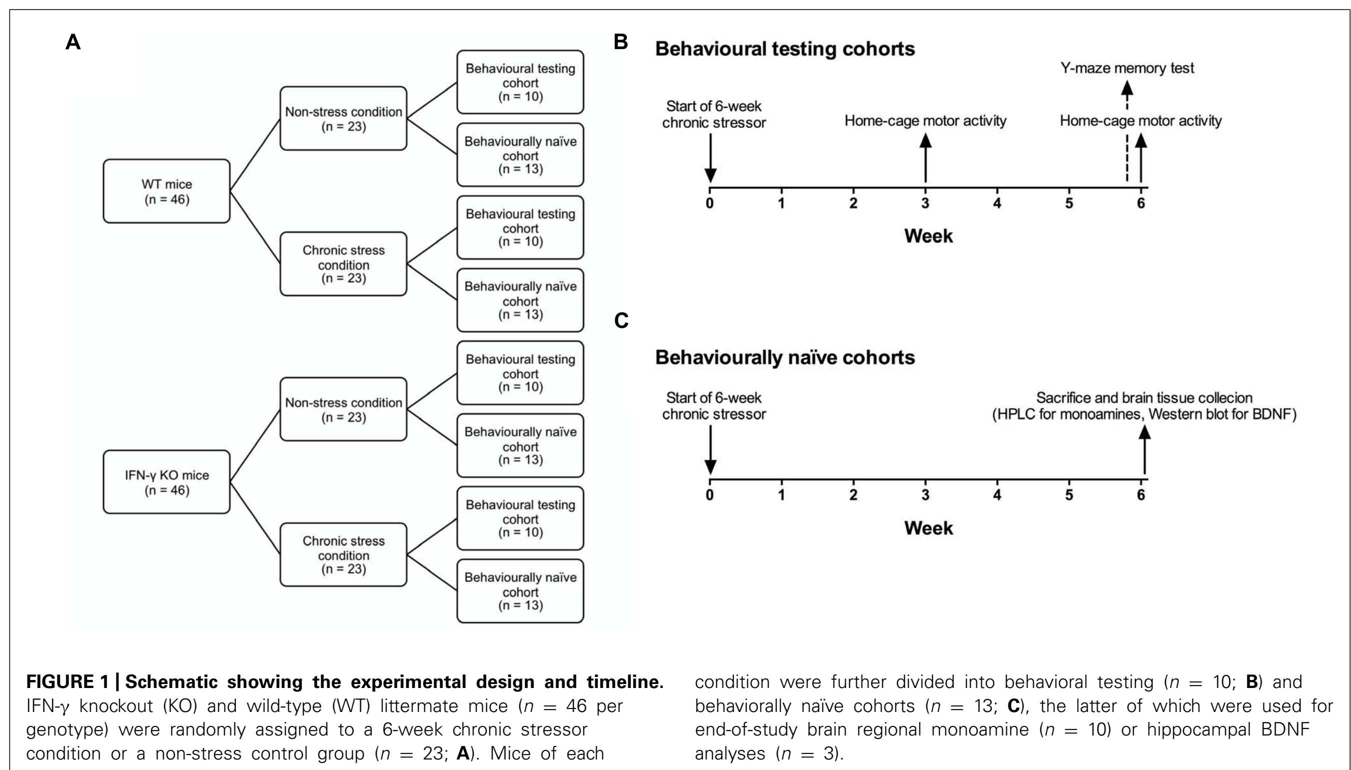



**FIGURE 1 | Schematic showing the experimental design and timeline.** IFN-γ knockout (KO) and wild-type (WT) littermate mice ($n$ = 46 per genotype) were randomly assigned to a 6-week chronic stressor condition or a non-stress control group ($n$ = 23; **A**). Mice of each condition were further divided into behavioral testing ($n$ = 10; **B**) and behaviorally naïve cohorts ($n$ = 13; **C**), the latter of which were used for end-of-study brain regional monoamine ($n$ = 10) or hippocampal BDNF analyses ($n$ = 3).

## CHRONIC STRESSOR REGIMEN

**Figure 1** presents a schematic of the experimental design and timeline. Animals of either genotype ($n = 46$) were randomly assigned to a 6-week chronic stressor condition or a non-stress control group ($n = 23$). Mice of each condition were further divided into behavioral testing ($n = 10$) and behaviorally naïve cohorts ($n = 13$), the latter of which were used for end-of-study brain regional monoamine ($n = 10$) or hippocampal BDNF analyses ($n = 3$). The chronic stressor regimen comprised the application of two stressors per day (or a single stressor on behavioral testing days) on a variable and unpredictable schedule, and consisted of both mild and moderate stressors per the method and rationale of Litteljohn et al. (2010). A list of the various stressors used is provided in **Table 1**. Following the first (morning) stressor, mice were returned to their home-cage until application of the second (afternoon) stressor. Animals assigned to the chronic stressor condition were housed in a holding room separate from, but otherwise identical to, their non-stressed counterparts.

## BEHAVIORAL ASSESSMENTS

### *Spontaneous home-cage activity*

Measurements of horizontal motor activity were obtained during complete, uninterrupted 12 h light/dark cycles using a Micromax infrared beam-break apparatus (AccuScan Instruments, Columbus, OH, USA) exterior to the home-cage. The same animals were tested on two separate occasions, corresponding to the midway point (Week 3: Day 21) and endpoint (Week 6: Day 42) of the stressor paradigm, and the data analyzed using a repeated measures analysis of variance (ANOVA). Testing commenced 60 min after termination of the morning stressor.

### *Spatial memory in the two-trial Y-maze*

During the final week of the experiment (Day 40), intermediate-term spatial recognition memory was assessed in a two-trial Y-maze task, in accordance with previously published methods (Dellu et al., 2000; Ferguson et al., 2000). The testing apparatus comprised three arms (30 cm × 8 cm × 15 cm) fashioned from black Plexiglas with an outer wood shell. The testing room was dimly lit and had on its walls various cardboard cut-outs of basic geometric shapes. During the first trial (acquisition phase) one arm of the maze was blocked with an opaque, removable panel. Mice were then placed individually in one of the two remaining accessible arms (i.e., the 'start' arm, which remained so in the second trial), with head directed away from the center of the maze. Animals were allowed to explore the open arms of the Y-maze for 5 min, after which they were returned to the home-cage. After 30 min, the second trial (retrieval phase) was conducted under identical experimental conditions to the first, excepting that the mice were now permitted free exploration for 5 min of all three arms of the maze (start, familiar and novel). The blocked arm (i.e., novel arm in the retrieval phase) varied between mice in a predetermined, pseudo-random manner, and the maze was cleaned with a dilute (2%) ethanol solution after each trial. Total arm entries (for each trial, defined as all four legs having entered a given arm) and % duration in the novel, start and familiar arms (Trial 2) were determined for each mouse. A discrimination index (DI) for novelty was calculated as follows: novel arm duration − (start arm duration + familiar arm duration)/2. A DI value not significantly different than zero (0) is understood to reflect a deficit in novelty discrimination (e.g., see Leconte et al., 2011). Y-maze testing occurred between 09:00 and 13:00

**Table 1 | List of stressors.**

| Stressor | Duration | Specifications |
|---|---|---|
| Social interaction | 60 min | Placement in a large cage (40 cm × 25 cm × 15 cm) divided into separate quadrants with three non-experimental male C57BL/6J mice (3–6 months old); this set-up allowed for interactions but not fighting |
| Soiled cage | 60 min | Introduction into a congener's soiled cage |
| Fox urine | 5 min | Exposure to 250 cc fox urine-infested air (Foxpert, St. Benjamin, QC, Canada) while in a novel, empty cage |
| Rat feces | 60 min | Introduction into an unfamiliar cage with fresh rat feces |
| Flat bottom restraint | 15 min | Restraint in semicircular Plexiglas tubes (4 cm × 12 cm) with tails taped to prevent mice from turning |
| Plastic bag restraint | 15 min | Restraint in tight-fitting triangular plastic bags equipped with a nose-hole for breathing |
| Footshock | — | 15 shocks, 500 ms duration at 30 s intervals, 0.3 mA, 60 Hz, a.c.) administered in individual shock chambers (30 cm × 14 cm × 15 cm) |
| Injection/handling | — | Intraperitoneal injection of 0.2 ml sterile physiological saline (Sigma Aldrich, USA) |
| Damp bedding | 60 min | 60 ml of water/l of sawdust bedding in novel cage |
| Tail hang | 30 s | — |
| Empty cage | 60 min | Introduction into an empty cage without sawdust or nestlet |
| Noise | 10 min | Intermittent background noise (40 dB) in isolated restraint chambers (30 cm × 14 cm × 15 cm) |
| Cage tilt | 60 min | 30° tilt of home-cage |
| Forced swim | 3 min | Forced swim in a glass cylinder (20 cm diameter × 25 cm high) containing tepid water (22 ± 1°C, 15 cm deep) |
| Light/dark cycle disruption | 12 h | Lights on during dark phase |

(i.e., 17–21 h after the previous day's afternoon stressor), after which mice in the stressor groups received a single afternoon stressor.

### BRAIN DISSECTION METHOD

Animals were rapidly decapitated between 09:00 and 11:00 on the day following the completion of the 6-week chronic stressor regimen (i.e., 17–19 h after the final stressor treatment, which for all animals was a 15-min flat-bottom restraint). Brains were excised and sectioned into sequential coronal slices using razor blades and a chilled stainless steel microdissecting block with adjacent slots arranged 0.5 mm apart. The locus coeruleus and nucleus accumbens were obtained by micropunch using a hollow 1.0 mm diameter biopsy needle (collected bilaterally), whereas the dorsal hippocampus was microdissected in its entirety using chilled razor blades. Brain tissue samples were taken with reference to the mouse brain atlas of Franklin and Paxinos (1997). A subset of the hippocampal samples was flash-frozen and stored at −80°C for later determination of BDNF content by Western blot ($n = 3$). The remaining samples were maintained in a homogenizing solution containing 14.17 g monochloroacetic acid, 0.0186 g EDTA, 5.0 ml methanol, and 500 ml high performance liquid chromatography (HPLC) grade water; and stored at −80°C for HPLC analysis.

### HPLC DETERMINATION OF CENTRAL AMINE AND METABOLITE CONCENTRATIONS

Levels of norepinephrine (NE), serotonin (5-HT) and dopamine (DA), and their respective primary metabolites, 3-methoxy-4-hydroxyphenylglycol (MHPG), 5-hydroxyindole acetic acid (5-HIAA), and 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were determined by HPLC within relevant brain punches according to previously reported methods (Liu et al., 2014). Tissue punches were homogenized by ultrasonic disruption (Sonic Dismembrator Model 100, Fisher Scientific) in the homogenizing solution in which they were initially frozen (with DHBA as an internal standard). The level of protein was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific 23225). Homogenized samples were centrifuged (12000 rpm for 3 min at 4°C), after which 50 μl of supernatant was injected, at a flow rate of 1 ml/min, into the automated HPLC system (Agilent 1100) with electrochemical detector (DECADE II SDC, Antec) and ZORBAX Eclipse XDB-C8 columns (Agilent: 4.6 mm inner diameter, 150 mm length, 5 μm particle size; thermostated at 40°C); the oxidation potential was maintained at 0.60 V. The mobile phase comprised: 90 mM sodium phosphate monobasic, 1.7 mM 1-octanesulfonic acid, 50 mM EDTA, 10% acetonitrile, 50 mM citric acid (monohydrate), 5 mM KCL, and HPLC-grade water. Monoamine and metabolite concentrations were expressed relative to the protein content of the samples, and final results presented as ng/mg protein.

### HIPPOCAMPAL BDNF PROTEIN DETERMINATION

Western immunoblotting was performed largely in accordance with our previously published methods (Mangano et al., 2011). Briefly, brain tissues ($n = 3$) were homogenized on ice in RIPA lysis buffer containing 50 mM Tris-base (pH 8.0), 150 mM NaCl, 1% Triton-X, 0.1% SDS, 0.5% sodium deoxycholate, and cOmplete Mini EDTA-free protease inhibitor (Roche, Basel, Switzerland). Lysates were centrifuged for 5 min (5000 rpm at 4°C) and supernatants collected. Total protein was then determined using a BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein from the hippocampus (50 μg/well) was diluted to a final volume of 35 μl in RIPA lysis buffer and 1X loading buffer (5% glycerol, 5% β-mercaptoethanol, 3% SDS, and 0.05% bromophenol blue), and samples heated in boiling water for 5 min. Proteins were separated by electrophoresis (120 V) on 12.5% sodium dodecyl sulfate-polyacrylamide gels and transferred overnight at 4°C (180 mA) onto PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were then blocked for 1-h with gentle agitation at room temperature in a Tris-buffered saline [TBS-T: 10 mM Tris-base (pH 8.0), 150 mM NaCl, 0.5% Tween-20] solution containing non-fat dry milk (5% w/v). Anti-BDNF primary antibody (1:500, sc-546, Santa Cruz Biotechnology, Dallas, TX, USA) was applied for 1.5-h at room temperature. After four successive 10-min washes in TBS-T, membranes were incubated with secondary antibody for 1-h at room temperature and with gentle shaking (goat anti-rabbit IgG peroxidase, 1:1000, A6154, Sigma). After another series of TBS-T washes, bands were visualized by exposing Kodak X-OMAT film (10 min for BDNF, 10 s for β-actin) to membranes treated with ECL substrate (Perkin Elmer, Waltham, MA, USA; for 1 min). The immunoblots were imaged using a Konica Minolta SRX-101A processor (Konica Minolta, Marunouchi, Chiyoda-ku, Tokyo), and band density quantified using AlphaEaseFC v.3.1.2 densitometry software (Alpha Innotech, San Leandro, CA, USA). After normalizing against β-actin (anti-β-actin; 1:5000, sc-47778, Santa Cruz), the BDNF/actin ratios were averaged across blots and the standard error of the mean determined for each treatment group.

### STATISTICAL ANALYSES

The monoamine and Western immunoblot data were analyzed by 2 (Genotype; WT vs. IFN-γ KO) × 2 (Treatment; non-stressed vs. stressed) ANOVAs followed where appropriate by Student–Newman–Keuls pairwise multiple comparisons ($p < 0.05$). The home-cage activity data were analyzed by a repeated measures ANOVA with Genotype and Treatment as the between-subjects variables and Time (Week 3 vs. Week 6) as the repeated measures variable. A mixed model ANOVA was also used for analyzing % duration in the three arms of the Y-maze; here, Genotype and Treatment were the between-subjects variables and Arm (novel vs. start vs. other) served as the within-subjects variable. Planned univariate $t$-tests facilitated comparisons of spatial memory performance in each of the experimental groups (% novel arm duration, DI) with that of a theoretical group performing at chance-level (33.33% and 0, respectively). In addition, Spearman's rank-order correlation coefficients (ρ) were calculated to assess the degree of association between locomotion (total arm entries) and memory performance (DI) in the Y-maze; separate analyses were conducted for the total sample (collapsing across IFN-γ KO and stress) and each of the four treatment groups. On account

of procedural error, one mouse was excluded from the home-cage activity assessments and two mice excluded from the Y-maze analyses. During the course of tissue dissection and monoamine determination a few samples were lost due to error or variability (>2.5 standard deviation from the mean); hence, the degrees of freedom for the statistical analyses varied within and across some brain regions and/or neurochemical substrates. Data were evaluated using a StatView (version 6.0) statistical software package and plotted with GraphPad Prism 6 (La Jolla, CA, USA).

## RESULTS

### CHRONIC STRESSOR TREATMENT TIME-DEPENDENTLY INFLUENCED HOME-CAGE ACTIVITY IN IFN-γ WILD-TYPE AND KNOCKOUT MICE

It is understood that psychologically relevant stressors can modulate motor functioning, and sometimes in diametrically opposed ways (Soblosky and Thurmond, 1986; Venzala et al., 2012). Indeed, psychomotor symptoms are a quite common occurrence in depression, as well as myriad other stressor-related psychiatric conditions (Buyukdura et al., 2011). Here, we report the outcomes of our time series analysis of home-cage locomotor activity. The repeated measures ANOVA revealed significant Genotype × Treatment and Treatment × Time interactions for home-cage activity ($Fs_{1,35}$ = 5.27 and 14.66, respectively, $p < 0.05$). As shown in **Figure 2**, while at the midway point of the experiment (Week 3) motor activity was significantly reduced by the chronic stressor, by the end of the experiment (Week 6) animals that were exposed to the stressor actually displayed increased home-cage activity ($p < 0.05$). Despite finding a significant Genotype × Treatment interaction for home-cage activity, follow-up analyses failed to reveal any statistically significant simple main effects. The existence of such a "crossover" interaction suggests that the early occurring, hypolocomotive effect of the chronic stressor predominated in the WT animals, whereas the later-occurring, activity-boosting effect of the stressor was most pronounced in the KOs; this interpretation is borne out by visual inspection of the data (see **Figure 2**). Moreover, the multiple Bonferroni-corrected pairwise comparisons revealed that, at Week 6, only the stressed IFN-γ KO mice displayed significantly higher levels of activity compared to their non-stressed counterparts ($p < 0.0018$).

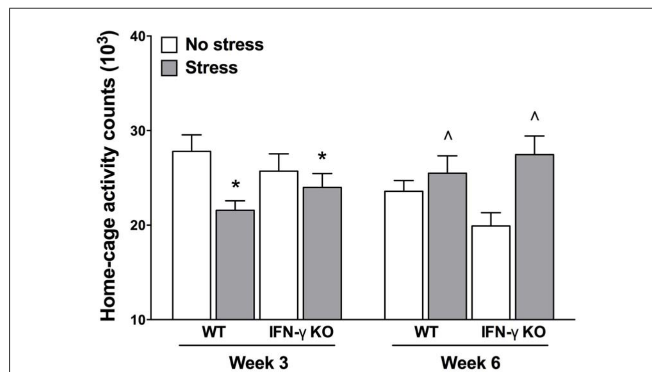



**FIGURE 2 | Influence of chronic stress on home-cage locomotor activity among IFN-γ wild-type and knockout mice.** Overall, chronic stressor exposure had the effect of reducing home-cage activity at Week 3 (mid-way through the experiment), but increasing locomotor activity at Week 6 (end of experiment). However, the early occurring hypolocomotive effect of stress was clearly most prominent in the WT animals, whereas the stressor's later-occurring hyperlocomotive effect was most evident in the KO mice (see accompanying text). Data are presented as mean ± SEM. *$p < 0.05$ relative to non-stressed mice (collapsed across genotype) at Week 3, and ˆ$p < 0.05$ relative to non-stressed mice (collapsed across genotype) at Week 6 (Two-way repeated measures ANOVA). WT, wild-type; KO, knockout.

### CHRONIC STRESS FACILITATED SPATIAL RECOGNITION MEMORY IN THE IFN-γ KNOCKOUT MICE

**Table 2** presents the total number of arm entries during the acquisition (Trial 1) and retention phases (Trial 2) of the Y-maze test. During the acquisition phase, the IFN-γ KOs made fewer total arm entries than the WT animals, regardless of stressor treatment ($F_{1,34} = 9.05$, $p < 0.01$). In contrast, during the retention phase the total number of arm entries did not differ significantly between groups ($Fs < 2.8$, see **Table 2**). Since the acquisition phase of the Y-maze task is comparatively anxiety-laden, it is not surprising that the IFN-γ KOs, for which we and others have previously described an anxious phenotype (Kustova et al., 1998; Litteljohn et al., 2010; Campos et al., 2014), should display reduced activity in this context.

Time spent exploring the novel arm of the Y-maze is considered a reliable index of spatial memory functioning in rodents (Dellu et al., 2000; Leconte et al., 2011). The initial mixed model ANOVA revealed a significant main effect of Arm ($F_{2,68} = 6.88$, $p < 0.01$), such that overall the mice spent significantly more time exploring the novel vs. start or familiar arms ($p < 0.05$). Yet, as shown in **Figure 3A** and confirmed by the planned univariate $t$-tests, of the four treatment groups, only the non-stressed WT controls and the chronically stressed IFN-γ KO mice performed significantly above chance-level (33.33%, $p < 0.05$). Equivalently, our analysis of DI scores revealed that whereas both the WT control and stressed KO animals discriminated the novel arm to a significant extent (DI scores greater than 0, $p < 0.05$, see **Figure 3B**), neither the non-stressed IFN-γ KO controls nor the chronically stressed WT mice had DI values significantly different than 0 ($p > 0.1$). However, the corresponding ANOVA failed to uncover any statistically significant between-group differences ($Fs < 3.75$), and the within-group

**Table 2 | Total number of arm entries during the Y-maze acquisition and retention phases.**

| | Treatment condition | | | |
|---|---|---|---|---|
| | **WT-No stress** | **WT-stress** | **KO-no stress** | **KO-stress** |
| Acquisition phase | 27.4 ± 1.1 | 25.1 ± 2.5 | 21.1 ± 1.4* | 20.7 ± 1.5* |
| Retention phase | 25.1 ± 1.8 | 20.1 ± 1.6 | 21.1 ± 1.7 | 20.6 ± 1.5 |

*Data are presented as mean ± SEM (n = 8–10). *p < 0.05 relative to wild-type mice (collapsed across the stressor treatment). WT, wild-type; KO, knockout.*

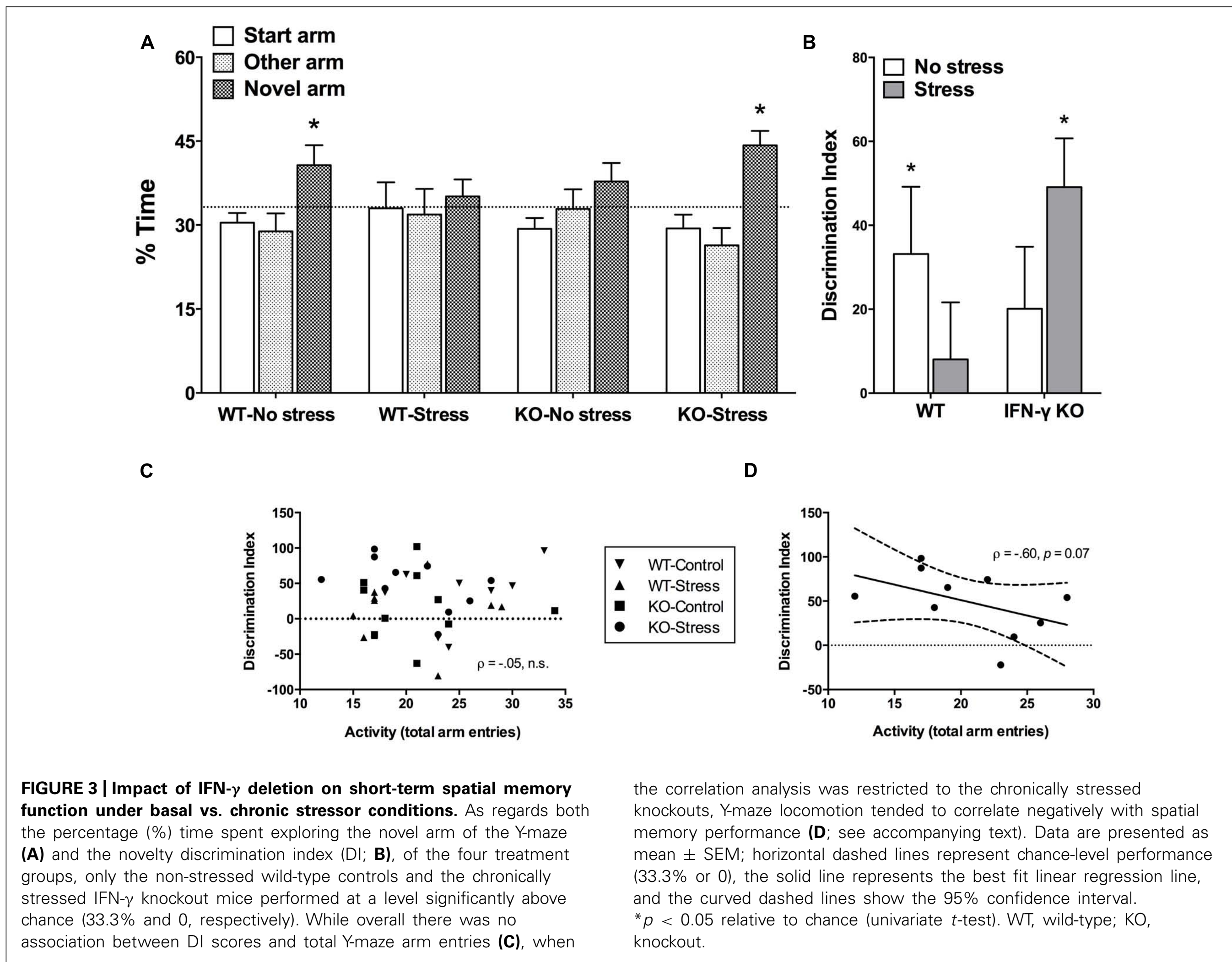



**FIGURE 3 | Impact of IFN-γ deletion on short-term spatial memory function under basal vs. chronic stressor conditions.** As regards both the percentage (%) time spent exploring the novel arm of the Y-maze **(A)** and the novelty discrimination index (DI; **B)**, of the four treatment groups, only the non-stressed wild-type controls and the chronically stressed IFN-γ knockout mice performed at a level significantly above chance (33.3% and 0, respectively). While overall there was no association between DI scores and total Y-maze arm entries **(C)**, when the correlation analysis was restricted to the chronically stressed knockouts, Y-maze locomotion tended to correlate negatively with spatial memory performance (**D**; see accompanying text). Data are presented as mean ± SEM; horizontal dashed lines represent chance-level performance (33.3% or 0), the solid line represents the best fit linear regression line, and the curved dashed lines show the 95% confidence interval. *$p < 0.05$ relative to chance (univariate *t*-test). WT, wild-type; KO, knockout.

variability of DI scores was clearly quite considerable (see **Figure 3B**).

In view of the stressor-induced and genotype-specific changes in home-cage activity, and despite the lack of significant treatment effects on total arm entries in the retention phase of the Y-maze, it was of interest to determine whether Y-maze locomotion correlated with spatial memory performance. In this regard, there was an utter lack of association between DI scores and total Y-maze arm entries when collapsing across the treatment groups ($\rho = -0.05$, $p > 0.70$, see **Figure 3C**). This was similarly the case when separate analyses were performed for the WT control, WT stressed and IFN-γ KO control groups ($\rho = 0.43$, 0.14, −0.14, respectively, $p > 0.25$). Yet, in the case of the chronically stressed KOs, though the negative correlation between DI scores and Y-maze activity was not statistically significant at the $\alpha = 0.05$ level ($\rho = -0.60$, $p = 0.07$), the $p$ value was considerably less than 0.10. While acknowledging the need for caution in interpreting this marginally significant trend, as seen in **Figure 3D** it would appear that, in the chronically stressed KOs only, reduced Y-maze locomotion tended to correspond with better spatial memory performance.

### BRAIN REGIONAL MONOAMINERGIC EFFECTS OF CHRONIC STRESS AND IFN-γ KNOCKOUT

Within the dorsal hippocampus, neither chronic stress nor IFN-γ deletion significantly affected the levels of 5-HT or its primary metabolite, 5-HIAA ($F$s < 2.7; see **Figures 4A,B**). However, as shown in **Figure 4C**, 5-HT turnover (i.e., the ratio of metabolite to parent amine) was significantly diminished overall among mice genetically lacking IFN-γ ($F_{1,34} = 5.75$, $p < 0.05$). A separate ANOVA revealed that the KOs also had diminished hippocampal NE levels relative to the WT mice, regardless of stressor history ($F_{1,33} = 7.15$, $p < 0.05$; see **Figure 4D**). In addition, concentrations of the primary NE metabolite, MHPG, varied according to the significant interaction of Genotype with Stress ($F_{1,33} = 5.05$, $p < 0.05$). As shown in **Figure 4E** and confirmed by the *post hoc* comparisons, whereas chronic stress had no effect on hippocampal MHPG levels in the WT animals, accumulation of the metabolite was robustly enhanced in the stressed KOs ($p < 0.05$, relative to KO controls). A significant Genotype × Stress interaction was likewise uncovered for NE turnover ($F_{1,32} = 6.24$, $p < 0.05$) such that the ratio of hippocampal MHPG to NE was markedly elevated in the

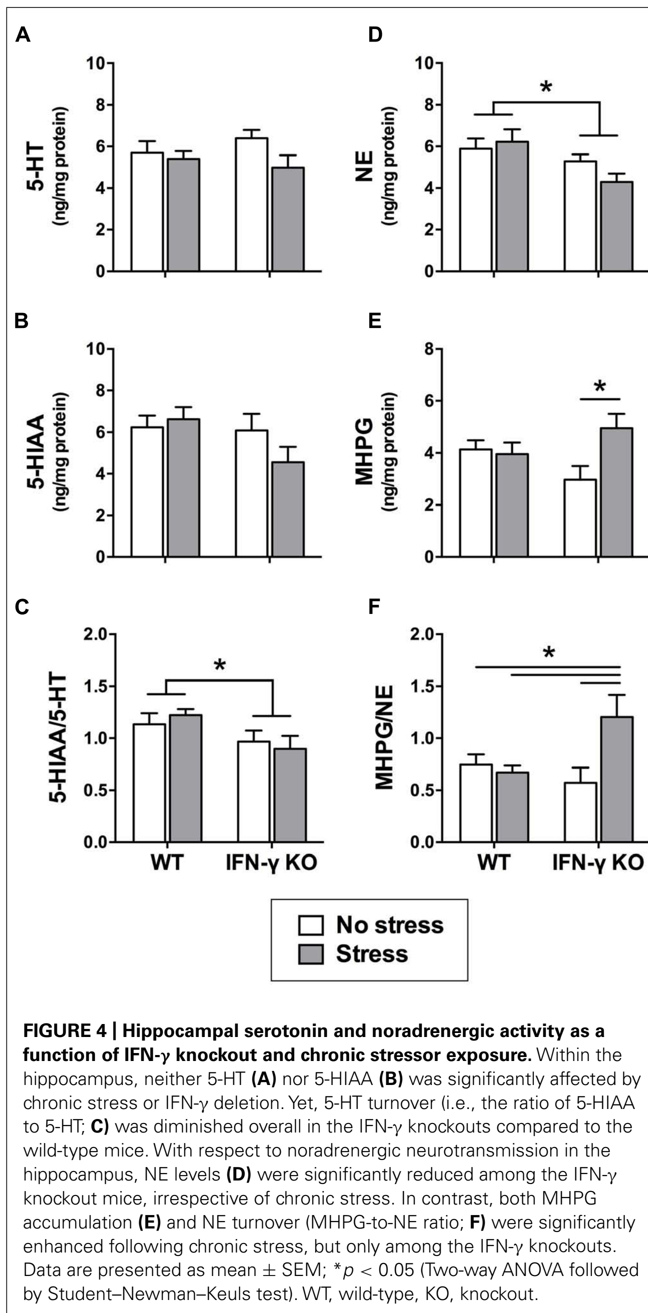



**FIGURE 4 | Hippocampal serotonin and noradrenergic activity as a function of IFN-γ knockout and chronic stressor exposure.** Within the hippocampus, neither 5-HT **(A)** nor 5-HIAA **(B)** was significantly affected by chronic stress or IFN-γ deletion. Yet, 5-HT turnover (i.e., the ratio of 5-HIAA to 5-HT; **C)** was diminished overall in the IFN-γ knockouts compared to the wild-type mice. With respect to noradrenergic neurotransmission in the hippocampus, NE levels **(D)** were significantly reduced among the IFN-γ knockout mice, irrespective of chronic stress. In contrast, both MHPG accumulation **(E)** and NE turnover (MHPG-to-NE ratio; **F)** were significantly enhanced following chronic stress, but only among the IFN-γ knockouts. Data are presented as mean ± SEM; *$p < 0.05$ (Two-way ANOVA followed by Student–Newman–Keuls test). WT, wild-type, KO, knockout.

stressed IFN-γ KOs compared to all other groups ($p < 0.05$; see **Figure 4F**).

Within the locus coeruleus, NE concentrations were significantly higher in the IFN-γ-deficient animals compared to their WT littermates, irrespective of chronic stressor exposure ($F_{1,35} = 8.48$, $p < 0.01$; see **Figure 5A**). But akin to what was observed in the hippocampus, locus coeruleus MHPG levels varied as a function of the interaction between Genotype and Stress ($F_{1,34} = 6.18$, $p < 0.05$). As depicted in **Figure 5B** and confirmed by the follow-up tests, among the IFN-γ KOs chronic stress induced a marked rise in MHPG concentrations ($p < 0.05$ compared to all other groups). In WT mice, however, MHPG levels were completely unaffected by the stressor. Notwithstanding these changes, the ANOVA for locus coeruleus NE turnover did not reveal any significant main or interaction effects of IFN-γ deletion and chronic stress ($F$s $< 1.4$; see **Figure 5C**).

Dopaminergic gating of information through the stressor-sensitive nucleus accumbens is considered to play an important role in both motor and memory function (Costall et al., 1984; Mele et al., 2004; Baker and Kalivas, 2005). It was therefore of interest in the present study to characterize the accumbal dopaminergic effects of chronic stress and IFN-γ deficiency. As shown in **Table 3**, neither of the experimental treatments (nor their interaction) significantly affected indices of dopaminergic neurotransmission in this brain region (DA, DOPAC, and HVA concentrations, as well as DA turnover; $F$s $< 3.4$).

### HIPPOCAMPAL BDNF EXPRESSION WAS UNCHANGED FOLLOWING CHRONIC STRESSOR EXPOSURE

A large body of evidence demonstrates that the neurotrophic factor, BDNF, is essential for hippocampus-dependent memory function and adaptive neuroplastic responses to stressors (Schmidt and Duman, 2010; Taliaz et al., 2010). We were therefore somewhat surprised to find that neither chronic stress nor IFN-γ deletion significantly influenced hippocampal BDNF protein expression ($F$s $< 1$, see **Figures 6A,B**). The Western immunoblot analysis likewise failed to reveal any significant main or interaction effects of the experimental treatments on the hippocampal protein concentrations of a neuronally secreted immature form of BDNF (i.e., proBDNF; $F$s $< 1$, see **Figures 6A,C**).

## DISCUSSION

Accumulating evidence suggests a role of IFN-γ in depression and other stressor-associated psychological disturbances (O'Connor et al., 2009a; Dahl et al., 2014); however, the question whether IFN-γ contributes to the pathological process in ecologically inspired chronic stressor models has gone largely untested. In what is to the best of our knowledge the only other study published to date on this subject, we previously reported that IFN-γ deficiency conferred protection in mice against some of the immune, stress hormone and limbic monoamine effects associated with chronic exposure to a psychologically relevant stressor (Littlejohn et al., 2010). Intriguingly, our results also suggested that IFN-γ may have divergent emotion-relevant actions under normal and chronic stressor conditions, as the KO animals basally showed enhanced anxiety-like behavior coupled with heightened corticosteroid levels and central amygdala NE and 5-HT usage. Such findings are actually consistent with the earlier results of Kustova et al. (1998), as well as the more recent ones of Campos et al. (2014), which indicated that mice genetically lacking IFN-γ are characterized basally by increased emotionality and anxiety. The results of the present investigation extend these findings by demonstrating that stressor context also appears to be crucial in determining the influence of IFN-γ on spatial memory function and related neurochemical systems. Indeed, under normal conditions mice genetically lacking IFN-γ exhibited impaired spatial memory, which we suggest might be related to altered hippocampal (and perhaps locus coeruleus) monoaminergic

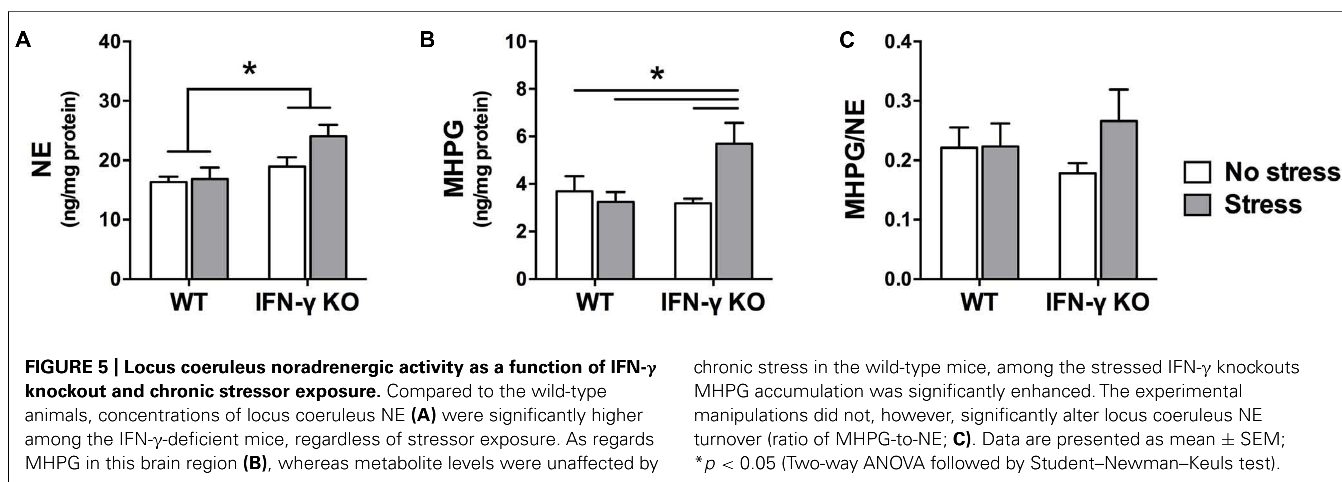



**FIGURE 5 | Locus coeruleus noradrenergic activity as a function of IFN-γ knockout and chronic stressor exposure.** Compared to the wild-type animals, concentrations of locus coeruleus NE **(A)** were significantly higher among the IFN-γ-deficient mice, regardless of stressor exposure. As regards MHPG in this brain region **(B)**, whereas metabolite levels were unaffected by chronic stress in the wild-type mice, among the stressed IFN-γ knockouts MHPG accumulation was significantly enhanced. The experimental manipulations did not, however, significantly alter locus coeruleus NE turnover (ratio of MHPG-to-NE; **C)**. Data are presented as mean ± SEM; $^*p < 0.05$ (Two-way ANOVA followed by Student–Newman–Keuls test).

**Table 3 | Dopaminergic activity within the nucleus accumbens as a function of chronic stress and IFN-γ deletion.**

| | Concentration (ng/mg protein) | | | DA turnover [(DOPAC+HVA)/DA] |
|---|---|---|---|---|
| | DA | DOPAC | HVA | |
| WT-no stress | 123.57 ± 14.76 | 15.43 ± 1.19 | 7.95 ± .43 | 0.216 ± 0.035 |
| WT-stress | 152.78 ± 17.56 | 15.98 ± 1.04 | 5.96 ± .91 | 0.173 ± 0.039 |
| KO-no stress | 156.33 ± 20.07 | 16.81 ± 1.36 | 5.50 ± .54 | 0.155 ± 0.017 |
| KO-stress | 186.87 ± 19.00 | 17.67 ± 1.61 | 6.28 ± .85 | 0.145 ± 0.022 |

*Data are presented as mean ± SEM. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid.*

neurotransmission but not BDNF signaling or nucleus accumbens dopaminergic activity. Contrastingly, under conditions of chronic stress IFN-γ deficiency appeared actually to facilitate memory function, and this pro-mnemonic effect coincided with enhanced hippocampal and locus coeruleus noradrenergic activity.

## IMPACT OF IFN-γ DEFICIENCY ON MEMORY AND RELATED BRAIN PROCESSES UNDER NORMAL CONDITIONS

It is now well recognized that pro-inflammatory cytokines can modulate cognitive processes, with pathological consequences probably at the forefront of attention. Yet, there is increasing evidence to suggest that cytokines and other pro-inflammatory stimuli (e.g., prostaglandins, amyloid-β peptide: Cowley et al., 2008; Puzzo et al., 2012) may under certain circumstances actually be beneficial for learning and memory; the key determinant here appears to be cytokine level (McAfoose and Baune, 2009; Yirmiya and Goshen, 2011). While highly elevated concentrations of pro-inflammatory cytokines, as can occur for instance in depression and many of its comorbid conditions (Anisman and Hayley, 2012; Dahl et al., 2014), generally provoke anti-mnemonic effects, it would appear that a certain basal physiological level of pro-inflammatory cytokine signal is required for normal memory function (Yirmiya and Goshen, 2011). Evidencing the latter, mice genetically lacking TNF-α or TNF receptor 1 displayed impaired spatial memory (TNF-R2 KO mice, however, displayed intact memory; Camara et al., 2013), and IL-1β signaling blockade produced a similar functional effect (Yirmiya et al., 2002; Goshen et al., 2007).

Consistent with such a view, we presently report that mice lacking IFN-γ showed disturbed spatial recognition memory in the basal state. Interestingly, Baron et al. (2008) revealed that *limited* central overexpression of IFN-γ resulted in improved hippocampus-dependent memory, whereas pathologically elevated concentrations of the cytokine have generally been associated with memory impairment (Lapter et al., 2009; Dutra et al., 2013; Too et al., 2014). Thus, when considered together, these data suggest that the hormetic-like dose-response pattern that was described elsewhere for memory and IL-1β (and several other immune actors; Yirmiya and Goshen, 2011) may very well be relevant too for IFN-γ. Further investigation is warranted to substantiate this possibility, and such efforts will do well to include a detailed time-and-dose-response analysis, as well as determinations of both circulating and brain regional IFN-γ concentrations in ecologically relevant animal disease models. Also, some caution should be exercised when interpreting the Y-maze behavioral data in the present study: while basally the KOs failed to perform significantly better than chance (consistent with impaired spatial memory), the ANOVA test did not reveal any significant between-group differences. As mentioned previously, there was considerable within-group variability in the behavioral data,

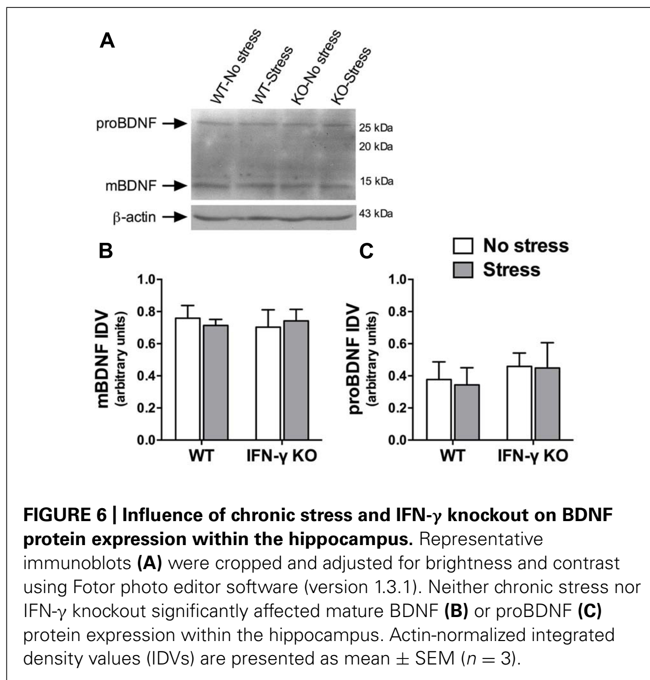



**FIGURE 6 | Influence of chronic stress and IFN-γ knockout on BDNF protein expression within the hippocampus.** Representative immunoblots **(A)** were cropped and adjusted for brightness and contrast using Fotor photo editor software (version 1.3.1). Neither chronic stress nor IFN-γ knockout significantly affected mature BDNF **(B)** or proBDNF **(C)** protein expression within the hippocampus. Actin-normalized integrated density values (IDVs) are presented as mean ± SEM ($n = 3$).

and this likely reflects the critical but often overlooked influence of individual differences in chronic stress susceptibility (e.g., Bergström et al., 2008). Therefore, it is our suggestion that the present findings be viewed as proof-of-principle for more comprehensive and larger-scale investigation into the prospective learning and memory effects of IFN-γ.

With respect to the possible neural substrate(s) subserving these memory changes, we submit that it may be particularly telling that the IFN-γ KOs overall displayed altered NE content within both the locus coeruleus and hippocampus (increased in the former brain region and reduced in the latter), as well as diminished hippocampal serotonin turnover. Each of these highly interconnected, stressor-sensitive brain regions plays a vital role in memory function, with the hippocampus considered especially critical for memory consolidation and spatial navigation (McGaugh, 2000; Suzuki, 2006), and the noradrenergic locus coeruleus mediating the potent cognition-modulatory effects of emotional arousal (Gibbs et al., 2010; Sara and Bouret, 2012). Moreover, human and animal studies alike have implicated both noradrenergic and serotonergic signaling in the memory process, and generally in a facilitatory capacity (Lee and Ma, 1995; Murchison et al., 2004; Meeter et al., 2006). It seems reasonable therefore to suggest that dysregulated monoaminergic neurotransmission, particularly in the hippocampus, could have been at least partially responsible for the memory deficit seen in these animals. Notably, memory impairment may have occurred both in spite of the observed increase of locus coeruleus NE (e.g., Gibbs et al., 2010) and independently of dopaminergic neurotransmission in the nucleus accumbens (which as will be recalled was unaffected by the experimental manipulations). Importantly, since the KO-specific hippocampal and locus coeruleus monoamine changes occurred irrespective of stressor challenge, it would appear that IFN-γ normally plays a role in the homeostatic regulation of these neurotransmitter systems (but presumably not the accumbal DA system). Alternatively, as the KOs were without IFN-γ signal from birth, it is possible that physiological concentrations of the cytokine are required for the normal maturation of these monoamine systems across development (Litteljohn et al., 2010).

Of course, such a reading cannot discount the possibility that other molecular and/or cellular processes might also have contributed to the presently described memory effects. Indeed, in our previous work (Litteljohn et al., 2010) we showed that mice genetically lacking IFN-γ had increased basal corticosterone levels and central amygdala monoamine activity, and we argued that such changes were relevant to the anxious phenotype already on record for these animals (Kustova et al., 1998). Yet, hippocampus-dependent memory is also subject to modulation by corticosterone and central amygdala monoaminergic neurotransmission – mainly retrieval in the case of the former and consolidation as regards the latter (de Quervain et al., 1998; Hermans et al., 2014) – and it is entirely plausible that one or both of these processes could have contributed to basal memory impairment among the IFN-γ KOs. Similarly, as a wealth of evidence has implicated hippocampal BDNF in memory function, with a reduction of neurotophin levels generally being tied to poor memory outcomes and an increase in BDNF levels usually signaling the opposite (Mizuno et al., 2000; Shirayama et al., 2002), we had speculated that any IFN-γ KO-associated decline in memory might also be attended by a reduction in hippocampal BDNF. Contrary to our expectations, however, it will be recalled that neither mature nor proBDNF protein levels were affected by IFN-γ deletion. These null BDNF results are in agreement with the recent enzyme-linked immunosorbent assay (ELISA)-based findings of Campos et al. (2014), and together our studies provide good evidence that altered hippocampal BDNF does not underlie the neurobehavioral phenotype of IFN-γ KO mice. That said, our results do not preclude the involvement of BDNF in prospective IFN-γ-associated proactive memory effects; in fact, Baron et al. (2008) provide evidence that the memory enhancement seen in mice overexpressing IFN-γ may at least partially be attributable to an upregulation of central BDNF.

#### INFLUENCE OF IFN-γ KNOCKOUT ON MEMORY AND RELATED NEUROCHEMICAL PROCESSES UNDER CHRONIC STRESSOR CONDITIONS

If a lack of IFN-γ can under normal conditions be seen to predispose to memory dysfunction, under conditions of chronic stress the result seems to be memory facilitation. Indeed, whereas neither the basal state KOs nor the chronically stressed WT mice demonstrated Y-maze performance that was significantly better than chance, both the stressor-treated IFN-γ-deficient mice and the non-stressed WT controls displayed intact spatial memory. These data are aligned somewhat with the findings of other studies indicating that chronic variable stress can paradoxically (and akin to what's been reported for acute as well as predictable chronic stress: e.g., Parihar et al., 2011; Uysal et al., 2012) enhance memory and learning (Bartolomucci et al., 2002; McLaughlin et al., 2005; Hawley et al., 2012); here we provide evidence suggesting that IFN-γ,

or rather a lack thereof, may be key. Interestingly, paralleling the behavioral data, our neurochemical analyses revealed that noradrenergic, but not serotonergic, metabolism was markedly and selectively augmented in the hippocampus and locus coeruleus among IFN-γ null mice that were exposed to the chronic stressor. Given the aforementioned importance of these brain regions in learning and memory (Gibbs et al., 2010), and the generally facilitative role ascribed to noradrenergic signaling in this regard (Lee and Ma, 1995), it appears likely that IFN-γ acts to restrict brain noradrenergic and, *consequently*, spatial memory responses to chronic stress.

Recent reports have documented elevations of circulating and brain IFN-γ levels among rodents exposed to chronic stressors (Liu et al., 2013; Wrona et al., 2014). And while these findings contrast with those of several other animal (e.g., Palumbo et al., 2012) as well as human studies (Segerstrom and Miller, 2004), there is a growing recognition that stress hormones can under certain circumstances augment brain inflammation (Sorrells et al., 2009). A number of routes exist by which IFN-γ could come to be influenced by, and hence contribute to, the central actions of psychological stressors (Litteljohn et al., 2010). One such potential mechanism involves a stressor-induced shift in the T-helper type-1 (Th1)/T-helper type-2 (Th2) cytokine balance in favor of the former (though stressor chronicity is a major influence here: Maes et al., 1998; Segerstrom and Miller, 2004). In this way, depression related pro-inflammatory Th1 responses (e.g., IDO activation and 5-HT depletion), of which IFN-γ is the principal effector, could become accentuated at the expense of anti-inflammatory Th2 ones (e.g., those mediated by IL-4 and IL-10; Najjar et al., 2013). Stressors can also provoke intestinal barrier dysfunction and mucosal inflammation (Vicario et al., 2010), which could lead to not only increased circulating and even central IFN-γ levels but also the potentiated trafficking of immune cells into the brain parenchyma (Tran et al., 2000; Schroder et al., 2004). Notably, the latter could also be realized through a stressor-induced disruption of blood–brain barrier integrity (Friedman et al., 1996; Northrop and Yamamoto, 2012). And finally, that brain-resident microglia are themselves capable of producing IFN-γ under the direction of endogenous cytokine signals (i.e., emanating from other glial cells and not necessarily brain-infiltrating leukocytes; Kawanokuchi et al., 2006) raises the intriguing possibility that stressors could act directly on microglial cells to influence central IFN-γ signaling.

Yet, it should be noted that by the end of the 6-week stressor regimen the chronically stressed KOs also displayed increased spontaneous locomotor activity; this was evident too in the stressed WT animals, but seemingly to a lesser degree. Several studies have linked hyperactivity in rodents to increased brainstem and hippocampal noradrenergic activity (though the role of NE in regulating motor brain circuitry is not straightforward and almost certainly involves cross-talk with brain DA and 5-HT systems; Suwabe et al., 2000; Ruocco et al., 2010; Lambertsen et al., 2012), and there is reason to think that a similar situation could be relevant to certain clinical contexts – for instance ADHD, impulse control disorders and (atypical) depression (Viggiano et al., 2004; Fan et al., 2012). Possibly, then, in the face of ongoing stress a lack of IFN-γ and the consequent potentiation of noradrenergic signaling could function as a double-edged sword – at once serving to facilitate the memory process (Tully and Bolshakov, 2010) and predisposing to hyperactivity, with the latter perhaps best viewed as a harbinger of impending allostatic overload. All the same, it is important to recognize that in the present study the motor-modulatory effect of stress was clearly task-specific. Indeed, whereas by the end of the 6-week experiment locomotion in the home-cage was increased among the stressor-exposed mice (predominantly in the KOs), Y-maze testing only 2 days prior revealed no such effect of stress (i.e., on total arm entries). Moreover, it will be recalled that, in the chronically stressed KOs only, Y-maze performance actually tended to correlate *negatively* with total arm entries (retention phase) – a phenomenon that was revealed elsewhere to be associated with good spatial memory performance under chronic stressor conditions (Conrad et al., 2003). Thus, if it can be allowed that enhanced NE utilization did, in fact, contribute to both the cognitive and home-cage motor changes observed in the stressed KOs, then these animals were presumably yet able to harness said noradrenergic drive to their mnemonic advantage and when the peculiarity of the circumstances demanded it.

In addition to monoaminergic imbalances, depression and other stressor-related behavioral disorders may involve disturbances of neuroplasticity, including changes in brain structure and neurotrophin systems (Calabrese et al., 2009; Schmidt and Duman, 2010; Hayley and Litteljohn, 2013). In particular, circulating levels of BDNF were found to be diminished in depressed subjects and to correlate with clinical recovery after treatment initiation (Shimizu et al., 2003; Huang et al., 2008). Similarly, animal studies revealed that chronic stress often diminishes central BDNF levels (Murakami et al., 2005; Luo et al., 2013), whereas BDNF-augmenting strategies (e.g., direct infusions, stimulators) typically induce antidepressant-like behavioral consequences (Shirayama et al., 2002; Schmidt and Duman, 2010; Ye et al., 2011). We were therefore somewhat surprised to find that hippocampal BDNF remained unchanged in the present study upon exposure to chronic stress. Interestingly, though in the minority, a number of studies have reported a lack of or even inverse relationship between hippocampal BDNF and chronic stress (Lucca et al., 2008; Larsen et al., 2010; Hanson et al., 2011). And while not contesting the crucial role of BDNF in memory function, it's worth noting that hippocampal BDNF has not always been found to positively correlate with memory performance (Muñoz et al., 2010; Bechara and Kelly, 2013). Conceivably, then, germline loss of IFN-γ may have altered the hippocampal neuroinflammatory milieu in such a way that, in the face of chronic stress, upregulated (compensatory) expression of alternative growth factors (e.g., GDNF, NGF, NT-3, IGF-1) and possibly even anti-inflammatory and/or anti-apoptotic messengers (e.g., IL-10 and bcl-2, respectively) could have positively affected memory-related processes independently of BDNF (Bian et al., 2012; Xuan et al., 2014).

Alternatively, since chronic stress has been shown to time-dependently regulate brain regional BDNF expression (Fanous et al., 2010; Xiao et al., 2011; Lakshminarasimhan and Chattarji, 2012; Capoccia et al., 2013), it is possible that our null BDNF findings might be related to the timing of mouse sacrifice relative

to stressor initiation and/or termination. Considering that in the present study animals were exposed to a rather lengthy 6-week stressor, a plausible scenario would have the stressor transiently suppressing BDNF levels, only for them to return to baseline by the time of sampling. This contention can be seen to derive at least some support from the home-cage activity data: whereas locomotor activity was significantly elevated after 6 weeks of exposure to stress (and predominantly in the KOs), at the mid-way point of the experiment the opposite was, in fact, noted. In effect, chronic stress time-dependently influenced home-cage motor activity and IFN-γ appeared to modulate the magnitude if not direction of such effects. Without time series data for the neurochemical endpoints we cannot exclude that hippocampal BDNF expression similarly changed across time; this is a definite weakness of the present study and a suggested worthwhile avenue of future research. In addition, we cannot speak to any potential effect of chronic stress or IFN-γ deficiency on BDNF expression at the mRNA level, nor can we rule out the possibility that a more sensitive assay such as ELISA could have detected presumably very subtle changes in BDNF protein expression. However, that Campos et al. (2014) failed to demonstrate an effect of IFN-γ deletion on ELISA-determined BDNF protein levels within the hippocampus and PFC would seem to lend credence to the present null findings, at least in regards to basal state BDNF expression. And finally, our use of genetically engineered mice on a C57BL/6J background might have been an issue, as this mouse strain is considered to be somewhat less stressor-sensitive than certain other strains (e.g., BALB/c; Tannenbaum and Anisman, 2003; Pothion et al., 2004). In this regard, Bergström et al. (2008) showed that chronic mild stress differentially modulates hippocampal BDNF expression in stressor-resilient and stressor-sensitive rats.

## CONCLUSION

To summarize, our data suggest that IFN-γ differentially modulates memory-related processes under normal and chronic stressor conditions. Specifically, in the basal state IFN-γ appears to facilitate hippocampus-dependent spatial memory, probably at least partially due to the cytokine's involvement in the homeostatic regulation of relevant brain monoamine systems. Under conditions of chronic stress, however, IFN-γ appears instead to restrict potentially adaptive brain noradrenergic and spatial memory responses. While our data do not support a role of hippocampal BDNF in this regard, they do not preclude the possible involvement of other structural and/or functional neuroplastic processes (e.g., IFN-γ-dependent alterations of hippocampal neurogenesis: Baron et al., 2008; Li et al., 2010; Campos et al., 2014); we advocate here for further investigation of this intriguing possibility. More generally, the current findings are aligned with a growing body of work indicating that stressor context can greatly influence the behavioral, immune and neurochemical effects of other cytokine and immune challenges (e.g., IFN-α, LPS, poly I:C; Anisman et al., 2007; Gandhi et al., 2007; Gibb et al., 2008). It should be noted, however, that compensatory neuronal or immunological changes stemming from a lack of IFN-γ during key developmental stages could have contributed to the present findings.

## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Darcy Litteljohn and Shawn Hayley. Performed the experiments: Darcy Litteljohn and Eric Nelson. Analyzed the data and wrote the paper: Darcy Litteljohn. Edited and approved the manuscript: Darcy Litteljohn and Shawn Hayley.

## ACKNOWLEDGMENTS

Thanks to Drs. Jerzy Kulczycki and Hymie Anisman for help with the monoamine analyses. Eric Nelson performed the Western blots and drafted the corresponding methods section. We also thank Ashley Brennan, Jordan Brennan, Geoffrey Crowe, Amie Cummings, Anudip Gill, Eugenia Petoukhov, Marzena Sieczkos, and Kristen Stewart for their assistance in conducting various aspects of the experiments. This research was supported by funds awarded to Darcy Litteljohn (Doctoral Research Award program) and Shawn Hayley (Canada Research Chairs program) from the Canadian Institutes of Health Research (CIHR).

## REFERENCES

Anisman, H., Gibb, J., and Hayley, S. (2008). Influence of continuous infusion of interleukin-1beta on depression-related processes in mice: corticosterone, circulating cytokines, brain monoamines, and cytokine mRNA expression. *Psychopharmacology (Berl.)* 199, 231–244. doi: 10.1007/s00213-008-1166-z

Anisman, H., and Hayley, S. (2012). Inflammatory factors contribute to depression and its comorbid conditions. *Sci. Signal.* 5:pe45. doi: 10.1126/scisignal.2003579

Anisman, H., Poulter, M. O., Gandhi, R., Merali, Z., and Hayley, S. (2007). Interferon-alpha effects are exaggerated when administered on a psychosocial stressor backdrop: cytokine, corticosterone and brain monoamine variations. *J. Neuroimmunol.* 186, 45–53. doi: 10.1016/j.jneuroim.2007.02.008

Baker, D. A., and Kalivas, P. W. (2005). "Sensitization and relapse: dopamine-glutamate interactions," in *Dopamine and Glutamate in Psychiatric Disorders*, eds W. J. Schmidt and M. E. A. Reith (Totowa, NJ: Humana Press), 355–370.

Baron, R., Nemirovsky, A., Harpaz, I., Cohen, H., Owens, T., and Monsonego, A. (2008). IFN-gamma enhances neurogenesis in wild-type mice and in a mouse model of Alzheimer's disease. *FASEB J.* 22, 2843–2852. doi: 10.1096/fj.08-105866

Bartolomucci, A., de Biurrun, G., Czéh, B., van Kampen, M., and Fuchs, E. (2002). Selective enhancement of spatial learning under chronic psychosocial stress. *Eur. J. Neurosci.* 15, 1863–1866. doi: 10.1046/j.1460-9568.2002.02043.x

Bechara, R. G., and Kelly, A. M. (2013). Exercise improves object recognition memory and induces BDNF expression and cell proliferation in cognitively enriched rats. *Behav. Brain Res.* 245, 96–100. doi: 10.1016/j.bbr.2013.02.018

Bergström, A., Jayatissa, M. N., Mørk, A., and Wiborg, O. (2008). Stress sensitivity and resilience in the chronic mild stress rat model of depression; an in situ hybridization study. *Brain Res.* 1196, 41–52. doi: 10.1016/j.brainres.2007.12.025

Bian, Y., Pan, Z., Hou, Z., Huang, C., Li, W., and Zhao, B. (2012). Learning, memory, and glial cell changes following recovery from chronic unpredictable stress. *Brain Res. Bull.* 88, 471–476. doi: 10.1016/j.brainresbull.2012.04.008

Brustolim, D., Ribeiro-dos-Santos, R., Kast, R. E., Altschuler, E. L., and Soares, M. B. (2006). A new chapter opens in anti-inflammatory treatments: the antidepressant bupropion lowers production of tumor necrosis factor-alpha and interferon-gamma in mice. *Int. Immunopharmacol.* 6, 903–907. doi: 10.1016/j.intimp.2005.12.007

Buyukdura, J. S., McClintock, S. M., and Croarkin, P. E. (2011). Psychomotor retardation in depression: biological underpinnings, measurement, and treatment. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 35, 395–409. doi: 10.1016/j.pnpbp.2010.10.019

Calabrese, F., Molteni, R., Racagni, G., and Riva, M. A. (2009). Neuronal plasticity: a link between stress and mood disorders. *Psychoneuroendocrinology* 34(Suppl. 1), S208–S216. doi: 10.1016/j.psyneuen.2009.05.014

Camara, M. L., Corrigan, F., Jaehne, E. J., Jawahar, M. C., Anscomb, H., Koerner, H., et al. (2013). TNF-α and its receptors modulate complex behaviours and

neurotrophins in transgenic mice. *Psychoneuroendocrinology* 38, 3102–3114. doi: 10.1016/j.psyneuen.2013.09.010

Campos, A. C., Vaz, G. N., Saito, V. M., and Teixeira, A. L. (2014). Further evidence for the role of interferon-gamma on anxiety- and depressive-like behaviors: involvement of hippocampal neurogenesis and NGF production. *Neurosci. Lett.* 578, 100–105. doi: 10.1016/j.neulet.2014.06.039

Capoccia, S., Berry, A., Bellisario, V., Vacirca, D., Ortona, E., Alleva, E., et al. (2013). Quality and timing of stressors differentially impact on brain plasticity and neuroendocrine-immune function in mice. *Neural Plast.* 2013:971817. doi: 10.1155/2013/971817

Chesler, D. A., and Reiss, C. S. (2002). The role of IFN-gamma in immune responses to viral infections of the central nervous system. *Cytokine Growth Factor Rev.* 13, 441–454. doi: 10.1016/S1359-6101(02)00044-8

Conrad, C. D., Grote, K. A., Hobbs, R. J., and Ferayorni, A. (2003). Sex differences in spatial and non-spatial Y-maze performance after chronic stress. *Neurobiol. Learn. Mem.* 79, 32–40. doi: 10.1016/S1074-7427(02)00018-7

Costall, B., Domeney, A. M., and Naylor, R. J. (1984). Locomotor hyperactivity caused by dopamine infusion into the nucleus accumbens of rat brain: specificity of action. *Psychopharmacology (Berl.)* 82, 174–180. doi: 10.1007/BF00427768

Cowley, T. R., Fahey, B., and O'Mara, S. M. (2008). COX-2, but not COX-1, activity is necessary for the induction of perforant path long-term potentiation and spatial learning in vivo. *Eur. J. Neurosci.* 27, 2999–3008. doi: 10.1111/j.1460-9568.2008.06251.x

Dahl, J., Ormstad, H., Aass, H. C., Malt, U. F., Bendz, L. T., Sandvik, L., et al. (2014). The plasma levels of various cytokines are increased during ongoing depression and are reduced to normal levels after recovery. *Psychoneuroendocrinology* 45, 77–86. doi: 10.1016/j.psyneuen.2014.03.019

Dalton, D. K., Pitts-Meek, S., Keshav, S., Figari, I. S., Bradley, A., and Stewart, T. A. (1993). Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science* 259, 1739–1742. doi: 10.1126/science.8456300

Dellu, F., Contarino, A., Simon, H., Koob, G. F., and Gold, L. H. (2000). Genetic differences in response to novelty and spatial memory using a two-trial recognition task in mice. *Neurobiol. Learn. Mem.* 73, 31–48. doi: 10.1006/nlme.1999.3919

de Quervain, D. J., Roozendaal, B., and McGaugh, J. L. (1998). Stress and glucocorticoids impair retrieval of long-term spatial memory. *Nature* 394, 787–790. doi: 10.1038/29542

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E. K., et al. (2010). A meta-analysis of cytokines in major depression. *Biol. Psychiatry* 67, 446–457. doi: 10.1016/j.biopsych.2009.09.033

Dutra, R. C., Moreira, E. L., Alberti, T. B., Marcon, R., Prediger, R. D., and Calixto, J. B. (2013). Spatial reference memory deficits precede motor dysfunction in an experimental autoimmune encephalomyelitis model: the role of kallikrein-kinin system. *Brain Behav. Immun.* 33, 90–101. doi: 10.1016/j.bbi.2013.06.002

Fan, X., Bruno, K. J., and Hess, E. J. (2012). Rodent models of ADHD. *Curr. Top. Behav. Neurosci.* 9, 273–300. doi: 10.1007/7854_2011_121

Fanous, S., Hammer, R. P. Jr., and Nikulina, E. M. (2010). Short- and long-term effects of intermittent social defeat stress on brain-derived neurotrophic factor expression in mesocorticolimbic brain regions. *Neuroscience* 167, 598–607. doi: 10.1016/j.neuroscience.2010.02.064

Fava, M. (2003). The role of the serotonergic and noradrenergic neurotransmitter systems in the treatment of psychological and physical symptoms of depression. *J. Clin. Psychiatry* 64(Suppl. 13), 26–29.

Ferguson, J. N., Young, L. J., Hearn, E. F., Matzuk, M. M., Insel, T. R., and Winslow, J. T. (2000). Social amnesia in mice lacking the oxytocin gene. *Nat. Genet.* 25, 284–288. doi: 10.1038/35020688

Franklin, K. B., and Paxinos, G. (1997). *The Mouse Brain in Stereotaxic Coordinates.* San Diego, CA: Academic Press.

Friedman, A., Kaufer, D., Shemer, J., Hendler, I., Soreq, H., and Tur-Kaspa, I. (1996). Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. *Nat. Med.* 2, 1382–1385. doi: 10.1038/nm1296-1382

Gabbay, V., Klein, R. G., Alonso, C. M., Babb, J. S., Nishawala, M., De Jesus, G., et al. (2009). Immune system dysregulation in adolescent major depressive disorder. *J. Affect. Disord.* 115, 177–182. doi: 10.1016/j.jad.2008.07.022

Gandhi, R., Hayley, S., Gibb, J., Merali, Z., and Anisman, H. (2007). Influence of poly I:C on sickness behaviors, plasma cytokines, corticosterone and central monoamine activity: moderation by social stressors. *Brain Behav. Immun.* 21, 477–489. doi: 10.1016/j.bbi.2006.12.005

Gibb, J., Hayley, S., Gandhi, R., Poulter, M. O., and Anisman, H. (2008). Synergistic and additive actions of a psychosocial stressor and endotoxin challenge: circulating and brain cytokines, plasma corticosterone and behavioral changes in mice. *Brain Behav. Immun.* 22, 573–589. doi: 10.1016/j.bbi.2007.12.001

Gibbs, M. E., Hutchinson, D. S., and Summers, R. J. (2010). Noradrenaline release in the locus coeruleus modulates memory formation and consolidation; roles for α- and β-adrenergic receptors. *Neuroscience* 170, 1209–1222. doi: 10.1016/j.neuroscience.2010.07.052

Goshen, I., Kreisel, T., Ounallah-Saad, H., Renbaum, P., Zalzstein, Y., Ben-Hur, T., et al. (2007). A dual role for interleukin-1 in hippocampal-dependent memory processes. *Psychoneuroendocrinology* 32, 1106–1115. doi: 10.1016/j.psyneuen.2007.09.004

Hamon, M., and Blier, P. (2013). Monoamine neurocircuitry in depression and strategies for new treatments. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 45, 54–63. doi: 10.1016/j.pnpbp.2013.04.009

Hanson, N. D., Owens, M. J., Boss-Williams, K. A., Weiss, J. M., and Nemeroff, C. B. (2011). Several stressors fail to reduce adult hippocampal neurogenesis. *Psychoneuroendocrinology* 36, 1520–1529. doi: 10.1016/j.psyneuen.2011.04.006

Hawley, D. F., Morch, K., Christie, B. R., and Leasure, J. L. (2012). Differential response of hippocampal subregions to stress and learning. *PLoS ONE* 7:e53126. doi: 10.1371/journal.pone.0053126

Hayley, S., and Litteljohn, D. (2013). Neuroplasticity and the next wave of antidepressant strategies. *Front. Cell. Neurosci.* 7:218. doi: 10.3389/fncel.2013.00218

Hermans, E. J., Battaglia, F. P., Atsak, P., de Voogd, L. D., Fernández, G., and Roozendaal, B. (2014). How the amygdala affects emotional memory by altering brain network properties. *Neurobiol. Learn. Mem.* 112, 2–16. doi: 10.1016/j.nlm.2014.02.005

Hill, M. N., Hellemans, K. G., Verma, P., Gorzalka, B. B., and Weinberg, J. (2012). Neurobiology of chronic mild stress: parallels to major depression. *Neurosci. Biobehav. Rev.* 36, 2085–2117. doi: 10.1016/j.neubiorev.2012.07.001

Hinwood, M., Morandini, J., Day, T. A., and Walker, F. R. (2012). Evidence that microglia mediate the neurobiological effects of chronic psychological stress on the medial prefrontal cortex. *Cereb. Cortex* 22, 1442–1454. doi: 10.1093/cercor/bhr229

Huang, T. L., Lee, C. T., and Liu, Y. L. (2008). Serum brain-derived neurotrophic factor levels in patients with major depression: effects of antidepressants. *J. Psychiatr. Res.* 42, 521–525. doi: 10.1016/j.jpsychires.2007.05.007

Jiang, H., Wang, Z., Wang, Y., Xie, K., Zhang, Q., Luan, Q., et al. (2013). Antidepressant-like effects of curcumin in chronic mild stress of rats: involvement of its anti-inflammatory action. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 47, 33–39. doi: 10.1016/j.pnpbp.2013.07.009

Kaster, M. P., Gadotti, V. M., Calixto, J. B., Santos, A. R., and Rodrigues, A. L. (2012). Depressive-like behavior induced by tumor necrosis factor-α in mice. *Neuropharmacology* 62, 419–426. doi: 10.1016/j.neuropharm.2011.08.018

Kawanokuchi, J., Mizuno, T., Takeuchi, H., Kato, H., Wang, J., Mitsuma, N., et al. (2006). Production of interferon-gamma by microglia. *Mult. Scler.* 12, 558–564. doi: 10.1177/1352458506070763

Koo, J. W., and Duman, R. S. (2008). IL-1beta is an essential mediator of the antineurogenic and anhedonic effects of stress. *Proc. Natl. Acad. Sci. U.S.A.* 105, 751–756. doi: 10.1073/pnas.0708092105

Krügel, U., Fischer, J., Radicke, S., Sack, U., and Himmerich, H. (2013). Antidepressant effects of TNF-α blockade in an animal model of depression. *J. Psychiatr. Res.* 47, 611–616. doi: 10.1016/j.jpsychires.2013.01.007

Kubera, M., Lin, A. H., Kenis, G., Bosmans, E., van Bockstaele, D., and Maes, M. (2001). Anti-Inflammatory effects of antidepressants through suppression of the interferon-gamma/interleukin-10 production ratio. *J. Clin. Psychopharmacol.* 21, 199–206. doi: 10.1097/00004714-200104000-00012

Kustova, Y., Sei, Y., Morse, H. C. Jr., and Basile, A. S. (1998). The influence of a targeted deletion of the IFNgamma gene on emotional behaviors. *Brain Behav. Immun.* 12, 308–324. doi: 10.1006/brbi.1998.0546

Kwant, A., and Sakic, B. (2004). Behavioral effects of infection with interferon-gamma adenovector. *Behav. Brain Res.* 151, 73–82. doi: 10.1016/j.bbr.2003.08.008

Lakshminarasimhan, H., and Chattarji, S. (2012). Stress leads to contrasting effects on the levels of brain derived neurotrophic factor in the hippocampus and amygdala. *PLoS ONE* 7:e30481. doi: 10.1371/journal.pone.0030481

Lambertsen, K. L., Gramsbergen, J. B., Sivasaravanaparan, M., Ditzel, N., Sevelsted-Møller, L. M., Oliván-Viguera, A., et al. (2012). Genetic KCa3.1-deficiency produces locomotor hyperactivity and alterations in cerebral monoamine levels. *PLoS ONE* 7:e47744. doi: 10.1371/journal.pone.0047744

Lapter, S., Marom, A., Meshorer, A., Elmann, A., Sharabi, A., Vadai, E., et al. (2009). Amelioration of brain pathology and behavioral dysfunction in mice with lupus following treatment with a tolerogenic peptide. *Arthritis Rheum.* 60, 3744–3754. doi: 10.1002/art.25013

Larsen, M. H., Mikkelsen, J. D., Hay-Schmidt, A., and Sandi, C. (2010). Regulation of brain-derived neurotrophic factor (BDNF) in the chronic unpredictable stress rat model and the effects of chronic antidepressant treatment. *J. Psychiatr. Res.* 44, 808–816. doi: 10.1016/j.jpsychires.2010.01.005

Leconte, C., Bihel, E., Lepelletier, F. X., Bouët, V., Saulnier, R., Petit, E., et al. (2011). Comparison of the effects of erythropoietin and its carbamylated derivative on behaviour and hippocampal neurogenesis in mice. *Neuropharmacology* 60, 354–364. doi: 10.1016/j.neuropharm.2010.09.025

Lee, E. H., and Ma, Y. L. (1995). Amphetamine enhances memory retention and facilitates norepinephrine release from the hippocampus in rats. *Brain Res. Bull.* 37, 411–416. doi: 10.1016/0361-9230(95)00039-9

Li, L., Walker, T. L., Zhang, Y., Mackay, E. W., and Bartlett, P. F. (2010). Endogenous interferon gamma directly regulates neural precursors in the non-inflammatory brain. *J. Neurosci.* 30, 9038–9050. doi: 10.1523/JNEUROSCI.5691-09.2010

Litteljohn, D., Cummings, A., Brennan, A., Gill, A., Chunduri, S., Anisman, H., et al. (2010). Interferon-gamma deficiency modifies the effects of a chronic stressor in mice: implications for psychological pathology. *Brain Behav. Immun.* 24, 462–473. doi: 10.1016/j.bbi.2009.12.001

Liu, G., Rustom, N., Litteljohn, D., Bobyn, J., Rudyk, C., Anisman, H., et al. (2014). Use of induced pluripotent stem cell derived neurons engineered to express BDNF for modulation of stressor related pathology. *Front. Cell. Neurosci.* 8:316. doi: 10.3389/fncel.2014.00316

Liu, W., Sheng, H., Xu, Y., Liu, Y., Lu, J., and Ni, X. (2013). Swimming exercise ameliorates depression-like behavior in chronically stressed rats: relevant to proinflammatory cytokines and IDO activation. *Behav. Brain Res.* 242, 110–116. doi: 10.1016/j.bbr.2012.12.041

Liu, Y., Ho, R. C., and Mak, A. (2012). Interleukin (IL)-6, tumour necrosis factor alpha (TNF-α) and soluble interleukin-2 receptors (sIL-2R) are elevated in patients with major depressive disorder: a meta-analysis and meta-regression. *J. Affect. Disord.* 139, 230–239. doi: 10.1016/j.jad.2011.08.003

Lucca, G., Comim, C. M., Valvassori, S. S., Pereira, J. G., Stertz, L., Gavioli, E. C., et al. (2008). Chronic mild stress paradigm reduces sweet food intake in rats without affecting brain derived neurotrophic factor protein levels. *Curr. Neurovasc. Res.* 5, 207–213. doi: 10.2174/156720208786413406

Luo, J., Zhang, L., Ning, N., Jiang, H., and Yu, S. Y. (2013). Neotrofin reverses the effects of chronic unpredictable mild stress on behavior via regulating BDNF, PSD-95 and synaptophysin expression in rat. *Behav. Brain Res.* 253, 48–53. doi: 10.1016/j.bbr.2013.07.014

Maes, M., Song, C., Lin, A., De Jongh, R., Van Gastel, A., Kenis, G., et al. (1998). The effects of psychological stress on humans: increased production of pro-inflammatory cytokines and a Th1-like response in stress-induced anxiety. *Cytokine* 10, 313–318. doi: 10.1006/cyto.1997.0290

Maes, M., Song, C., Lin, A. H., Bonaccorso, S., Kenis, G., De Jongh, R., et al. (1999). Negative immunoregulatory effects of antidepressants: inhibition of interferon-gamma and stimulation of interleukin-10 secretion. *Neuropsychopharmacology* 20, 370–379. doi: 10.1016/S0893-133X(98)00088-8

Mahar, I., Bambico, F. R., Mechawar, N., and Nobrega, J. N. (2014). Stress, serotonin, and hippocampal neurogenesis in relation to depression and antidepressant effects. *Neurosci. Biobehav. Rev.* 38, 173–192. doi: 10.1016/j.neubiorev.2013.11.009

Mangano, E. N., Peters, S., Litteljohn, D., So, R., Bethune, C., Bobyn, J., et al. (2011). Granulocyte macrophage-colony stimulating factor protects against substantia nigra dopaminergic cell loss in an environmental toxin model of Parkinson's disease. *Neurobiol. Dis.* 43, 99–112. doi: 10.1016/j.nbd.2011.02.011

McAfoose, J., and Baune, B. T. (2009). Evidence for a cytokine model of cognitive function. *Neurosci. Biobehav. Rev.* 33, 355–366. doi: 10.1016/j.neubiorev.2008.10.005

McGaugh, J. L. (2000). Memory – a century of consolidation. *Science* 287, 248–251. doi: 10.1126/science.287.5451.248

McLaughlin, K. J., Baran, S. E., Wright, R. L., and Conrad, C. D. (2005). Chronic stress enhances spatial memory in ovariectomized female rats despite CA3 dendritic retraction: possible involvement of CA1 neurons. *Neuroscience* 135, 1045–1054. doi: 10.1016/j.neuroscience.2005.06.083

Meeter, M., Talamini, L., Schmitt, J. A., and Riedel, W. J. (2006). Effects of 5-HT on memory and the hippocampus: model and data. *Neuropsychopharmacology* 31, 712–720. doi: 10.1038/sj.npp.1300869

Mele, A., Avena, M., Roullet, P., De Leonibus, E., Mandillo, S., Sargolini, F., et al. (2004). Nucleus accumbens dopamine receptors in the consolidation of spatial memory. *Behav. Pharmacol.* 15, 423–431. doi: 10.1097/00008877-200409000-00017

Miller, A. H., Maletic, V., and Raison, C. L. (2009). Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. *Biol. Psychiatry* 65, 732–741. doi: 10.1016/j.biopsych.2008.11.029

Mizuno, M., Yamada, K., Olariu, A., Nawa, H., and Nabeshima, T. (2000). Involvement of brain-derived neurotrophic factor in spatial memory formation and maintenance in a radial arm maze test in rats. *J. Neurosci.* 20, 7116–7121.

Muñoz, P. C., Aspé, M. A., Contreras, L. S., and Palacios, A. G. (2010). Correlations of recognition memory performance with expression and methylation of brain-derived neurotrophic factor in rats. *Biol. Res.* 43, 251–258.

Murakami, S., Imbe, H., Morikawa, Y., Kubo, C., and Senba, E. (2005). Chronic stress, as well as acute stress, reduces BDNF mRNA expression in the rat hippocampus but less robustly. *Neurosci. Res.* 53, 129–139.

Murchison, C. F., Zhang, X. Y., Zhang, W. P., Ouyang, M., Lee, A., and Thomas, S. A. (2004). A distinct role for norepinephrine in memory retrieval. *Cell* 117, 131–143. doi: 10.1016/S0092-8674(04)00259-4

Myint, A. M., Bondy, B., Baghai, T. C., Eser, D., Nothdurfter, C., Schüle, C., et al. (2013). Tryptophan metabolism and immunogenetics in major depression: a role for interferon-γ gene. *Brain Behav. Immun.* 31, 128–33. doi: 10.1016/j.bbi.2013.04.003

Myint, A. M., O'Mahony, S., Kubera, M., Kim, Y. K., Kenny, C., Kaim-Basta, A., et al. (2007). Role of paroxetine in interferon-alpha-induced immune and behavioural changes in male Wistar rats. *J. Psychopharmacol.* 21, 843–850. doi: 10.1177/0269881107077165

Najjar, S., Pearlman, D. M., Alper, K., Najjar, A., and Devinsky, O. (2013). Neuroinflammation and psychiatric illness. *J. Neuroinflammation* 10, 43. doi: 10.1186/1742-2094-10-43

Northrop, N. A., and Yamamoto, B. K. (2012). Persistent neuroinflammatory effects of serial exposure to stress and methamphetamine on the blood-brain barrier. *J. Neuroimmune Pharmacol.* 7, 951–968. doi: 10.1007/s11481-012-9391-y

O'Connor, J. C., André, C., Wang, Y., Lawson, M. A., Szegedi, S. S., Lestage, J., et al. (2009a). Interferon-gamma and tumor necrosis factor-alpha mediate the upregulation of indoleamine 2,3-dioxygenase and the induction of depressive-like behavior in mice in response to bacillus Calmette–Guerin. *J. Neurosci.* 29, 4200–4209. doi: 10.1523/JNEUROSCI.5032-08.2009

O'Connor, J. C., Lawson, M. A., André, C., Moreau, M., Lestage, J., Castanon, N., et al. (2009b). Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. *Mol. Psychiatry* 14, 511–522. doi: 10.1038/sj.mp.4002148

Oxenkrug, G., Perianayagam, M., Mikolich, D., Requintina, P., Shick, L., Ruthazer, R., et al. (2011). Interferon-gamma (+874) T/A genotypes and risk of IFN-alpha-induced depression. *J. Neural Transm.* 118, 271–274. doi: 10.1007/s00702-010-0525-1

Palumbo, M. L., Trinchero, M. F., Zorrilla-Zubilete, M. A., Schinder, A. F., and Genaro, A. M. (2012). Glatiramer acetate reverts stress-induced alterations on adult neurogenesis and behavior. Involvement of Th1/Th2 balance. *Brain Behav. Immun.* 26, 429–438. doi: 10.1016/j.bbi.2011.12.006

Parihar, V. K., Hattiangady, B., Kuruba, R., Shuai, B., and Shetty, A. K. (2011). Predictable chronic mild stress improves mood, hippocampal neurogenesis and memory. *Mol. Psychiatry* 16, 171–183. doi: 10.1038/mp.2009.130

Pittenger, C., and Duman, R. S. (2008). Stress, depression, and neuroplasticity: a convergence of mechanisms. *Neuropsychopharmacology* 33, 88–109. doi: 10.1038/sj.npp.1301574

Pothion, S., Bizot, J. C., Trovero, F., and Belzung, C. (2004). Strain differences in sucrose preference and in the consequences of unpredictable chronic mild stress. *Behav. Brain Res.* 155, 135–146. doi: 10.1016/j.bbr.2004.04.008

Puzzo, D., Privitera, L., and Palmeri, A. (2012). Hormetic effect of amyloid-β peptide in synaptic plasticity and memory. *Neurobiol. Aging* 33, 1484.e15–1484.e24. doi: 10.1016/j.neurobiolaging.2011.12.020

Ruocco, L. A., Carnevale, U. A., Treno, C., Sadile, A. G., Melisi, D., Arra, C., et al. (2010). Prepuberal subchronic methylphenidate and atomoxetine induce different long-term effects on adult behaviour and forebrain dopamine, norepinephrine and serotonin in Naples high-excitability rats. *Behav. Brain Res.* 210, 99–106. doi: 10.1016/j.bbr.2010.02.020

Sara, S. J., and Bouret, S. (2012). Orienting and reorienting: the locus coeruleus mediates cognition through arousal. *Neuron* 76, 130–141. doi: 10.1016/j.neuron.2012.09.011

Schildkraut, J. J. (1965). The catecholamine hypothesis of affective disorders: a review of supporting evidence. *Am. J. Psychiatry* 122, 509–522.

Schmidt, F. M., Lichtblau, N., Minkwitz, J., Chittka, T., Thormann, J., Kirkby, K. C., et al. (2014). Cytokine levels in depressed and non-depressed subjects, and masking effects of obesity. *J. Psychiatr. Res.* 55, 29–34. doi: 10.1016/j.jpsychires.2014.04.021

Schmidt, H. D., and Duman, R. S. (2010). Peripheral BDNF produces antidepressant-like effects in cellular and behavioral models. *Neuropsychopharmacology* 12, 2378–2391. doi: 10.1038/npp.2010.114

Schroder, K., Hertzog, P. J., Ravasi, T., and Hume, D. A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* 75, 163–189. doi: 10.1189/jlb.0603252

Segerstrom, S. C., and Miller, G. E. (2004). Psychological stress and the human immune system: a meta-analytic study of 30 years of inquiry. *Psychol. Bull.* 130, 601–630. doi: 10.1037/0033-2909.130.4.601

Shimizu, E., Hashimoto, K., Okamura, N., Koike, K., Komatsu, N., Kumakiri, C., et al. (2003). Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. *Biol. Psychiatry* 54, 70–75. doi: 10.1016/S0006-3223(03)00181-1

Shirayama, Y., Chen, A. C., Nakagawa, S., Russell, D. S., and Duman, R. S. (2002). Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. *J. Neurosci.* 22, 3251–3261.

Simon, N. M., McNamara, K., Chow, C. W., Maser, R. S., Papakostas, G. I., Pollack, M. H., et al. (2008). A detailed examination of cytokine abnormalities in major depressive disorder. *Eur. Neuropsychopharmacol.* 18, 230–233. doi: 10.1016/j.euroneuro.2007.06.004

Soblosky, J. S., and Thurmond, J. B. (1986). Biochemical and behavioral correlates of chronic stress: effects of tricyclic antidepressants. *Pharmacol. Biochem. Behav.* 24, 1361–1368. doi: 10.1016/0091-3057(86)90196-6

Sorrells, S. F., Caso, J. R., Munhoz, C. D., and Sapolsky, R. M. (2009). The stressed CNS: when glucocorticoids aggravate inflammation. *Neuron* 64, 33–39. doi: 10.1016/j.neuron.2009.09.032

Sukoff Rizzo, S. J., Neal, S. J., Hughes, Z. A., Beyna, M., Rosenzweig-Lipson, S., Moss, S. J., et al. (2012). Evidence for sustained elevation of IL-6 in the CNS as a key contributor of depressive-like phenotypes. *Transl. Psychiatry* 2:e199. doi: 10.1038/tp.2012.120

Suwabe, A., Kubota, M., Niwa, M., Kobayashi, K., and Kanba, S. (2000). Effect of a 5-HT(1A) receptor agonist, flesinoxan, on the extracellular noradrenaline level in the hippocampus and on the locomotor activity of rats. *Brain Res.* 858, 393–401. doi: 10.1016/S0006-8993(00)01941-7

Suzuki, W. A. (2006). Encoding new episodes and making them stick. *Neuron* 50, 19–21. doi: 10.1016/j.neuron.2006.03.029

Taliaz, D., Stall, N., Dar, D. E., and Zangen, A. (2010). Knockdown of brain-derived neurotrophic factor in specific brain sites precipitates behaviors associated with depression and reduces neurogenesis. *Mol. Psychiatry* 15, 80–92. doi: 10.1038/mp.2009.67

Tannenbaum, B., and Anisman, H. (2003). Impact of chronic intermittent challenges in stressor-susceptible and resilient strains of mice. *Biol. Psychiatry* 53, 292–303. doi: 10.1016/S0006-3223(02)01487-7

Too, L. K., Ball, H. J., McGregor, I. S., and Hunt, N. H. (2014). The pro-inflammatory cytokine interferon-gamma is an important driver of neuropathology and behavioural sequelae in experimental pneumococcal meningitis. *Brain Behav. Immun.* 40, 252–268. doi: 10.1016/j.bbi.2014.02.020

Tran, E. H., Prince, E. N., and Owens, T. (2000). IFN-gamma shapes immune invasion of the central nervous system via regulation of chemokines. *J. Immunol.* 164, 2759–2768. doi: 10.4049/jimmunol.164.5.2759

Tully, K., and Bolshakov, V. Y. (2010). Emotional enhancement of memory: how norepinephrine enables synaptic plasticity. *Mol. Brain* 3:15. doi: 10.1186/1756-6606-3-15

Uysal, N., Sisman, A. R., Dayi, A., Ozbal, S., Cetin, F., Baykara, B., et al. (2012). Acute footshock-stress increases spatial learning-memory and correlates to increased hippocampal BDNF and VEGF and cell numbers in adolescent male and female rats. *Neurosci. Lett.* 514, 141–146. doi: 10.1016/j.neulet.2012.02.049

Venzala, E., García-García, A. L., Elizalde, N., Delagrange, P., and Tordera, R. M. (2012). Chronic social defeat stress model: behavioral features, antidepressant action, and interaction with biological risk factors. *Psychopharmacology (Berl.)* 224, 313–325. doi: 10.1007/s00213-012-2754-5

Vicario, M., Guilarte, M., Alonso, C., Yang, P., Martínez, C., Ramos, L., et al. (2010). Chronological assessment of mast cell-mediated gut dysfunction and mucosal inflammation in a rat model of chronic psychosocial stress. *Brain Behav. Immun.* 24, 1166–1175. doi: 10.1016/j.bbi.2010.06.002

Viggiano, D., Ruocco, L. A., Arcieri, S., and Sadile, A. G. (2004). Involvement of norepinephrine in the control of activity and attentive processes in animal models of attention deficit hyperactivity disorder. *Neural Plast.* 11, 133–149. doi: 10.1155/NP.2004.133

Wrona, D., Listowska, M., Kubera, M., Glac, W., Grembecka, B., Plucińska, K., et al. (2014). Effects of chronic desipramine pretreatment on open field-induced suppression of blood natural killer cell activity and cytokine response depend on the rat's behavioral characteristics. *J. Neuroimmunol.* 268, 13–24. doi: 10.1016/j.jneuroim.2013.10.001

Xiao, L., Shu, C., Tang, J., Wang, H., Liu, Z., and Wang, G. (2011). Effects of different CMS on behaviors, BDNF/CREB/Bcl-2 expression in rat hippocampus. *Biomed. Aging Pathol.* 1, 138–146. doi: 10.1016/j.biomag.2010.10.006

Xuan, A. G., Pan, X. B., Wei, P., Ji, W. D., Zhang, W. J., Liu, J. H., et al. (2014). Valproic acid alleviates memory deficits and attenuates amyloid-β deposition in transgenic mouse model of Alzheimer's disease. *Mol. Neurobiol.* doi: 10.1007/s12035-014-8751-4 [Epub ahead of print].

Ye, Y., Wang, G., Wang, H., and Wang, X. (2011). Brain-derived neurotrophic factor (BDNF) infusion restored astrocytic plasticity in the hippocampus of a rat model of depression. *Neurosci. Lett.* 503, 15–19. doi: 10.1016/j.neulet.2011.07.055

Yirmiya, R., and Goshen, I. (2011). Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain Behav. Immun.* 25, 181–213. doi: 10.1016/j.bbi.2010.10.015

Yirmiya, R., Winocur, G., and Goshen, I. (2002). Brain interleukin-1 is involved in spatial memory and passive avoidance conditioning. *Neurobiol. Learn. Mem.* 78, 379–389. doi: 10.1006/nlme.2002.4072

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

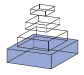
*Received: 04 September 2014; accepted: 03 November 2014; published online: 20 November 2014.*

*Citation: Litteljohn D, Nelson E and Hayley S (2014) IFN-γ differentially modulates memory-related processes under basal and chronic stressor conditions. Front. Cell. Neurosci.* ***8****:391. doi: 10.3389/fncel.2014.00391*

*This article was submitted to the journal Frontiers in Cellular Neuroscience.*

*Copyright © 2014 Litteljohn, Nelson and Hayley. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*

GENERAL COMMENTARY
published: 23 August 2013
doi: 10.3389/fncel.2013.00132



# Hippocampal Lipocalin 2: an emotional link between neurons and glia

**Oliver von Bohlen und Halbach[1]* and Hermona Soreq[2]**

[1] Institute of Anatomy and Cell Biology, Universitätsmedizin Greifswald, Greifswald, Germany
[2] The Edmond and Lily Safra Center of Brain Science, The Hebrew University of Jerusalem, Jerusalem, Israel
*Correspondence: oliver.vonbohlen@uni-greifswald.de

**Edited by:**
Silvia Alboni, University of Modena and Reggio Emilia, Italy

**Keywords: lipocalin-2, astrocytes, hippocampus, synaptic plasticity, mouse**

**A commentary on**

**Lipocalin-2 is involved in emotional behaviors and cognitive function**
*by Ferreira, A. C., Pinto, V., Dá Mesquita, S., Novais, A., Sousa, J. C., Correia-Neves, M., et al. (2013). Front. Cell Neurosci. 7:122. doi: 10.3389/fncel.2013.00122*

Lipocalins are a diverse family of secreted proteins that are involved in a variety of cellular processes. A member of the lipocalin protein family is lipocalin-2. Lipocalin-2 mRNA and protein expression in the adult brain, under physiological conditions, is thought to be rather low, especially in the hippocampus (Chia et al., 2011). Moreover, lipocalin-2 seems to be expressed, at least in the adult brain, by glia cells (Chia et al., 2011; Marques et al., 2012). Lipocalin-2 has been found to be upregulated in astrocytes after neuronal injury induced e.g. by kainate (Chia et al., 2011) and it has been shown that reactive astrocytes secrete lipocalin2 to promote neuron death (Bi et al., 2013).

Based on the expression of lipocalin 2 in the brain, the data shown in the paper "Lipocalin-2 is involved in emotional behaviors and cognitive function" by Ferreira and colleagues are very interesting. The data may hint a strong interplay between glia cell function and neuronal functioning. The authors demonstrate that lack of lipocalin-2 alters behavior and cognitive functions and induces morphological changes (dendritic length and spine-densities) and that absence of lipocalin-2 impairs synaptic plasticity. These interesting findings offer the opportunity to analyze the interplay of astrocytes and neurons in more detail. There are different scenarios that may account for the effects seen in the lipocalin-2 deficient mice:

(1) The observed changes may be mediated by a receptor capable of binding lipocalin, like the 24p3R (Devireddy et al., 2005), which is known to be expressed by neurons (Ip et al., 2011).
(2) Gliotransmission and the tripartite synapse (Santello et al., 2012) are known to play important roles for electrophysiological properties of neurons. Deletion of lipocalin-2 may affect the population of astrocytes, leading to changes in the activity-dependent synaptic plasticity.
(3) Deficiency for lipocalin-2 may have an effect upon embryonic brain development and/or modify other regulatory molecules, such as microRNAs whose other targets may be affected differently due to these changes.

In conclusion, lipocalin-2 regulation connects between cell types, behavioral paradigms and possibly developmental ones.

## REFERENCES

Bi, F., Huang, C., Tong, J., Qiu, G., Huang, B., Wu, Q., et al. (2013). Reactive astrocytes secrete lcn2 to promote neuron death. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4069–4074. doi: 10.1073/pnas.1218497110

Chia, W. J., Dawe, G. S., and Ong, W. Y. (2011). Expression and localization of the iron-siderophore binding protein lipocalin 2 in the normal rat brain and after kainate-induced excitotoxicity. *Neurochem. Int.* 59, 591–599. doi: 10.1016/j.neuint.2011.04.007

Devireddy, L. R., Gazin, C., Zhu, X., and Green, M. R. (2005). A cell-surface receptor for lipocalin 24p3 selectively mediates apoptosis and iron uptake. *Cell* 123, 1293–1305. doi: 10.1016/j.cell.2005.10.027

Ip, J. P., Nocon, A. L., Hofer, M. J., Lim, S. L., Muller, M., and Campbell, I. L. (2011). Lipocalin 2 in the central nervous system host response to systemic lipopolysaccharide administration. *J. Neuroinflammation* 8:124. doi: 10.1186/1742-2094-8-124

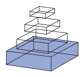
Marques, F., Mesquita, S. D., Sousa, J. C., Coppola, G., Gao, F., Geschwind, D. H., et al. (2012). Lipocalin 2 is present in the EAE brain and is modulated by natalizumab. *Front. Cell Neurosci.* 6:33. doi: 10.3389/fncel.2012.00033

Santello, M., Cali, C., and Bezzi, P. (2012). Gliotransmission and the tripartite synapse. *Adv. Exp. Med. Biol.* 970, 307–331. doi: 10.1007/978-3-7091-0932-8_14

*Received: 02 August 2013; accepted: 06 August 2013; published online: 23 August 2013.*
*Citation: von Bohlen und Halbach O and Soreq H (2013) Hippocampal Lipocalin 2: an emotional link between neurons and glia. Front. Cell. Neurosci. **7**:132. doi: 10.3389/fncel.2013.00132*
*This article was submitted to the journal Frontiers in Cellular Neuroscience.*
*Copyright © 2013 von Bohlen und Halbach and Soreq. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 07 August 2014
doi: 10.3389/fncel.2014.00214



# Interleukin-18 alters protein expressions of neurodegenerative diseases-linked proteins in human SH-SY5Y neuron-like cells

***Elina M. Sutinen*[1,2], *Minna A. Korolainen*[3], *Jukka Häyrinen*[4], *Irina Alafuzoff*[5], *Steven Petratos*[6], *Antero Salminen*[1,7], *Hilkka Soininen*[1,2,7], *Tuula Pirttilä*[1,2,7†] and *Johanna O. Ojala*[1,2]***

[1] Department of Neurology, Institute of Clinical Medicine, University of Eastern Finland, Kuopio, Finland
[2] Brain Research Unit, Clinical Research Centre, University of Eastern Finland, Kuopio, Finland
[3] Institute of Biotechnology, University of Helsinki, Helsinki, Finland
[4] School of Medicine, Institute of Biomedicine, University of Eastern Finland, Kuopio, Finland
[5] Rudbecklaboratoriet, Department of Immunology, Genetics and Pathology, Molecular and Morphological Pathology, Uppsala University, Uppsala, Sweden
[6] Regenerative Neuroscience and Development Laboratory, Department of Medicine, Central Clinical School, Monash University, Prahran, VIC, Australia
[7] Department of Neurology, Kuopio University Hospital, Kuopio, Finland

***Edited by:***
*Silvia Alboni, University of Modena and Reggio Emilia, Italy*

***Reviewed by:***
*Brian David Gulbransen, Michigan State University, USA*
*Robert Weissert, University of Regensburg, Germany*

****Correspondence:***
*Johanna O. Ojala, Brain Research Unit, Clinical Research Centre, University of Eastern Finland, Mediteknia, PO Box 1627, Kuopio, FI-70211, Finland*
*e-mail: johanna.ojala@uef.fi*

*† Deceased 2010.*

Chronic inflammation and oxidative stress (OS) are present in Alzheimer's disease (AD) brains in addition to neuronal loss, Amyloid-β (Aβ) plaques and hyperphosphorylated tau-protein neurofibrillary tangles (NFTs). Previously we showed that levels of the pro-inflammatory cytokine, interleukin-18 (IL-18), are elevated in post-mortem AD brains. IL-18 can modulate the tau kinases, Cdk5 and GSK3β, as well as Aβ-production. IL-18 levels are also increased in AD risk diseases, including type-2 diabetes and obesity. Here, we explored other IL-18 regulated proteins in neuron-like SH-SY5Y cells. Differentiated SH-SY5Y cells, incubated with IL-18 for 24, 48, or 72 h, were analyzed by two-dimensional gel electrophoresis (2D-DIGE). Specific altered protein spots were chosen and identified with mass spectrometry (MS) and verified by western immunoblotting (WIB). IL-18 had time-dependent effects on the SH-SY5Y proteome, modulating numerous protein levels/modifications. We concentrated on those related to OS (DDAH2, peroxiredoxins 2, 3, and 6, DJ-1, BLVRA), Aβ-degradation (MMP14, TIMP2), Aβ-aggregation (Septin-2), and modifications of axon growth and guidance associated, collapsin response mediator protein 2 (CRMP2). IL-18 significantly increased antioxidative enzymes, indicative of OS, and altered levels of glycolytic α- and γ-enolase and multifunctional 14-3-3γ and -ε, commonly affected in neurodegenerative diseases. MMP14, TIMP2, α-enolase and 14-3-3ε, indirectly involved in Aβ metabolism, as well as Septin-2 showed changes that increase Aβ levels. Increased 14-3-3γ may contribute to GSK3β driven tau hyperphosphorylation and CRMP2 Thr514 and Ser522 phosphorylation with the Thr555-site, a target for Rho kinase, showing time-dependent changes. IL-18 also increased caspase-1 levels and vacuolization of the cells. Although our SH-SY5Y cells were not aged, as neurons in AD, our work suggests that heightened or prolonged IL-18 levels can drive protein changes of known relevance to AD pathogenesis.

**Keywords: Interleukin-18, inflammation, neurodegenerative diseases, proteomics, oxidative stress related proteins, CRMP2, DDAH, MMP14**

**Abbreviations:** Aβ, Amyloid-β; ACN, Acetonitrile; AD, Alzheimer's disease; ADMA, N(G),N(G)-dimethyl-L-arginine; APP, Amyloid-β precursor protein; ATRA, all-trans retinoic acid; BACE-1, β-site APP cleaving enzyme 1; BDNF, brain derived neurotrophic factor; BLVR, biliverdin reductase; BR, bilirubin; BV, biliverdin; Cdk5, cyclin dependent kinase-5; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; CRMP2, collapsin response mediator protein 2; Cu/Zn-SOD, Cu/Zn-superoxide dismutase-1; DDAH, N(G), N(G)-dimethylarginine dimethylaminohydrolase; DDAHr, DDAH2 antibody reactive protein; 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis; DJ-1, Parkinson disease protein 7; DTT, dithiothreitol; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; ENOA, enolase α; ENOG, enolase γ; ERK, extracellular signal-regulated kinase; ESI, electrospray ionization mass spectrometry; FA, formic acid; GSK3β, glycogen synthase kinase-3β; HO, heme oxygenase; HPLC, High-performance liquid chromatography; IAA, iodoacetamide; ICE, interleukin-1β convertin enzyme; IDO,indoleamine 2,3-dioxygenase; IGIF, interferon-γ inducing factor; IL-18BP, interleukin-18 binding protein; IFNγ, interferon-γ; LC-MS, liquid chromatography-mass spectrometry; LRRK2, leucine-rich repeat serine/threonine-protein kinase 2; MALDI; matrix-assisted laser desorption ionization; MAPK, mitogen activated protein kinase; MMA, N(G)-monomethyl-L-arginine; MS, mass spectrometry; MScl, multiple sclerosis; MMP14/MT1-MMP, membrane-type-1 matrix metalloproteinase; M-W; Mann–Whitney *U*-test; MW, molecular weight; NFT, neurofibrillary tangles; NOS, nitric oxide synthase; OS, oxidative stress; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PD, Parkinson disease; pI, isoelectric point; PI3K, phophatidylinositol 3-kinase; PKC, protein kinase C; PMF, peptide mass fingerprint; PRX, peroxiredoxin; PS-1, presenilin 1; ROS, reactive oxygen species; RT, room temperature; SDS, sodium dodecyl sulfate; s.e.m., standard error of mean; Sem3A, semaphorin- 3A; SEPT2, Septin-2; TBS, tris buffered saline; TCEP, tris (2-carboxyethyl) phosphine hydrochloride; TFA, trifluoroacetic acid; TIMP2, tissue inhibitor of metalloproteinases 2; TLR4; toll-like receptor 4; TOF, time of flight; tPA, tissue-type plasminogen activator; T-t, Student's *t*-test; uPA, urokinase-type plasminogen activator; WIB, western immunoblotting.

## INTRODUCTION

The most common form of dementia is sporadic, late-onset Alzheimer's disease (AD), the etiology of which is still unknown. As well as contributory environmental factors and increased incidence in females, well-established risk factors for sporadic AD include aging, type-2 diabetes and obesity, which all involve increased inflammation levels (Bendlin et al., 2010; Hamer et al., 2012). The neuropathological hallmarks of AD include extracellular neuritic amyloid-β (Aβ) plaques and intraneuronal neurofibrillary tangles (NFTs) containing hyperphosphorylated tau (Braak and Braak, 1997). In addition to neuronal loss, chronic inflammation, oxidative stress (OS), and excitotoxic damage characterize AD brains (Braak and Braak, 1991; von Bernhardi and Eugenín, 2012; Morales et al., 2014). However, the pathological sequelae and the interaction of these mechanisms during the course of the disease are largely unknown.

Inflammation in the brain is primarily driven by activated microglia and astrocytes, leading to the over-production of inflammatory cytokines and other inflammatory products in association with increased reactive oxygen species (ROS) (Morales et al., 2014). The interleukin-1 family member interleukin-18 (IL-18), also known as IL-1γ or interferon-γ-inducing factor (IGIF), is an inflammatory cytokine that is produced mainly by activated microglia in the brain. Previously we showed that expression of this upstream cytokine is elevated in many AD brain regions (Ojala et al., 2009). IL-18, indirectly via interferon-γ (IFNγ) induction, can also increase indoleamine 2,3-dioxygenase (IDO), in turn producing neuroregulatory and excitotoxic tryptophan catabolites that are proposed to contribute to AD neuropathology (Guillemin et al., 2005; Anderson and Ojala, 2010). As with IL-1β, IL-18 is cleaved by caspase-1 [interleukin-1β converting enzyme (ICE)] to an active secreted form. In comparison to IL-1β, IL-18 expression is higher, partly due to its longer half-life (Ojala et al., 2009), suggesting that it may exert a more significant impact in the brain than the more extensively researched IL-1β. The normal function of brain IL-18 is unknown, but it may have a role in central nervous system development as well as contributing to brain immune and inflammatory processes (Ojala et al., 2008; Alboni et al., 2010). Interestingly, increased IL-18 levels have also been detected in type-2 diabetes (Aso et al., 2003), obesity (Esposito et al., 2002), and ischemic heart disease (Mallat et al., 2001). IL-18 is also induced during physical/emotional stress responses (Sugama and Conti, 2008; Yaguchi et al., 2010), and is a susceptibility factor for depression (Haastrup et al., 2012), another condition that is intimately associated with neurodegenerative processes (Diniz et al., 2013).

Here we tested time-related impacts of pro-inflammatory IL-18 in pure human neuron-like cells. We hypothesized that IL-18 has a role in the development of neuropathological changes related to several neurodegenerative diseases including AD. This is based on our recent findings showing IL-18 effects on tau and its phosphorylation (Ojala et al., 2008), as well as on amyloid precursor protein (APP) processing and Aβ production (Sutinen et al., 2012) in differentiated SH-SY5Y neuron-like cells. The main aim of the present study was to discover other relevant molecules and/or mechanisms in these human cells that are regulated by IL-18 and that may have general importance in the pathogenesis of neurodegenerative diseases, including AD. We used two-dimensional difference-in-gel electrophoresis (2D-DIGE) and mass spectrometry (MS) to find IL-18 regulated targets. The findings were verified by western immunoblotting (WIB).

Our study showed that IL-18 induces time-dependent effects on protein expression profiles in differentiated neuron-like SH-SY5Y cells. The most significant protein changes were found at 24 h but were also evident at 48 and 72 h post-IL-18 treatment, vs. untreated control cells. Following the detection of altered proteins, we focused on targets related to OS. The most interesting targets, DDAH, Septin-2 and MMP14 were also studied from frontal and occipital lobes of AD-patients and healthy controls, as well as the impact of Aβ on their expression. We also evaluated the vacuolization of the neuron-like SH-SY5Y cells and their caspase-1 expression.

## MATERIALS AND METHODS

### CELL CULTURE AND TREATMENTS

SH-SY5Y neuroblastoma cells (DSMZ, Germany) were cultured in Dulbeccos's medium (BioWhittaker/Cambrex, Belgium) containing 4.5 g/L glucose, 5% fetal bovine serum (FBS; HyClone/Pierce; Logan, UT, USA), 2 mM L-glutamine (Cambrex/Lonza), 100 U/ml penicillin and 10 μg/mL streptomycin (Cambrex/Lonza) in a humidified atmosphere 5% $CO_2$ at 37°C. The cells were plated as $3 \times 10^5$ cells/well into the 6-well plate (Nunc™, Denmark), and differentiated within 3 days of treatment with 10 μM all-trans retinoic acid (ATRA; Sigma-Aldrich, St. Louis, MO, USA) followed by 4 days of 50 ng/mL human recombinant brain derived neurotrophic factor (BDNF, Alomone labs, Israel) (Sutinen et al., 2012). Bioactive recombinant human IL-18 (MBL Medical Biological laboratories Co, Ltd., Japan) was added to the culture medium at 150 ng/mL for 24, 48, and 72 h. BDNF was also present during the IL-18 treatments. Since the concentration of IL-18 and BDNF is not known in the very close proximity of the release site, we used an excess concentration (physiological 40–80 ng/ml), also due to their recombinant origin and effort to force the time frame of the experiment to 72 h due to the technical reasons. Also IL-18 binding protein (IL-18BPa/Fc Chimera; R&D Systems) and Aβ42 (Bachem) were used in some experiments.

After the treatment, the washed cells were lysed by using M-PER® Mammalian Protein Extraction Reagent (Pierce). The culture medium was also collected. The protein content was determined with DC Protein assay (BioRad) and samples were stored in −80°C.

### PROTEIN LABELING

Protein samples (135 μg) were prepared from lysates of control and IL-18 treated cells, then purified using the 2-D clean up-kit (GE Healthcare) in accordance with the manufacturer's protocol. Samples of culture medium from the controls and IL-18 treated (500 μL) wells were concentrated to 15 μL by using Nanosep 3K omega centrifugal devices (Pall Corporation). CyDye DIGE Fluor, minimal labeling kit (GE Healthcare) was used for labeling of the lysate or medium proteins. Internal standards, consisting of an

aliquot of all the samples to be used in a particular experiment, was mixed and labeled with Cy2 dye. Control and IL-18 treated samples were randomized and half of these samples were labeled with Cy3 and the other half with Cy5. All dyes were incubated for 30 min in the dark on ice in accordance with the manufacturer's protocol (GE Healthcare). Labeling was terminated by incubation with 1 μL of 10 mM L-lysine for 10 min on ice.

### TWO-DIMENSIONAL GEL ELECTROPHORESIS (2D-DIGE)

The first dimension 4–7 NL ImmobilineTM DryStrips, 24 cm (GE Healthcare) were rehydrated over night by using DeStreakTM rehydration solution (GE Healthcare) and IPG buffer pH 4–7 (GE Healthcare). The labeled samples were formulated for the strips by adding an equal volume of 2× buffer consisting of 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), 2% IPG buffer and 2% dithiothreitol (DTT) (GE Healthcare), followed by incubation on ice for 15 min, after which the samples were cup-loaded into the rehydrated strips. The first dimension was run by applying 500 V (500 kVh), 1000 V (5200 kVh), 8000 V (16500 kVh), and finally 8000 V (42200 kVh) by using an EttanTM IPGphor unit (Amersham). The focused strips were equilibrated in 2% sodium dodecyl sulfate (SDS) equilibration buffer containing 6 M urea, 50 mM Tris pH 8.8, 30% glyserin and 0.002% bromophenol blue, first with added 1% DTT at RT for 12 min, followed by buffer supplemented with 2.5% iodoacetamide (IAA) for another 12 min at room temperature (RT). After the equilibration, the strips were loaded into the 12.5% SDS-polyacrylamide gels (1 mm) in an Ettan DALT-separation unit (Amersham) and run at 12 W/gel for 30 min and 128 W/gel until the bromophenol blue front reached the bottom of the gel. Each gel run contained six separate experiments from the same time-point. Since the minimal labeling technique (Unlü et al., 1997) labels each protein with a maximum of only one dye molecule per protein (Swatton et al., 2004) and all of the dyes have identical molecular weights (MW) and isoelectric points (pI) but differ in their excitation and emission wavelengths, the MW of the proteins were minimally affected. The 2D images were visualized by Typhoon 9400 Variable Mode Imager scanner (GE Healthcare) using 488, 523, and 633 nm lasers for Cy2, Cy3, and Cy5 dyes.

### SPOT ANALYSIS

All the protein-spots in the gel images were quantified by using the DeCyder 6.5.11 software (GE Healthcare). A DeCyder differential in-gel analysis module was used for pair wise comparisons of each sample (Cy3 and Cy5) to the Cy2 mixed standard present in each gel. The DeCyder biological variation analysis module was then used to simultaneously match all the protein-spot images from the six gels and to calculate average abundance ratios across the samples. The protein-spots in the gels were compared to the 2D map databases (SWISS-2DPAGE and SWISS-PROT).

### MASS SPECTROMETRY (MS)

Differentiated proteins within the gels were stained with PlusOne™ Silver Staining Kit (GE Healthcare) according to manufacturer's instructions. The proteins of interest were cut out manually from the silver stained gels, shrank with acetonitrile (ACN) and cleaved with trypsin (Sequencing grade Modified Trypsin V5111; Promega, Madison, WI, USA) at +37°C overnight. Protein MS identification was done in collaboration with the protein chemistry research facility in University of Helsinki, Viikki, and Department of Biosciences, University of Eastern Finland, Kuopio. In the University of Helsinki, the cleaved samples were purified with ZipTip-method and eluted directly from the tips onto a MTP 384 ground steel sample plate with saturated α-cyano-4-hyrdoxycinnamic acid in 0.1% trifluoroacetic acid (TFA) and 60% ACN. Matrix-assisted laser desorption ionization—time of flight (MALDI-TOF) mass spectrometer mass spectra for peptide mass fingerprints (PMF) and MALDI-TOF/TOF mass spectra for identification by fragment ion analysis were acquired in positive ion reflectron mode using an Ultraflex TOF/TOF instrument (Bruker Daltonik GmbH, Bremen, Germany). The PMF spectra were processed with FlexAnalysis (version 3.0) and spectra were internally calibrated by using trypsin peaks. Separation of the protein digests liquid chromatography-MS/MS (LC-MS/MS) were done on a C18 column by using a nanoscale high-performance liquid chromatography (HPLC) system (Ultimate 3000; Dionex, Sunnyvale, CA). The samples were concentrated and desalted on a C18 precolumn (PROTECOL; SGE Analytical Science, Griesheim, Germany) at a flow rate 7 μL/min (0.1% TFA). Peptides were eluted with a linear gradient of ACN in 0.1% formic acid (FA) at a flow rate of 200 nL/min. MS/MS of tryptic peptides was conducted on a hybrid quadrupole/TOF mass spectrometer with NanosprayII source (QSTAR Elite; Applied Biosystems, Foster city, CA). The Nanospray was generated using a PicoTip needle. The peak list was generated with ProteinPilot-software (Applied Biosystem).

At the University of Eastern Finland, the tryptic peptides were separated using the Ultimate/Famos/Switchos capillary liquid chromatography system (LC Packings, Amsterdam, Netherlands). The samples were filtered on-line through PEEK encapsulated titanium filter (VICI/ Valco, Houston, TX, USA) and trapped onto PepMap 100 C18 pre-column (Dionex, Sunnyvale, CA, USA) in 0.1% FA (Sigma Aldrich, Steinhelm, Germany) with 2% ACN (Sigma Aldrich) at a flow rate of 30 μL/min using an Applied Biosystems 400 Solvent Delivery System (Applied Biosystems). After the 3 min of loading and clean-up, the precolumn was automatically switched in-line with a C18 Mass Spec analytical column (Grace Vydac, Hesperia, CA, USA). The peptides were eluted using a linear ACN gradient starting from 0.1% FA with 5% ACN to 30% of 0.1% FA with 95% ACN in 35 min, and finally to 100% of 0.1% FA with 95% ACN. The LC flow was connected to the mass spectrometer by a nanoelectrospray ionization MS (ESI) ion source (MDS Sciex, South San Francisco, CA, USA) using distally coated 10 μm PicoTip emitters (New Objective, MS Wil GmbH, Switzerland). Mass spectra were recorded in a positive mode on a QSTAR XL hybrid quadrupole TOF instrument (Applied Biosystems), using information-dependent acquisition for obtaining MS/MS data.

### PROTEIN IDENTIFICATION

Database searches with obtained data were performed with Mascot search engine against the SwissProt database (http://www.matrixscience.com). The search criteria for both Mascot searches were human-specific taxonomy, trypsin digestion with

one missed cleavage allowed, carbamidomethyl modification of cysteine as a fixed modification and oxidation of methionine as a variable modification. For the PMF spectra the maximum peptide mass tolerance was ±50 ppm. For the LC-MS/MS spectra both the maximum precursor ion mass tolerance and MS/MS fragment ion mass tolerance were 0.2 Da and peptide charge state of +2 or +3 was used.

#### HUMAN SAMPLES

DDAH2, SEPT2 and MMP14 were also examined from post-mortem human brain samples (occipital and frontal lobes as pairs from the same individual). Control samples included 3 women and 3 men (age 83.3 years, SD ±5.7; Braak 1.17 ±0.75, obduction delay 5.7 h ± 2.5; cause of death: four cardiac problems, one pneumonia, one pulmonary embolism). The AD patients included 7 women and 2 men (age 83.1 years, SD ±8.1; Braak 5.22 ±0.67, obduction delay 5.3 h ± 3.0; cause of death: five pneumonia, two cardiac reasons, two pulmonary embolisms). The samples were stored at −80°C until used. The brain tissue samples were homogenized on ice into the lysis buffer containing 10 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 1% Triton X-100, 2 mM EDTA, and Complete Protease inhibitors (Roche Diagnostics; Mannheim, Germany) using a pellet pestle (Kontes; Vineland, NJ, USA). Protein levels were estimated with the DC protein assay kit (Bio-Rad Laboratories; Hercules, CA, USA) according to manufacturer's protocol.

The study followed the recommendations for biomedical research involving humans (Declaration of Helsinki of The World Medical Association 1964 including the revisions and clarifications up to Tokyo 2004 and CIOMS international ethical guidelines for biomedical research involving human subjects), as well as the law concerning information protection. Further, the human studies were approved by the Ethics Committee of Kuopio University Hospital. The written informed consents were obtained from the subjects or their representatives by the clinicians at the Department of Neurology, Kuopio University Hospital. Permission to obtain the post-mortem tissue was provided by the National Board of Medical Legal Affairs/National Supervisory Authority for Welfare and Health.

#### WESTERN IMMUNOBLOTTING (WIB)

For WIB, equal amounts of protein (25 or 35 μg) from cell lysates or human brain samples, and equal volumes of cell culture media (120 μL) were electrophoresed in 12.5% SDS-polyacrylamide gels. Proteins were transferred to Hybond-P PVDF membrane (Amersham Biosciences/GE Healthcare). Full-Range and Low-Range Rainbow Molecular Weight Markers (GE Healthcare) as well as Spectra™ Multicolor Broad Range Protein Ladder (Fermentas) were used for the detection of target proteins correct MW. The antibodies were commercial and evaluated by the suppliers and end users (**Table 1**). However, we performed some characterization studies for a number of antibodies that required validation due to a partially limited use of some of them in the literature. Those studies included different cell lines known to express or had low expression of the targets as well as known inducers (Supplementary data 1). A peptide inhibition assays were also performed to determine the specificity of CRMP2 Thr555 (Supplementary data 2). The membranes were blocked with 5% non-fat milk (Valio, Finland) in phosphate buffered saline (PBS) containing 0.05% Tween-20 (Fluka/Sigma-Aldrich; Steinheim, Germany) or alternatively in Tris buffered saline (TBS) containing 0.1% Tween-20 for 1–2 h. The milk-blocked membranes were immune detected for 1–2 h at RT or over night at +4°C with selected primary antibodies (diluted in 1:400), and washed with PBS/0.05% Tween-20 or TBS/0.1% Tween-20 for 3 × 5 min. The primary antibodies are collected in **Table 1**. The antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), ECM Biosciences, Abcam (Cambridge, UK), Millipore, Sigma-Aldrich and GE Healthcare. The secondary antibodies were used as 1:10000 dilution and 1.5–2 h incubation time.

After the incubations, the membranes were washed as described for 4 × 5 min followed by 15 min wash with PBS or TBS. The targets were detected with Immobilon Western Chemiluminescent HRP Substrate (Millipore; Billerica, MA, USA) and documented to Fujifilm Super RX (Fuji Photo Film Co., Ltd.). The x-ray films were scanned and analyzed with MCID M5-image analysis system (Imaging Research Inc.; Ontario, Canada). The same membrane was used in three to four different antibody reactions after stripping with four times wash with TBS/0.1% Tween-20 at RT followed by shaking in stripping buffer (62.7 mM Tris pH 6.8, 2% SDS, 0.7% β-mercaptoethanol) at +50°C for 30 min and washing six times with TBS/0.1% Tween-20 at RT.

#### STATISTICS

The separate experiments and samples ($n = 5$–17) were analyzed with parametric paired Student's *t*-test (T-t) and non-parametric Mann–Whitney *U*-test (M-W).

## RESULTS

### 2D-DIGE ANALYSIS OF DIFFERENTIATED SH-SY5Y NEURON-LIKE CELLS TREATED WITH IL-18

#### *IL-18 altered protein profiles in SH-SY5Y cells in time related manner*

Time-dependent changes in the protein levels were apparent in differentiated SH-SY5Y cells after 150 ng/mL IL-18 treatments for 24, 48, or 72 h, compared to respective untreated control cells (**Table 2**). The altered protein profiles with greatest differences were identified in the cells exposed to IL-18 for 24 h, approximately 4.2% of the total protein spots were differentially expressed. From this total of 81 differentially expressed proteins, 27 proteins were up- and 54 down-regulated by IL-18 treatment. IL-18 addition for 48 and 72 h also showed significant protein changes vs. untreated cells (48 h: total 65; 45 increased, 20 decreased, approximately 3.3%; 72 h: total 51; 31 increased, 20 decreased, approximately 2.7%). The number of protein spots was greatest at the 48 h time-point (1965 spots) (**Figure 1A**), suggesting enhanced translational modifications occurring in early expressed proteins in comparison to later proteins. Total protein spot number was lowest in non-differentiated, untreated SH-SY5Y neuroblastoma cells, being 1754 (**Figure 1B**). IL-18 also induced some changes in the culture medium at the 72 h time-point (**Figure 1C**). However, the separation of secreted proteins from cell debris of the closed cell culture system proved difficult.

**Table 1 | The list of antibodies used in the study.**

| | Provider | Cat N:o | Raised in | Purification | Sequence | Certification for specificity (company), user, our studies |
|---|---|---|---|---|---|---|
| **PRIMARY ANTIBODY** | | | | | | |
| PRX2 (N-13) | Santa Cruz Bio. | sc-23967 | goat pc | affinity pur. | h, near the N-terminus | (WB); Petrak et al., 2007; suppl.1 |
| PRX3 (12B) | Santa Cruz Bio. | sc-59661 | mouse mc | | h, full length PRX3 | (WB, IF); McCommis et al., 2011; suppl.1 |
| PRX6 (E-18) | Santa Cruz Bio. | sc-55013 | goat pc | affinity pur. | h, internal region | (WB); suppl.1 |
| DJ-1 (N-20) | Santa Cruz Bio. | sc-27004 | goat pc | affinity pur. | h, near N-terminus | (WB, IF); Batelli et al., 2008; suppl.1 |
| BLVRA (2E4) | Santa Cruz Bio. | sc-100511 | mouse mc | | h, recombinant BLVRA | (WB); suppl.1 |
| DDAH2 (C-19) | Santa Cruz Bio. | sc-26071 | goat pc | affinity pur. | h, C-terminus | (WB); Park et al., 2008; suppl.1 |
| TIMP2 (H-140) | Santa Cruz Bio. | sc-5539 | rabbit pc | | h, aa 81-220 | (WB, IHC); Polyakova et al., 2008; suppl.1 |
| MT-MMP-1 (H-72) | Santa Cruz Bio. | sc-30074 | rabbit pc | | h, aa 511-582 | (WB, IHC); Saygili et al., 2011; suppl.1 |
| SEPT2 (N-12) | Santa Cruz Bio. | sc-20408 | goat pc | affinity pur. | h, near the N-terminus | (WB, IHC); Maimaitiyiming et al., 2008; suppl.1 |
| α Enolase (L-27) | Santa Cruz Bio. | sc-100812 | mouse mc | | h, recombinant α-enolase | (WB, IF, IHC); Yu et al., 2010; suppl.1 |
| γ Enolase (N-14) | Santa Cruz Bio. | sc-31859 | goat pc | affinity pur. | h, near N-terminus | (WB), Yamamoto et al., 2011; suppl.1 |
| 14-3-3-γ (C-16) | Santa Cruz Bio. | sc-731 | rabbit pc | affinity pur. | h, C-terminus | (WB, IF, IHC); Schindler et al., 2006; Shiga et al., 2006; suppl.1 |
| 14-3-3-ε (E-20) | Santa Cruz Bio. | sc-31962 | goat pc | affinity pur. | h, internal region | (WB); suppl.1 |
| CRMP2 (clone 1B1) | Santa Cruz Bio. | sc-101348 | mouse mc | | h, synthetic peptide derived from the C-terminus | (WB); Chi et al., 2009; suppl.1 |
| CRMP2 (Ser522), phospho-specific | ECM Biosci. | CP2191 | rabbit pc | affinity pur. | h, P-CRMP (S522) synthetic peptide (coupled to carrier protein) corresponding to aa:s surrounding S522 | (WB); Yoneda et al., 2012 |
| CRMP2 (Thr555), phospho-specific | ECM Biosci. | CP2251 | rabbit pc | affinity pur. | h, P-CRMP2 (T555) synthetic peptide (couple to carrier protein) corresponding to aa:s surrounding T555 | (WB, ICC); suppl.2 |
| CRMP2 (Thr514) | Abcam | ab62478 | rabbit pc | affinity pur. | h, synthetic phosphopeptide derived from the phosphorylation site of T514 (T-V-$T^p$-P-A) | (WB, ICC, IHC), Lim et al., 2014 |
| Caspase 1 (14F468) | Santa Cruz Bio. | sc-56036 | mouse mc | | h, aa 371-390 | (WB); Zhang et al., 2012; suppl.1 |
| Anti-Aβ[4-10]-antibody (W0-2) | Millipore | MABN10 | mouse mc | protein G pur. | h, amyloid β-peptide | (WB, IHC); Ida et al., 1996 |
| Anti-Alzheimer precursor protein A4 (22C11) | Millipore | MAB348 | mouse mc | purified Ig | pur. rc Alzheimer preucrsor A4 (pre4695) fusion protein | (WB, IHC); Mudher et al., 2001 |
| Actin (H-196) | Santa Cruz Bio. | sc-7210 | rabbit pc | | h, aa 180-375 | (WB, intracell. FCM anal.); Calegari et al., 2011 |
| α-Tubulin (B-5-1-2, mouse ascites fluid) | Sigma-Aldrich | T 5168 | mouse mc | | sarkosyl resistant filaments from sea urchin sperm axonemes | (WB, ICC); Czymai et al., 2010 |
| **SECONDARY ANTIBODY** | | | | | | |
| anti-rabbit | GE Healthcare | NA9340 | donkey | affinity pur. | rabbit IgG, peroxidase-linked species specific $(Fab')_2$ fragment | (WB, IHC) |
| anti-mouse | GE Healthcare | NA931 | sheep | affinity pur. | mouseit IgG, peroxidase-linked species specific (whole antibody) | (WB, IHC) |
| anti-goat IgG, $(Fab')_2$-HRP | Santa Cruz Bio. | sc-3851 | donkey | affinity pur. | goat IgG, conjugated with HRP | (WB) |

*The references are listed in supplementary data.*

*h, human; mc, monoclonal; pc, polyclonal, suppl., supplementary data.*

*pur., purified; Ig, immunoglobulin; aa, amino acid; rc, recombinant; HRP, horse radish peroxidase.*

*WB, western immunoblot; ICC, immunocytochemistry; IF, immunofluorescence; IHC, immunohistochemistry; FCM, flow cytometry.*

**Table 2 | IL-18 treatment induced protein changes in differentiated SH-SY5Y cell lysates, including alteration in levels and modifications, vs. to untreated control at different time-points (DeCyder spot detection -software).**

| Time-point | Changed protein spots | Up-regulated | Down-regulated | Total number of spots |
|---|---|---|---|---|
| 24 h | 81 (~4.2%) | 27 | 54 | 1934 |
| 48 h | 65 (~3.3%) | 45 | 20 | 1965 |
| 72 h | 51 (~2.7%) | 31 | 20 | 1866 |

Although the culture medium was taken gently from the cultures and centrifuged prior to use, the medium likely contained some ruptured cell contents due the delicate nature of the neurites. Therefore, due to the great variation in the number of protein spots, the medium was not possible to analyse in detail with this number of experiment repeats ($n = 6$). Nevertheless, the appearance of the IL-18 treated cells at the 72 h time-point prior to harvest is shown in **Figure 2**. The 150 ng/ml IL-18 treated cells were more vacuolized [4.1 vacuoles/ cell, standard error of mean (s.e.m.) ± 0.17; $n = 90$; $p \leq 0.000$ (T-t, M-W)] as well as 100 ng/ml treated cells [3.5 ±0.15; $n = 90$; $p \leq 0.000$ (T-t, M-W)] compared to untreated control cells (2.4 ± 0.12; $n = 90$). When 150 ng ml/ IL-18 treated cells were compared to 100 ng/ml treated ones, the increase of vacuole number was about 15.1% [$p = 0.019$ (T-t); $p = 0.026$ (M-W)]. Cell debris was also apparent indicating apoptotic cells (**Figure 2**).

Some of the proteins exhibiting change under 2D-DIGE were selected (**Figures 1A,C**) and identified by MS. From the 79 identified proteins, 29% were involved in proliferation/ differentiation, about 18% in cell signaling, 16% in transport and 13% in the regulation of oxidation. Proteins of interest were selected as shown in **Table 3**. From these proteins, 14 were of known relevance to neurodegenerative diseases and so were further analyzed by WIB.

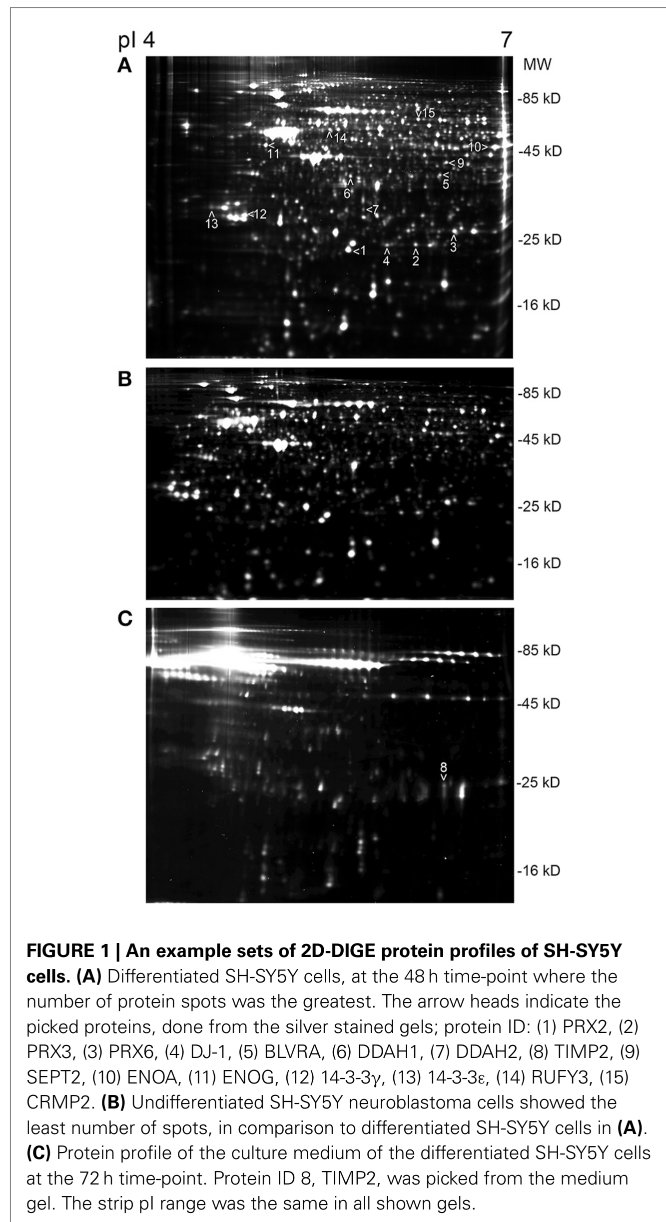



**FIGURE 1 | An example sets of 2D-DIGE protein profiles of SH-SY5Y cells. (A)** Differentiated SH-SY5Y cells, at the 48 h time-point where the number of protein spots was the greatest. The arrow heads indicate the picked proteins, done from the silver stained gels; protein ID: (1) PRX2, (2) PRX3, (3) PRX6, (4) DJ-1, (5) BLVRA, (6) DDAH1, (7) DDAH2, (8) TIMP2, (9) SEPT2, (10) ENOA, (11) ENOG, (12) 14-3-3γ, (13) 14-3-3ε, (14) RUFY3, (15) CRMP2. **(B)** Undifferentiated SH-SY5Y neuroblastoma cells showed the least number of spots, in comparison to differentiated SH-SY5Y cells in **(A)**. **(C)** Protein profile of the culture medium of the differentiated SH-SY5Y cells at the 72 h time-point. Protein ID 8, TIMP2, was picked from the medium gel. The strip pI range was the same in all shown gels.

## WESTERN IMMUNOBLOT ANALYSES OF 2D-DIGE IDENTIFIED PROTEINS WITH NEURODEGENERATION RELEVANCE

### *Oxidative stress related proteins*

Due to the modest changes in expression level observed in 2D-DIGE, the results were verified by WIB with an increased number of repeats along with an additional 6 h time-point. Of the identified proteins, our interest focused on those involved in the oxidative damage evident in AD brains. The WIB data have been collated in **Table 4**. Protein levels of cytoplasmic peroxiredoxin (PRX) 2 were reduced by 24.8% at the 48 h time-point, with mainly mitochondrial PRX3 showing a pronounced 542% increase at 6 h in IL-18 treated cells, compared to untreated controls. Cytoplasmic vesicle-localized PRX6 (Acidic calcium-independent phospholipase A2) was also increased by 124% at 6 h upon IL-18 treatment. The atypical peroxiredoxin-like peroxidase DJ-1 (Park-7), which also prevents synuclein aggregation (Baulac et al., 2009), was reduced by 24% in 48 h treated cells. The levels of another type of antioxidant, cytoplasmic biliverdin reductase A (BLVRA), showed a 91% increase at the 6 h treatment time-point. DDAH2, involved in the nitric oxide (NO) pathway as well as in apoptosis regulation (Wang et al., 2009), was increased by 40% at 48 h in IL-18 treated cells vs. untreated controls. IL-18 showed a concentration dependent DDAH2 increase at 72 h (**Figure 3A**). DDAH1 also showed an increase at the 72 h time-point in the 2D-DIGE analyses, but its expression was not further analyzed by WIB.

A concentration of 150 ng/ml of IL-18 was used in these experiments given our previous work showing that IL-18 does not seem to trigger apoptosis at a concentration of 100 ng/ml (Ojala et al., 2008). This may be due to increased antioxidative protective enzymes as well as the anti-apoptotic Bcl-xL, which we have shown previously to be increased by IL-18 (Sutinen et al., 2012). Also the presence of BDNF during these experiments will have protective effects, given our preliminary test showing that the removal of BDNF led to cells detaching from the culture wells as well as increasing vulnerability to cell death. Here we

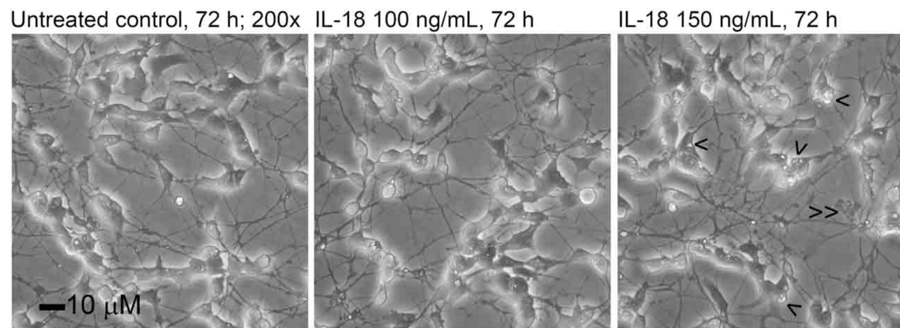



**FIGURE 2 | The appearance of the differentiated SH-SY5Y cells treated with 100 or 150 ng/ml of IL-18 for 72 h.** The number of vacuoles (>) was higher in 150 ng/ml treated cells (about 4.1/ cell) than in untreated control (2.4/ cell) or 100 ng/ml treated cells (3.5/ cell). Some cell debris is also apparent (> >).

**Table 3 | Proteins of human SH-SY5Y cells, identified with mass spectrometry and studied further with western immunoblots.**

| Id no | Protein name | Gene name | SwissProt | ID type | No. of peptides | Sequence coverage (%) | Mowse score | MW | pI |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Peroxiredoxin-2 | PRDX2_HUMAN | P32119 | ESI | 4 | 19 | 87 | 21892 | 5.67 |
| 2 | Thioredoxin-dependent peroxidase reductase | PRDX3_HUMAN | P30048 | MALDI | 7 | 44 | 57 | 27693 | 5.77 |
| 3 | Peroxiredoxin-6 | PRDX6_HUMAN | P30041 | MALDI | 23 | 83 | 272 | 25035 | 6.00 |
| 4 | Protein DJ-1 (Park 7) | PARK7_HUMAN | Q99497 | ESI | 7 | 38 | 149 | 19891 | 6.32 |
| 5 | Biliverdin reductase A | BIEA_HUMAN | P53004 | MALDI | 14 | 41 | 159 | 33428 | 6.06 |
| 6 | N(G), N(G)-dimethylarginine dimethylaminohydrolase | DDAH1_HUMAN | O94760 | MALDI | 30 | 66 | 235 | 31122 | 5.53 |
| 7 | N(G), N(G)-dimethylarginine dimethylaminohydrolase 2 | DDAH2_HUMAN | O95865 | MALDI | 7 | 39 | 96 | 29644 | 5.66 |
| 8 | Metalloproteinase inhibitor 2 | TIMP2_HUMAN | P16035 | ESI | 1 | 6 | 40 | 24399 | 6.48 |
| 9 | Septin-2 | SEPT2_HUMAN | Q15019 | MALDI | 15 | 49 | 145 | 41487 | 6.15 |
| 10 | Alpha-enolase | ENOA_HUMAN | P06733 | MALDI | 22 | 56 | 154 | 47169 | 7.01 |
| 11 | Gamma-enolase | ENOG_HUMAN | P09104 | MALDI | 15 | 49 | 177 | 47269 | 4.91 |
| 12 | 14-3-3 protein gamma | 1433G_HUMAN | P61981 | ESI | 4 | 17 | 36 | 28303 | 4.80 |
| 13 | 14-3-3 protein epsilon | 1433E_HUMAN | P62258 | ESI | 16 | 45 | 345 | 29174 | 4.63 |
| 14 | Protein RUFY3 (single axon-regulated protein) | RUFY3_HUMAN | Q7L099 | ESI | 11 | 22 | 57 | 52965 | 5.36 |
| 15 | Dihydropyrimidinase-related protein 2 (CRMP2) | DPYL2_HUMAN | Q16555 | MALDI | 13 | 25 | 132 | 58163 | 5.77 |

used caspase-1 as a marker for apoptosis initiation showing that IL-18 increased caspase-1 in a concentration dependent manner (**Figure 3A**). Increased caspase-1 expression was also detectable in samples from our previous study (Sutinen et al., 2012), where the cells were treated with 1 μM Aβ42 with or without 100 ng/ml IL-18 or with IL-18 alone (**Figure 4A**). IL-18 as 100 ng/ml for 72 h increased caspase-1 protein expression by 34.9% [s.e.m. ±10.9; $n = 5$; $p = 0.005$ (M-W)] and together with 1 μM Aβ42 by 44.5% (±22.2; $n = 5$; $p = 0.095$), whereas 1 μM Aβ42 as such by 23.3% (±22.9; $n = 5$; $p = 0.095$).

### *Amyloid-β related proteins*

We found changes in Aβ metabolism and its aggregation affecting targets. APP levels showed an IL-18 concentration depended reduction in 72 h treated cells (**Figure 3A**). IL-18 as 150 ng/ml for 72 h reduced protein levels of APP [mean -47.5%; s.e.m. ±9.7; $n = 5$; $p = 0.005$ (M-W)]. Further, as shown previously in a separate experiment (Sutinen et al., 2012), IL-18 treatment induced expression of Aβ and this effect was blocked by IL-18BP in 72 h treated cells (**Figure 4C**). Also Septin-2 (SEPT2), capable of interacting with Aβ aggregates (Pissuti Damalio et al., 2012), decreased by 28% at 24 h following IL-18 treatment but increased to 90.0% after 72 h of treatment. The increase at 72 h was concentration dependent (**Figure 3A**). Aβ42 at 1 μM (4.5 kD) with or without 100 ng/ml IL-18 increased the higher MW form of SEPT2 (approximately 46 kD), compared to untreated control (41 kD) (**Figure 4A**), suggesting interactions of Aβ with SEPT2.

Increased tissue inhibitor of metalloproteinase-2 (TIMP2) levels are evident in AD and, through the inhibition of MMPs, can regulate Aβ degradation (Liao and Van Nostrand, 2010; Merlo and Sortino, 2012). In our studies TIMP2 increased by 77% at the 24 h time-point and by 70% at 48 h in IL-18 treated cells. A slight increase in TIMP2 was also detectable in the culture medium after 48 or 72 h following IL-18 treatment (**Figure 3D**). Although not

**Table 4 | Protein expression level changes of the selected targets in differentiated SH-SY5Y cells.**

| | 6 h | | | | 24 h | | | | 48 h | | | | 72 h | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean % ± s.e.m. | *n* | *p*, T-t | *p*, M-W | Mean ± s.e.m. | *n* | *p*, T-t | *p*, M-W | Mean ± s.e.m. | *n* | *p*, T-t | *p*, M-W | Mean ± s.e.m. | *n* | *p*, T-t | *p*, M-W |
| PRX2 | | | | | −1.1 ± 17.4 | 5 | 0.952 | 0.095 | −24.8 ± 8.6 | 5 | **0.044** | 0.095 | 3.0 ± 24.3 | 5 | 0.909 | 0.577 |
| PRX3 | 542.3 ± 72.8 | 6 | **0.001** | **0.002** | 43.8 ± 21.8 | 15 | 0.074 | 0.096 | 90.4 ± 61.4 | 15 | 0.179 | 0.319 | 12.2 ± 14.5 | 15 | 0.431 | 0.319 |
| PRX6 | 123.8 ± 37.5 | 11 | **0.008** | **0.001** | 2.3 ± 10.3 | 16 | 0.825 | 0.519 | 2.7 ± 12.0 | 16 | 0.823 | 0.519 | 10.6 ± 14.6 | 17 | 0.481 | 0.348 |
| DJ-1 | 13.9 ± 14.6 | 5 | 0.396 | 1.000 | 2.5 ± 14.3 | 6 | 0.868 | 0.305 | −23.9 ± 7.5 | 6 | **0.025** | **0.040** | 9.2 ± 22.0 | 6 | 0.695 | 0.305 |
| BLVRA | 90.8 ± 29.7 | 11 | **0.012** | **0.007** | 38.8 ± 21.8 | 17 | 0.096 | 0.348 | −7.6 ± 9.7 | 17 | 0.445 | 0.118 | 33.2 ± 34.3 | 17 | 0.347 | 0.754 |
| DDAH2 | 75.8 ± 60.9 | 6 | 0.269 | 1.000 | 37.5 ± 29.5 | 11 | 0.116 | 0.247 | 40.3 ± 18.9 | 11 | **0.016** | **0.007** | 9.5 ± 22.6 | 11 | 0.582 | 0.247 |
| TIMP2 | 216.9 ± 109.7 | 5 | 0.119 | 0.095 | 76.6 ± 20.2 | 10 | **0.004** | **0.001** | 70.3 ± 21.7 | 10 | **0.010** | **0.001** | 37.3 ± 29.7 | 10 | 0.240 | 1.000 |
| MMP14 | 29.4 ± 11.3 | 6 | **0.048** | **0.002** | 28.8 ± 19.2 | 10 | 0.169 | 1.000 | 15.4 ± 17.4 | 11 | 0.396 | 0.247 | 89.3 ± 39.3 | 11 | **0.046** | **0.007** |
| SEPT2 | 81.3 ± 48.6 | 4 | 0.392 | 0.219 | −28.2 ± 16.6 | 9 | **0.039** | **0.003** | 10.6 ± 19.3 | 10 | 0.913 | 0.419 | 90.0 ± 40.9 | 9 | 0.097 | **0.034** |
| ENOA | 32.4 ± 40.5 | 6 | 0.459 | 1.000 | −5.4 ± 5.1 | 12 | 0.313 | 0.459 | 13.7 ± 10.2 | 12 | 0.204 | 0.459 | −26.2 ± 9.1 | 12 | **0.015** | **<0.000** |
| ENOG | | | | | 46.4 ± 14.7 | 5 | **0.034** | 0.095 | 73.3 ± 54.6 | 5 | 0.251 | 0.095 | 9.2 ± 23.0 | 5 | 0.711 | 0.577 |
| 14-3-3γ | 111.8 ± 50.2 | 6 | 0.076 | **0.040** | 22.5 ± 17.6 | 8 | 0.241 | 0.369 | 29.9 ± 14.1 | 8 | 0.071 | 0.073 | 11.3 ± 16.8 | 8 | 0.521 | 1.000 |
| 14-3-3ε | −16.1 ± 15.5 | 6 | 0.347 | 0.305 | 42.5 ± 12.1 | 10 | **0.006** | **0.012** | 21.5 ± 20.2 | 11 | 0.312 | 0.700 | −7.1 ± 10.1 | 11 | 0.498 | 0.700 |

*The results are expressed as a Mean %.*

*Mean %: IL-18 treated/untreated control x100-100.*

*s.e.m., standard error of mean; n, number of separate repeats.*

*p, p-value [T-test (paired), T-t; Mann–Whitney U-test, M-W]; significant p-values marked as bold (0.05, 0.01, 0.001).*

detected in 2D-DIGE we examined membrane type 1 metalloproteinase (MT1-MMP/MMP-14), which is a target for TIMP2 and can activate MMP2, possibly important in Aβ degradation (Merlo and Sortino, 2012). Protein levels of MMP14 increased by 29% following 6 h of IL-18 and by 89% in the 72 h treated cells, showing an IL-18 concentration dependent effect (**Figure 3A**). However, whereas IL-18 slightly increased the soluble form of MMP14 in the culture medium, 1 μM Aβ42 treatment for 72 h reduced MMP14 levels (**Figure 4B**).

#### *Other neurodegenerative diseases related proteins*

The other interesting targets identified included enolase and 14-3-3 proteins, known to be altered in AD (Fountoulakis et al., 1999; Butterfield and Bader Lange, 2009). Carbon-oxygen lyase α-enolase (ENOA), involved in glucose metabolism, declined by 26% over 72 h of IL-18 treatment of the cells whereas γ-enolase (ENOG), a neurotrophic-like factor promoting growth, differentiation, survival and regeneration of neurons (Hafner et al., 2012), increased by 46% following 24 h of treatment, compared to controls. 14-3-3ε, involved in brain development and neuronal migration (Toyo-oka et al., 2003), increased by 43% in 24 h IL-18 treated cells, whereas the 14-3-3γ isoform, which displays similar functions, increased by 112%, but only at 6 h of IL-18 treatment.

Increased expression of RUFY3 (single axon-regulated protein) (Mori et al., 2007) was also detected after 72 h in IL-18 treated cells under 2D-DIGE, but this finding was not verified by WIB. Collapsin response mediator protein 2 (CRMP2; Dihydropyrimidinase-related protein 2) was examined given its variety of roles in neurons including development, polarity and neuron projection morphogenesis, axon growth and guidance, neuronal growth cone collapse, and cell migration (Higurashi et al., 2012). We found CRMP2 (62.3 kD, isoform 1; 58.2 kD isoform 2) to be modified at least at its Ser522, Thr514, and Thr555 sites (**Figures 3A,B**). Also, a higher MW-form of CRMP2, induced by 150 ng/ml IL-18 treatment, was detectable (**Figure 3C**). CRMP2 is modified by phosphorylation of several sites, and possibly also in other ways.

#### *Protein levels of DDAH2, SEPT2, and MMP14 were altered in the AD brain*

Based on the above, the most interesting targets were examined from frontal and occipital lobe post-mortem samples of AD patients and healthy age matched controls. An example set of DDAH, SEPT2 and MMP14 is shown in **Figure 5**, and it describes general changes in occipital and frontal lobe samples from one control and one AD patient, examined from the same WIB membrane. As collected in **Table 5**, we found that in occipital lobe, DDAH2 (approximately 29 kD) and SEPT2 levels reduced about 77% in AD patients, whereas DDAH2 antibody reactive protein (DDAHr; approximately 55 kD) increased in frontal lobe almost 140% compared to healthy controls. In healthy controls, DDAHr (approximately 55 kD) was about 50% less in frontal lobe than in occipital lobe. This 55 kD DDAHr form requires further studies. In AD patients, MMP14 was increased around 80% compared to healthy controls, although its general level in AD patients seemed to be less than in healthy controls in both lobes. Therefore, the general slight reduction of MMP14 levels in AD may alter

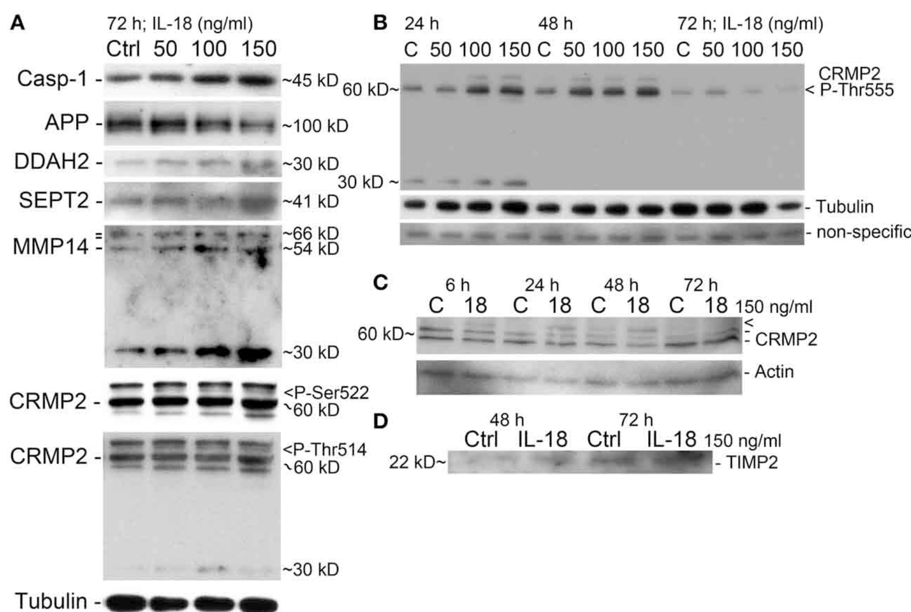



**FIGURE 3 | Western immunoblot verification of the selected targets. (A)** IL-18 induced our apoptosis marker caspase-1 in a concentration dependent manner, with APP level reduced in 72 h treated cells. DDAH2 and SEPT2 levels are increased as well as processed MMP14 (54 kD) and precursor MMP14 (above, 66 kD, propeptide 64 kD). The 30 kD form may be some a non-typical cleavage form of MMP14 (antibody detects aa 511–582 at C-terminus). CRMP2 P-Ser522 specific antibody detected a faint concentration dependent band (>). CRMP2 P-Thr514 also increase (>), with the intensity of CRMP2 different to that of CRMP-2 P-Ser522. Also an additional band about 30 kD was detectable. All the results are from the same membrane. **(B)** CRMP2 also showed increased phosphorylation at Thr555 at 24 and 48 h time-points, after which it reduced, as detected by specific antibody. The band about 30 kD, was evident only at 24 h. Tubulin shows some co-regulation. Non-specific band indicates evenness of sample loading. **(C)** CRMP2 was studied also with non-phosphor specific antibody after 150 ng/ml IL-18 treatment. CRMP2 (isoform 1: 62 kD, isoform 2: 58 kD) were unaffected, but high MW-form above was IL-18 responsive (>), which may be a modified form of CRMP2. CRMP is phosphorylatable at several sites but other modifications may also exist. Actin was used as a loading control. **(D)** IL-18 at 150 ng/ml also increased TIMP2 levels in the culture medium of SH-SY5Y cells vs. untreated control.

for instance the Aβ degradation pathways. The specific role of MMP14 in AD pathogenesis requires further investigation. Due to the biological variations of human samples, more definite results of the given targets require higher number of AD and control samples.

In conclusion, the results of many neuronal cell types including post mortem AD samples do not correlate well in our living one cell type neuronal model in this number of repeats. However, our model may describe the events in neurons *in vivo* when acute high levels or even chronic lower levels of IL-18 are present. Our earlier concentration studies have suggested that lower doses of IL-18 (50 ng/ml) also have similar effects than higher 100 or 150 ng/ml doses, but in a longer timeframe, apparent also in some level in **Figures 3A,B**. For the maintenance of the cells, longer than 72 h incubation time would require the culture medium change leading to distortions of the experiment as well as the results.

## DISCUSSION

In the present study, we found that IL-18 had time-dependent effects on protein expression profiles in differentiated neuron-like SH-SY5Y cells. As summarized in **Figure 6**, our study shows that IL-18 either by itself or through induction of OS, differentially induces protective enzymes, as well as proteins associated with Aβ degradation vs. accumulation and apoptosis. IL-18 was shown to regulate targets affecting glucose metabolism, survival and regeneration, neuronal differentiation, and brain development. Many of these proteins have previously been studied in different AD brain regions, and are linked to AD/dementia (Korolainen et al., 2010). The findings are also in line with our earlier studies showing increased IL-18 in the brain of AD patients (Ojala et al., 2009), in turn contributing to many time-dependent protein changes, which can contribute to the pathogenesis of AD, including via NFT and Aβ-plaque formation (Ojala et al., 2008; Sutinen et al., 2012).

OS plays an important role in the pathogenesis of numerous age-related neurodegenerative diseases including multiple sclerosis (MScl) and Parkinson's disease (PD) (Anderson and Maes, 2014), as well as AD, by inducing cell damage and wider inflammatory processes (Krapfenbauer et al., 2003). Uncontrolled OS can lead to apoptosis but it can also cause sublethal damages that induce regenerative mechanisms (Ojala et al., 2008), leading to protein alterations typical of AD. However, Aβ itself can also induce OS (Opazo et al., 2002) and, as we showed, IL-18 can induce Aβ-production. Oxidatively modified proteins in turn are associated with tau and Aβ pathology (von Bernhardi and Eugenín, 2012). The brain is highly sensitive to OS due to its richness of fatty acids that can undergo peroxidation and its

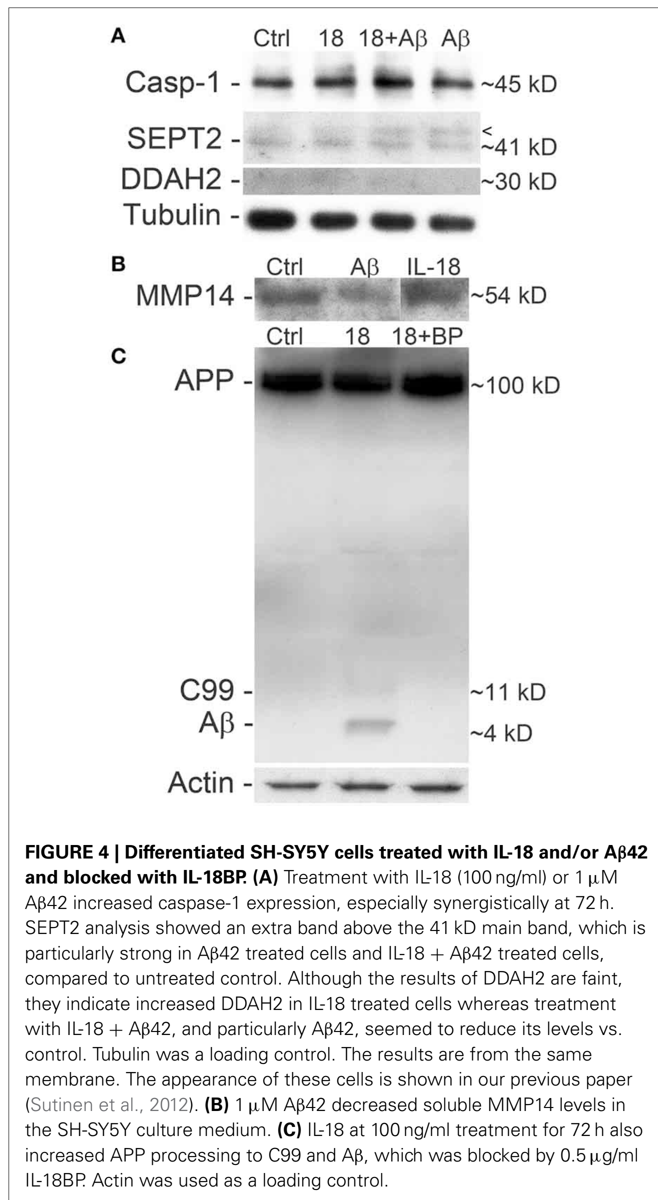



**FIGURE 4 | Differentiated SH-SY5Y cells treated with IL-18 and/or Aβ42 and blocked with IL-18BP. (A)** Treatment with IL-18 (100 ng/ml) or 1 μM Aβ42 increased caspase-1 expression, especially synergistically at 72 h. SEPT2 analysis showed an extra band above the 41 kD main band, which is particularly strong in Aβ42 treated cells and IL-18 + Aβ42 treated cells, compared to untreated control. Although the results of DDAH2 are faint, they indicate increased DDAH2 in IL-18 treated cells whereas treatment with IL-18 + Aβ42, and particularly Aβ42, seemed to reduce its levels vs. control. Tubulin was a loading control. The results are from the same membrane. The appearance of these cells is shown in our previous paper (Sutinen et al., 2012). **(B)** 1 μM Aβ42 decreased soluble MMP14 levels in the SH-SY5Y culture medium. **(C)** IL-18 at 100 ng/ml treatment for 72 h also increased APP processing to C99 and Aβ, which was blocked by 0.5 μg/ml IL-18BP. Actin was used as a loading control.

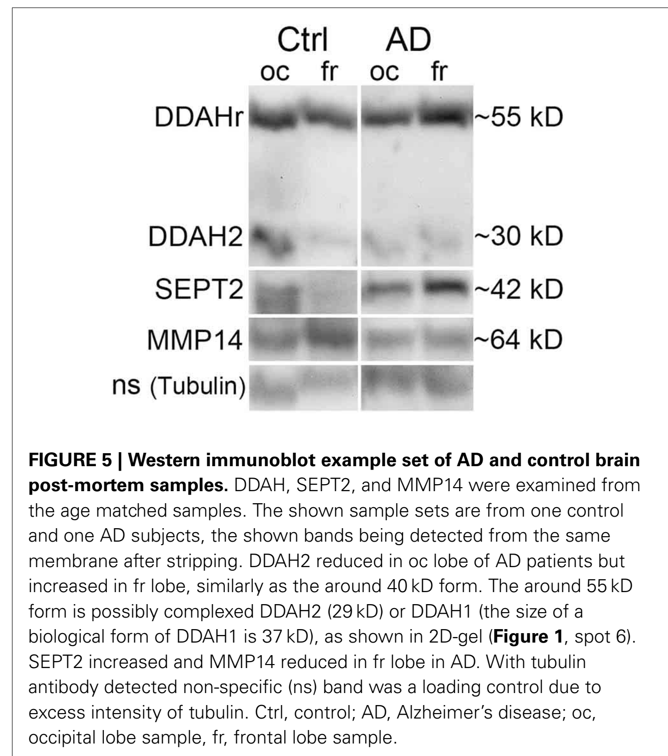



**FIGURE 5 | Western immunoblot example set of AD and control brain post-mortem samples.** DDAH, SEPT2, and MMP14 were examined from the age matched samples. The shown sample sets are from one control and one AD subjects, the shown bands being detected from the same membrane after stripping. DDAH2 reduced in oc lobe of AD patients but increased in fr lobe, similarly as the around 40 kD form. The around 55 kD form is possibly complexed DDAH2 (29 kD) or DDAH1 (the size of a biological form of DDAH1 is 37 kD), as shown in 2D-gel (**Figure 1**, spot 6). SEPT2 increased and MMP14 reduced in fr lobe in AD. With tubulin antibody detected non-specific (ns) band was a loading control due to excess intensity of tubulin. Ctrl, control; AD, Alzheimer's disease; oc, occipital lobe sample, fr, frontal lobe sample.

high oxygen demand, coupled to a relative scarcity of robust antioxidant systems (Lee et al., 2010). Among the multiple mechanisms and triggers by which OS can accumulate, inflammatory cytokines can sustain oxidative and nitrosative stress, leading eventually to increased levels of oxidation-altered metabolites. As such, understanding of the consequences of inflammation and OS is important since it may elucidate the initial events underlying AD.

We found expression changes in PRXs after the IL-18 treatments. PRXs (PRX1-6) are a family of multifunctional antioxidant thioredoxin-dependent peroxidases that are differentially localized intracellularly, with further variation across different cell types (Hanschmann et al., 2013). The major functions of PRXs involve cellular protection against OS, including ROS-mediated DNA-fragmentation, modulation of intracellular signaling cascades that utilize hydrogen peroxide as a second messenger molecule and the regulation of cell proliferation. PRX2 is expressed exclusively in neurons (Krapfenbauer et al., 2003). PRX3 levels are decreased in the AD brain, and since mitochondria are generally also extensively damaged in AD, leakage may contribute to its reduction (Krapfenbauer et al., 2003). PRX6 is increased in astrocytes, diffuse plaques and in neuritic plaques, but not in AD neurons (Power et al., 2008). It can reduce hydrogen peroxide as well as short chain organic, fatty acids and phospholipid hydroperoxides, indicating its importance in the regulation of phospholipid turnover (Fisher, 2011). PRX2 deficient neurons are particularly sensitive to hydrogen peroxide induced cell death. PRX2, like anti-apoptotic Bcl-2 and Bcl-xL proteins, can also interact with presenilin-1 (PS-1), thereby being involved in processing of APP to Aβ. This binding can provide protection against neuronal apoptosis (Alberici et al., 1999; Zhou et al., 2002).

DJ-1 reduced by IL-18 treatment. DJ-1, a susceptibility gene in PD, can scavenge hydrogen peroxide and function as a redox-sensitive chaperone (Batelli et al., 2008; Baulac et al., 2009). It can also regulate oxidant status via the modulation of Cu/Zn-superoxide dismutase-1 (Cu/Zn-SOD) levels (Wang et al., 2011), as well as preventing α-synuclein aggregation (Batelli et al., 2008). DJ-1 is also detectable in tau inclusions (Rizzu et al., 2004) and further, it is required to correct mitochondrial morphology and function, as well as the autophagy of dysfunctional mitochondria. Therefore, loss of its function generally leads to neurodegeneration, likely mediated by mitochondria (Irrcher et al., 2010). As a summary, the decrease in DJ-1, induced by IL-18, is likely to have wide ranging effects, including mitochondrial function and antioxidant regulation.

**Table 5 | Expression level changes of the selected target proteins in occipital and frontal lobes of AD patients and healthy controls.**

| | Mean ± s.e.m. | *n* | Mean ± s.e.m. | *n* | Mean % | *p*, T-t | *p*, M-W | Mean % | *p*, T-t | *p*, M-W |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Ctrl, oc** | | **AD, oc** | | **AD/Ctrl, oc** | | | **Ctrl, fr/oc** | | |
| DDAHr ~55 kD | 1.60 ± 0.40 | 6 | 1.39 ± 0.28 | 9 | −12.7 | 0.673 | 0.749 | −49.0 | **0.049** | 0.109 |
| DDAH2 ~29 kD | 5.44 ± 2.23 | 4 | 1.22 ± 0.65 | 5 | −77.6 | 0.084 | **0.050** | −78.9 | 0.055 | 0.083 |
| SEPT2 | 4.57 ± 1.92 | 4 | 1.03 ± 0.43 | 5 | −77.5 | 0.084 | **0.027** | −56.3 | 0.145 | 0.248 |
| MMP14 | 1.54 ± 0.62 | 6 | 0.94 ± 0.12 | 9 | −38.8 | 0.272 | 0.631 | 48.8 | 0.168 | 0.200 |
| | **Ctrl, fr** | | **AD, fr** | | **AD/Ctrl, fr** | | | **AD, fr/oc** | | |
| DDAHr ~55 kD | 0.82 ± 0.15 | 6 | 1.95 ± 0.32 | 9 | 138.9 | **0.018** | **0.010** | 39.6 | 0.107 | 0.145 |
| DDAH2 ~29 kD | 1.15 ± 0.52 | 4 | 1.01 ± 0.40 | 5 | −11.9 | 0.837 | 1.000 | −17.3 | 0.394 | 0.917 |
| SEPT2 | 1.99 ± 1.10 | 4 | 1.26 ± 0.50 | 5 | −37.1 | 0.531 | 0.806 | 21.8 | 0.371 | 0.754 |
| MMP14 | 2.29 ± 0.40 | 6 | 1.71 ± 0.36 | 9 | −25.3 | 0.309 | 0.262 | 81.6 | **0.029** | **0.019** |

*Mean: calculated from the numerical band densities [Graph density × area (G/DxA), expressed as a percentage of the total area scanned] after normalization with tubulin; numerical value describes the relative intensity of the examined band.*

*Mean %: AD/Ctrl or frontal/occipital x100-100.*

*s.e.m., standard error of mean; n, number of individuals.*

*P, p-value [T-test, T-t (paired); Mann–Whitney U-test, M-W]; significant p-values marked as bold (0.05, 0.01, 0.001).*

*Ctrl, healthy control; AD, Alzheimer's disease; oc, occipital lobe; fr, frontal lobe.*

IL-18 treatment increased BLVRA levels, as evident also in AD (Barone et al., 2011). Evolutionarily conserved BLVR and heme oxygenase (HO) have an essential role in the fight against OS. BLVR is expressed in all tissues as BLVRA (dominant in adults) and BLVRB (dominant in fetal state) (Kim and Park, 2012). Multifunctional BLVR is regulated by a number of factors including biliverdin and phosphorylation, which in turn are regulated by OS and free radicals (Wegiel and Otterbein, 2012). HO is needed to cleave the heme ring to form biliverdin (BV), with soluble BLVR converting BV to the potent antioxidant bilirubin (BR) (Jansen et al., 2010). Both BR and BV are potent scavengers of peroxyl radicals (Jansen and Daiber, 2012). BR can also interact with and neutralize NO radicals leading to the formation of NO-bilirubin (Barone et al., 2009), whereas BV, through NO-dependent nuclear translocation of BLVR, inhibits Toll-like receptor-4 (TLR4) expression (Wegiel et al., 2011), another factor known to regulate AD susceptibility (Chen et al., 2012). Interestingly, due to the lipophilic nature of BR, it has suggested to be particularly effective against lipophilic ROS (Jansen and Daiber, 2012). BLVR is also known to function as a Ser/Thr/Tyr-kinase and transcription factor as well as in cell-signaling, mediated by protein kinase C (PKC), mitogen activated protein kinase (MAPK), and phophatidylinositol 3-kinase (PI3K) (Lerner-Marmarosh et al., 2005; Gibbs et al., 2012; Kim and Park, 2012). Therefore, the increase in BLVR by IL-18 is likely to modulate neuronal antioxidants, transcription and intracellular signaling pathways.

Protein levels of DDAH were increased in IL-18 treated cells as well as the higher MW DDAHr within the frontal lobes of AD patients. DDAH controls cellular methylarginine concentration by hydrolyzing N(G),N(G)-dimethyl-L-arginine (ADMA), and N(G)-monomethyl-L-arginine (MMA) which act as inhibitors for nitric oxide synthase (NOS) (Selley, 2004; Pope et al., 2009). Catabolization of these endogenous inhibitors by DDAH may increase NOS activity and NO production with resultant increases in nitrosative stress. NO also has an important role in synaptic events regulating learning and memory, and its dysregulation may lead to synaptic dysfunction (Selley, 2004). Further, NO is a vasodilator and can regulate blood pressure (Dayoub et al., 2008). DDAH1 localizes uniformly in the cytosol and nucleus, whereas DDAH2 is in the cytosol (Birdsey et al., 2000), but may also translocate to mitochondria upon IL-1β stimulation (Cillero-Pastor et al., 2012). Interestingly, ADMA/DDAH pathway can regulate angiogenesis (Fiedler et al., 2009). Overall, the increase observed in DDAH1 detected in 2D-gels and DDAH2 (29 kD) following IL-18 treatment will alter NOS, NO, and nitrosative stress, as well as NO related signaling processes. DDAHr (around 55 kD) requires further studies.

TIMP2 and MMP14 levels were time-dependently increased in IL-18 treated cells. TIMP2 inactivates matrix MMPs by covering their catalytic zinc cofactor site (Brew and Nagase, 2010). On the other hand, MMP14 is a physiological activator for several MMPs, including for pro-MMP2, complexed with TIMP2 (Lehti et al., 1998; Itoh et al., 2001). MMPs can also be activated by the free radical peroxynitrite (ONOO-) (Ridnour et al., 2012). IL-18 inducible MMP2 and MMP9 are expressed in SH-SY5Y cells (Ishida et al., 2004; Chandrasekar et al., 2006; Merlo and Sortino, 2012), and further, MMP2, -9 as well as a soluble MMP14 form can participate in the degradation of Aβ (Liao and Van Nostrand, 2010; Merlo and Sortino, 2012). However, increased PRX6 expression is associated with increased levels of MMP9 and the urokinase-type plasminogen activator (uPA) as well as decreased TIMP2 (Chang et al., 2007; Lee et al., 2009b). Both uPA and tissue-type plasminogen activator (tPA) are also expressed in SH-SY5Y cells (Neuman et al., 1989), and both of them, via plasminogen, increase the protease plasmin, also able to degrade Aβ (Tucker et al., 2000) and regulate MMP levels. Active plasmin can also remodel the extracellular matrix (ECM) directly, as well as indirectly via the activation of MMPs (Baramova et al., 1997). In AD patients, MMP14 levels are higher

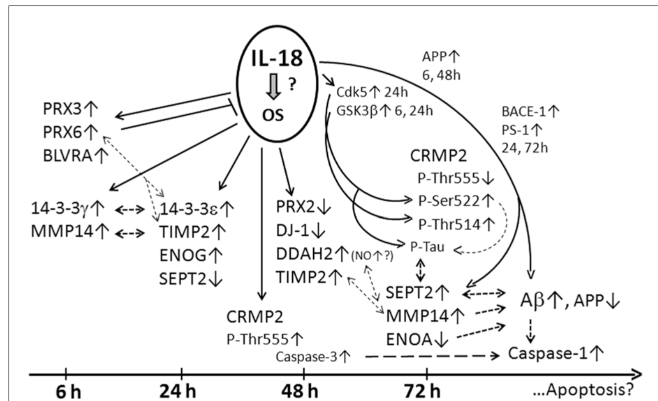



**FIGURE 6 | Summary figure of the WIB results.** IL-18 may induce OS due to increase of antioxidative **PRX3**, -6, and **BLVRA** in SH-SY5Y cells. **14-3-3γ** (modulator of apoptosis; intracellular trafficking; cell cycle control; signal transduction; and metabolism), **14-3-3ε** (anti-apoptotic; a component of the prion/amyloid deposits of Gerstmann-Straüssler-Scheinker disease). 14-3-3ε can bind PRX6, translocating PRX6 to lysosomes when MAPK is activated. Both 14-3-3γ and -ε can prevent α-synuclein inclusion formation. 14-3-3 proteins can complex with tau and GSK3β promoting tau phosphorylation and NFT formation. 14-3-3ε can increase MMP2. **TIMP2** (MMP inactivator), **MMP14** (a physiological activator for pro-MMP2 complexed with TIMP2). IL-18 inducible MMP2 and MMP9 are also expressed in SH-SY5Y cells. MMP2, -9 and soluble MMP14 can participate in Aβ degradation. Increased PRX6 is linked with increased MMP9 and uPA, and decreased TIMP2 expression. uPA and tPA are expressed in SH-SY5Y cells, and both, via plasminogen, increase protease plasmin, also able degrading Aβ and regulating MMP levels. Active plasmin can remodel directly and indirectly, via activation of MMPs, the ECM. **ENOG** (glycolysis; a neurotrophic-like factor promoting growth, differentiation, survival, and regeneration of neurons). The neurotrophic activity is mediated by the C-terminus which activates PI3K/Akt and MAPK/ERK. Cathepsin X, which activity increases during aging and interacts also with Aβ-plaques, cleaves the C-terminus of ENOG and ENOA, impairing the survival and neuritogenesis of neuronal cells. **DJ-1** (scavenges $H_2O_2$; modulates Cu/Zn-SOD; a redox-sensitive chaperone; prevents α-SNC aggregation; detectable in tau inclusions; loss of function leads to neurodegeneration, likely mediated by mitochondria). DDAH (regulates apoptosis; controls cellular methylarginine concentration by hydrolyzing NOS inhibitors ADMA and MMA). **DDAH** can increase NOS activity, NO production and resultant increase in nitrosative stress. NO also regulates learning and memory. ONOO- can activate MMPs. **ENOA** (glycolysis; plasminogen receptor). Interaction of plasminogen with ENOA enhances its activation to plasmin by tPA or uPA. Modifications of ENOA can lead to its catalytic inactivation, which can alter its role as a plasminogen receptor, effecting ECM remodeling, neuronal survival and Aβ degradation. **SEPT2** (interacts with Aβ; linked to pathological features of Aβ plaques and NFTs; filament-forming cytoskeletal GTPase that can modulate cytokinesis, cell division, exocytosis, and membrane trafficking). Generally, Septins form hetero-oligomeric complexes which assemble into filaments, mediated by the GTPase activity. **CRMP2** (mediates Sem3A signaling in the nervous system; interacts e.g., with tubulin, Numb, kinesin1, and Sra1; promotes axon elongation; phosphorylated by several Ser/Thr kinases (ROCK: Thr555, Cdk5: Ser522, GSK3β: Ser518, Thr514,509). Ser522-phosphorylation is essential for sequential phophorylations by GSK3β at Ser518 and Thr514,509. These phosphorylation steps are required in Sem3A signaling. The phosphorylations disrupt the interaction of CRMP2 with tubulin or Numb. CRMP2 hyperphosphorylation is an early event in AD progression and occurs downstream of APP, but neither excessive Aβ42 peptide nor neurotoxicity alone are sufficient to promote it. CRMP2 hyperphosphorylated at Thr509 and Ser518,522 is also present in NFTs. IL-18 can induce **Cdk5** and **GSK3β** and **caspase-3** (Ojala et al., 2008) as well as **APP**, **BACE-1**, and **PS-1** (Sutinen et al., 2012) marked as smaller font. Interactions are shown with dashed lines.

in the frontal lobe compared to the occipital lobe, but seemed to be less than in healthy controls. As such, IL-18 regulation of PRX6, TIMP2, NO, MMPs and MMP14 may be associated with the regulation of Aβ production as well as plasmin and MMP mediated Aβ degradation.

Whereas ENOG levels increased, ENOA levels declined time-dependently in IL-18 treated cells. Homo- or heterodimeric ENOs are glycolytic enzymes, which are also associated with hypoxia and ischemia as well as AD (Sultana et al., 2007; Butterfield and Bader Lange, 2009). ENOA is consistently found to be up-regulated in mild cognitive impairment and AD, in which glucose hypometabolism and upregulation of glycolytic enzymes are predominant features (Castegna et al., 2002). ENOA can be glycosylated (Owen et al., 2009), oxidized (Castegna et al., 2002), glutathionylated (Newman et al., 2007), or nitrated (Reed et al., 2009), which, when elevated, lead to its catalytic inactivation. Depending on the pathophysiological condition of the cells, ENOA can also function as a plasminogen receptor on the surface of several cell types (Díaz-Ramos et al., 2012). Interaction of plasminogen with ENOA enhances its activation to plasmin by tPA or uPA (Sinniger et al., 1999). However, ENOA modifications that lead to its catalytic inactivation are also likely to alter its role as a plasminogen receptor (Gentile et al., 2009), with consequences for ECM remodeling, neuronal survival and Aβ degradation. Although ENOG levels are increased markedly, for instance in cardiovascular disease, cerebral trauma, brain tumors, and Creutzfeldt-Jakob disease, it can also function as a neurotrophic-like factor promoting growth, differentiation, survival, and regeneration of neurons after translocation and binding to the plasma membrane (Aksamit et al., 2001; Hafner et al., 2010, 2012). Its neurotrophic activity is regulated by the C-terminal peptide which activates the PI3K/Akt and MAPK/ERK signaling pathways (Hafner et al., 2012). Cathepsin X, the activation of which increases in an age-dependent manner and is also associated with Aβ-plaques (Wendt et al., 2007), cleaves the C-terminal amino acids of ENOG as well as ENOA, impairing the survival and neuritogenesis of neuronal cells (Obermajer et al., 2009). Whether cathepsin X is inducible by IL-18 is unknown.

We found time-dependent changes in the expression of 14-3-3 proteins. Generally, the 14-3-3 family of proteins is a highly conserved group of molecules that play important roles in apoptosis, intracellular trafficking, cell cycle control, signal transduction, and metabolism (Steinacker et al., 2011). 14-3-3ε can protect against ischemic cerebral infarction and neuronal apoptosis (Wu et al., 2009), but it is also a component of the prion protein amyloid deposits of Gerstmann-Straüssler-Scheinker disease (Di Fede et al., 2007) indicating a role in amyloid regulation in other neurological conditions. 14-3-3ε can also bind PRX6, which leads to translocation of PRX6 to lysosomal organelles in the presence of MAPK activity (Sorokina et al., 2011). 14-3-3γ and 14-3-3ε also have binary interactions with leucine-rich repeat Ser/Thr-protein kinase 2 (LRRK2), detected in α-synuclein positive Lewy bodies, as well as in Hirano bodies in AD (Perry et al., 2008; Li et al., 2011). Both 14-3-3γ and -ε can prevent α-synuclein inclusion formation (Yacoubian et al., 2010) and via its association with phosphorylated Bad prevent its apoptotic actions (Koh, 2008). 14-3-3 proteins can also bind simultaneously to tau and GSK3β and promote tau phosphorylation and formation of NFTs

(Yuan et al., 2004). Further, 14-3-3ε can increase MMP2 expression via p38 MAPK signaling (Lee et al., 2009a). As such, the different 14-3-3 isoforms have variable effects that may contribute to AD degenerative processes, as well as having wider beneficial effects, which will be modulated by IL-18.

IL-18 regulated SEPT2 expression in time-dependent manner. There was also an indication that SEPT2 levels are increased in the frontal lobe compared to occipital lobe of AD patients, although this requires further investigation. SEPT2, capable of interacting with Aβ aggregates, has been linked with NFTs and pathological features of Aβ plaques in AD (Kinoshita et al., 1998; Pissuti Damalio et al., 2012). SEPT2 is a member of the septin family, which is a conserved group of filament-forming cytoskeletal GTPases. The members form hetero-oligomeric complexes which assemble into filaments in a GTPase activity required manner. As such the septins have a wide range of physiological effects, including roles in the regulation of cytokinesis, cell division, exocytosis, and membrane trafficking (Peterson and Petty, 2010; Mostowy and Cossart, 2012).

The other interesting targets, found in 2D-DIGE, were RUFY3 and CRMP2. RUFY3 is implicated in single axon formation by developing neurons due to its ability to inhibit PI3K in minor neuronal processes, thereby preventing the formation of additional axons (Mori et al., 2007). We also found time-dependent changes in phosphorylation of CRMP2. CRMP2 is a member of the CRMP family and promotes axon elongation in primary hippocampal neurons or SH-SY5Y cells (Cole et al., 2004). CRMP2 seems to be involved in neurodegenerative mechanisms common to AD and quite possibly also other neuroinflammatory conditions, such as MScl (Petratos et al., 2008, 2012). Generally, CRMPs are the major phosphoproteins in the developing brain, and they mediate Semaphorin3A (Sem3A) signaling in the nervous system. They interact with many factors, including tubulin, Numb, kinesin1, and Sra1 (Uchida et al., 2009). For instance CRMP2 is phosphorylated by several Ser/Thr kinases, such as Rho kinase (ROCK), Cdk5, and GSK3β especially at its C-terminal sites. As we showed previously, IL-18 can induce Cdk5 and GSK3β (Ojala et al., 2008), suggesting a role for IL-18 in the regulation of CRMP2. ROCK phosphorylates CRMP2 at Thr555 and Cdk5 at Ser522, with Ser522-phosphorylation being essential for sequential phophorylations by GSK3β at Ser518, Thr514, and Thr509. This sequential phosphorylation of CRMP2 by Cdk5 and GSK3β is a necessary step in Sem3A signaling (Uchida et al., 2009), with the CRMP2 phosphorylations disrupting the interaction with other factors including tubulin or Numb. Further, Sem3A or ROCK pathways seem to independently regulate growth cone collapse (Arimura et al., 2000), whereas phosphorylation sites and levels of CRMP2 may control axon growth by coordinating the stability and configuration of growth cone microtubules (Hur et al., 2011). However, abnormal CRMP2 hyperphosphorylation is an early event in AD progression (Cole et al., 2007) and occurs downstream of APP, but neither excessive Aβ42 peptide nor neurotoxicity alone are sufficient to promote it (Williamson et al., 2011). CRMP2, hyperphosphorylated at Thr509, Ser518, and Ser522, is also present in NFTs (Gu et al., 2000). With axonal growth cones being dynamic extensions of developing axons that seek appropriate synaptic targets, it is plausible that hyperphosphorylated forms of CRMP2 that are evident in AD may represent a blockade of such growth mechanisms in dystrophic/swollen neurites.

In conclusion, the increase in antioxidative enzymes BLVRA, PRX3 and -6 as well as generally neuroprotective 14-3-3 proteins is likely driven either by IL-18 itself or by induction of OS (**Figure 6**). DDAH2 increases at a later time-point suggest increased nitrosative stress, and reduced DJ-1 increased mitochondrial stress, which combined to Aβ production may lead to activation of caspase-1 and apoptosis. Increased TIMP2 as well as decreased ENOA and 14-3-3ε may indirectly lead to increased Aβ accumulation, whereas increased SEPT2 may enhance Aβ aggregation. Changes in CRMP2 phosphorylation suggest impaired interactions with tubulin. The most interesting novel targets were examined in AD brain samples, where DDAHr (about 55 kD) was increased in frontal lobes of AD patients and general levels of MMP14 appeared to be decreased with this number of repeats. The role of SEPT2 in AD requires further studies. The SH-SY5Y findings are in line with our earlier studies, in which we have shown that IL-18 can induce several time-dependent protein changes that have a role in the pathogenesis of AD including NFT and Aβ-plaque formation (Ojala et al., 2008; Sutinen et al., 2012). Further, in the present study increased antioxidative enzymes seems to be followed by APP processing to Aβ (Sutinen et al., 2012). IL-18 also increased concentration dependently caspase-1 expression, indicating initiation of the apoptotic pathway. Whether APP processing to Aβ in relation to OS or its regulating enzymes is a cause or a consequence of the activation of apoptotic pathway in neurons requires further studies. However, in aged normal human neurons especially, the poorly functioning defense systems may lead to apoptosis and/or enhanced processing of APP to Aβ. Overall, our work strongly suggests that increased IL-18 plays an important role in AD, particularly in overlapping the biological underpinnings of the diseases that increase AD risk, such as obesity, type-2 diabetes and depression as well as when wider AD risk gene alleles are present.

## AUTHOR CONTRIBUTIONS

Johanna O. Ojala carried out the cell culture, Elina M. Sutinen and Johanna O. Ojala the laboratory work including WIBs, and Elina M. Sutinen, Johanna O. Ojala, Minna A. Korolainen, and Jukka Häyrinen the gel- and mass spectrometry analyses; Johanna O. Ojala, Tuula Pirttilä, Elina M. Sutinen, and Minna A. Korolainen contributed to the design of the study; Irina Alafuzoff provided the human samples; Tuula Pirttilä, Elina M. Sutinen, Minna A. Korolainen, and Hilkka Soininen provided the research support. Johanna O. Ojala and Elina M. Sutinen wrote the manuscript. Steven Petratos, Antero Salminen, and Hilkka Soininen had intellectual input to the manuscript. All authors read and approved the final version of the manuscript.

## ACKNOWLEDGMENTS

This study was funded by the Academy of Finland (grant 110320, Prof. Tuula Pirttilä), Finnish Cultural Foundation; North Savo Regional Fund (Elina M. Sutinen), Academy of Finland (grant 127684, Minna A. Korolainen) and cNeuPro (Minna A. Korolainen). The authors thank Drs. Tuula Nyman and Juha

Piltti for their skillful technical assistance and George Anderson for checking the language of the manuscript. We also thank the patients and the relatives of the patients that have provided permission to use the samples, and those who have taken care of those valuable samples.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2014.00214/abstract

## REFERENCES

Aksamit, A. J. Jr., Preissner, C. M., and Homburger, H. A. (2001). Quantitation of 14-3-3 and neuron-specific enolase proteins in CSF in Creutzfeldt-Jakob disease. *Neurology* 57, 728–730. doi: 10.1212/WNL.57.4.728

Alberici, A., Moratto, D., Benussi, L., Gasparini, L., Ghidoni, R., Gatta, L. B., et al. (1999). Presenilin 1 protein directly interacts with Bcl-2. *J. Biol. Chem.* 274, 30764–30769. doi: 10.1074/jbc.274.43.30764

Alboni, S., Cervia, D., Sugama, S., and Conti, B. (2010). Interleukin 18 in the CNS. *J. Neuroinflammation* 7, 9. doi: 10.1186/1742-2094-7-9

Anderson, G., and Maes, M. (2014). Neurodegeneration in Parkinson's disease: interactions of oxidative stress, tryptophan catabolites and depression with mitochondria and sirtuins. *Mol. Neurobiol.* 49, 771–783. doi: 10.1007/s12035-013-8554-z

Anderson, G., and Ojala, J. (2010). Alzheimer's and seizures: interleukin-18, indoleamine 2,3-dioxygenase and quinolinic acid. *Int. J. Tryptophan Res.* 3, 169–173. doi: 10.4137/IJTR.S4603

Arimura, N., Inagaki, N., Chihara, K., Ménager, C., Nakamura, N., Amano, M., et al. (2000). Phosphorylation of collapsin response mediator protein-2 by Rho-kinase. Evidence for two separate signaling pathways for growth cone collapse. *J. Biol. Chem.* 275, 23973–23980. doi: 10.1074/jbc.M001032200

Aso, Y., Okumura, K., Takebayashi, K., Wakabayashi, S., and Inukai, T. (2003). Relationships of plasma interleukin-18 concentrations to hyperhomocysteinemia and carotid intimal-media wall thickness in patients with type 2 diabetes. *Diabetes Care* 26, 2622–2627. doi: 10.2337/diacare.26.9.2622

Baramova, E. N., Bajou, K., Remacle, A., L'Hoir, C., Krell, H. W., Weidle, U. H., et al. (1997). Involvement of PA/plasmin system in the processing of pro-MMP-9 and in the second step of pro-MMP-2 activation. *FEBS Lett.* 405, 157–162. doi: 10.1016/S0014-5793(97)00175-0

Barone, E., Di Domenico, F., Cenini, G., Sultana, R., Cini, C., Preziosi, P., et al. (2011). Biliverdin reductase–a protein levels and activity in the brains of subjects with Alzheimer disease and mild cognitive impairment. *Biochim. Biophys. Acta* 1812, 480–487. doi: 10.1016/j.bbadis.2011.01.005

Barone, E., Trombino, S., Cassano, R., Sgambato, A., De Paola, B., Di Stasio, E., et al. (2009). Characterization of the S-denitrosylating activity of bilirubin. *J. Cell Mol. Med.* 13, 2365–2375. doi: 10.1111/j.1582-4934.2008.00680.x

Batelli, S., Albani, D., Rametta, R., Polito, L., Prato, F., Pesaresi, M., et al. (2008). DJ-1 modulates alpha-synuclein aggregation state in a cellular model of oxidative stress: relevance for Parkinson's disease and involvement of HSP70. *PLoS ONE* 3:e1884. doi: 10.1371/journal.pone.0001884

Baulac, S., Lu, H., Strahle, J., Yang, T., Goldberg, M. S., Shen, J., et al. (2009). Increased DJ-1 expression under oxidative stress and in Alzheimer's disease brains. *Mol. Neurodegener.* 4, 12. doi: 10.1186/1750-1326-4-12

Bendlin, B. B., Carlsson, C. M., Gleason, C. E., Johnson, S. C., Sodhi, A., Gallagher, C. L., et al. (2010). Midlife predictors of Alzheimer's disease. *Maturitas* 65, 131–137. doi: 10.1016/j.maturitas.2009.12.014

Birdsey, G. M., Leiper, J. M., and Vallance, P. (2000). Intracellular localization of dimethylarginine dimethylaminohydrolase overexpressed in an endothelial cell line. *Acta Physiol. Scand.* 168, 73–79. doi: 10.1046/j.1365-201x.2000.00672.x

Braak, H., and Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol.* 82, 239–259. doi: 10.1007/BF00308809

Braak, H., and Braak, E. (1997). Frequency of stages of Alzheimer-related lesions in different age categories. *Neurobiol. Aging* 18, 351–357. doi: 10.1016/S0197-4580(97)00056-0

Brew, K., and Nagase, H. (2010). The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. *Biochim. Biophys. Acta* 1803, 55–71. doi: 10.1016/j.bbamcr.2010.01.003

Butterfield, D. A., and Bader Lange, M. L. (2009). Multifunctional roles of enolase in Alzheimer's disease brain: beyond altered glucose metabolism. *J. Neurochem.* 111, 915–933. doi: 10.1111/j.1471-4159.2009.06397.x

Castegna, A., Aksenov, M., Thongboonkerd, V., Klein, J. B., Pierce, W. M., Booze, R., et al. (2002). Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part II: dihydropyrimidinase-related protein 2, alpha-enolase and heat shock cognate 71. *J. Neurochem.* 82, 1524–1532. doi: 10.1046/j.1471-4159.2002.01103.x

Chandrasekar, B., Mummidi, S., Mahimainathan, L., Patel, D. N., Bailey, S. R., Imam, S. Z., et al. (2006). Interleukin-18-induced human coronary artery smooth muscle cell migration is dependent on NF-kappaB- and AP-1-mediated matrix metalloproteinase-9 expression and is inhibited by atorvastatin. *J. Biol. Chem.* 281, 15099–15109. doi: 10.1074/jbc.M600200200

Chang, X. Z., Li, D. Q., Hou, Y. F., Wu, J., Lu, J. S., Di, G. H., et al. (2007). Identification of the functional role of peroxiredoxin 6 in the progression of breast cancer. *Breast Cancer Res.* 9, R76. doi: 10.1186/bcr1789

Chen, Y. C., Yip, P. K., Huang, Y. L., Sun, Y., Wen, L. L., Chu, Y. M., et al. (2012). Sequence variants of toll like receptor 4 and late-onset Alzheimer's disease. *PLoS ONE* 7:e50771. doi: 10.1371/journal.pone.0050771

Cillero-Pastor, B., Mateos, J., Fernández-López, C., Oreiro, N., Ruiz-Romero, C., and Blanco, F. J. (2012). Dimethylarginine dimethylaminohydrolase 2, a newly identified mitochondrial protein modulating nitric oxide synthesis in normal human chondrocytes. *Arthritis Rheum.* 64, 204–212. doi: 10.1002/art.30652

Cole, A. R., Knebel, A., Morrice, N. A., Robertson, L. A., Irving, A. J., Connolly, C. N., et al. (2004). GSK-3 phosphorylation of the Alzheimer epitope within collapsin response mediator proteins regulates axon elongation in primary neurons. *J. Biol. Chem.* 279, 50176–50180. doi: 10.1074/jbc.C400412200

Cole, A. R., Noble, W., van Aalten, L., Plattner, F., Meimaridou, R., Hogan, D., et al. (2007). Collapsin response mediator protein-2 hyperphosphorylation is an early event in Alzheimer's disease progression. *J. Neurochem.* 103, 1132–1144. doi: 10.1111/j.1471-4159.2007.04829.x

Dayoub, H., Rodionov, R. N., Lynch, C., Cooke, J. P., Arning, E., Bottiglieri, T., et al. (2008). Overexpression of dimethylarginine dimethylaminohydrolase inhibits asymmetric dimethylarginine-induced endothelial dysfunction in the cerebral circulation. *Stroke* 39, 180–184. doi: 10.1161/STROKEAHA.107.490631

Díaz-Ramos, A., Roig-Borrellas, A., García-Melero, A., and López-Alemany, R. (2012). α-Enolase, a multifunctional protein: its role on pathophysiological situations. *J. Biomed. Biotechnol.* 2012, 156795. doi: 10.1155/2012/156795

Di Fede, G., Giaccone, G., Limido, L., Mangieri, M., Suardi, S., Puoti, G., et al. (2007). The ε isoform of 14-3-3 protein is a component of the prion protein amyloid deposits of Gerstmann-Straüssler-Scheinker Disease. *J. Neuropathol. Exp. Neurol.* 66, 124–130. doi: 10.1097/nen.0b013e3180302060

Diniz, B. S., Butters, M. A., Albert, S. M., Dew, M. A., and Reynolds, C. F. 3rd (2013). Late-life depression and risk of vascular dementia and Alzheimer's disease: systematic review and meta-analysis of community-based cohort studies. *Br. J. Psychiatry* 202, 329–335. doi: 10.1192/bjp.bp.112.118307

Esposito, K., Pontillo, A., Ciotola, M., Di Palo, C., Grella, E., Nicoletti, G., et al. (2002). Weight loss reduces interleukin-18 levels in obese women. *J. Clin. Endocrinol. Metab.* 87, 3864–3866. doi: 10.1210/jcem.87.8.8781

Fiedler, L. R., Bachetti, T., Leiper, J., Zachary, I., Chenm, L., Renné, T., et al. (2009). The ADMA/DDAH pathway regulates VEGF-mediated angiogenesis. *Arterioscler. Thromb. Vasc. Biol.* 29, 2117–2124. doi: 10.1161/ATVBAHA.109.194035

Fisher, A. B. (2011). Peroxiredoxin 6: a bifunctional enzyme with glutathione peroxidase and phospholipase A2 activities. *Antioxid. Redox Signal.* 15, 831–844. doi: 10.1089/ars.2010.3412

Fountoulakis, M., Cairns, N., and Lubec, G. (1999). Increased levels of 14-3-3 gamma and epsilon proteins in brain of patients with Alzheimer's disease and Down syndrome. *J. Neural. Transm. Suppl.* 57, 323–335.

Gentile, F., Pizzimenti, S., Arcaro, A., Pettazzoni, P., Minelli, R., D'Angelo, D., et al. (2009). Exposure of HL-60 human leukaemic cells to 4-hydroxynonenal promotes the formation of adduct(s) with alpha-enolase devoid of plasminogen binding activity. *Biochem. J.* 422, 285–294. doi: 10.1042/BJ20090564

Gibbs, P. E., Tudor, C., and Maines, M. D. (2012). Biliverdin reductase: more than a namesake - the reductase, its peptide fragments, and biliverdin regulate activity of the three classes of protein kinase C. *Front. Pharmacol.* 3:31. doi: 10.3389/fphar.2012.00031

Gu, Y., Hamajima, N., and Ihara, Y. (2000). Neurofibrillary tangle-associated collapsin response mediator protein-2 (CRMP-2) is highly phosphorylated on Thr-509, Ser-518, and Ser-522. *Biochemistry* 39, 4267–4275. doi: 10.1021/bi992323h

Guillemin, G. J., Smythe, G., Takikawa, O., and Brew, B. J. (2005). Expression of indoleamine 2,3-dioxygenase and production of quinolinic acid by human microglia, astrocytes, and neurons. *Glia* 49, 15–23. doi: 10.1002/glia.20090

Haastrup, E., Bukh, J. D., Bock, C., Vinberg, M., Thørner, L. W., Hansen, T., et al. (2012). Promoter variants in IL18 are associated with onset of depression in patients previously exposed to stressful-life events. *J. Affect. Disord.* 136, 134–138. doi: 10.1016/j.jad.2011.08.025

Hafner, A., Obermajer, N., and Kos, J. (2010). γ-1-syntrophin mediates trafficking of γ-enolase towards the plasma membrane and enhances its neurotrophic activity. *Neurosignals* 18, 246–258. doi: 10.1159/000324292

Hafner, A., Obermajer, N., and Kos, J. (2012). γ-Enolase C-terminal peptide promotes cell survival and neurite outgrowth by activation of the PI3K/Akt and MAPK/ERK signalling pathways. *Biochem. J.* 443, 439–450. doi: 10.1042/BJ20111351

Hamer, M., Sabia, S., Batty, G. D., Shipley, M. J., Tabák, A. G., Singh-Manoux, A., et al. (2012). Physical activity and inflammatory markers over 10 years: follow-up in men and women from the Whitehall II cohort study. *Circulation* 126, 928–933. doi: 10.1161/CIRCULATIONAHA.112.103879

Hanschmann, E. M., Godoy, J. R., Berndt, C., Hudemann, C., and Lillig, C. H. (2013). Thioredoxins, glutaredoxins, and peroxiredoxins-molecular mechanisms and health significance: from cofactors to antioxidants to redox signaling. *Antioxid. Redox Signal.* 19, 1539–1605. doi: 10.1089/ars.2012.4599

Higurashi, M., Iketani, M., Takei, K., Yamashita, N., Aoki, R., Kawahara, N., et al. (2012). Localized role of CRMP1 and CRMP2 in neurite outgrowth and growth cone steering. *Dev. Neurobiol.* 72, 1528–1540. doi: 10.1002/dneu.22017

Hur, E. M., Saijilafu, Lee, B. D., Kim, S. J., Xu, W. L., and Zhou, F. Q. (2011). GSK3 controls axon growth via CLASP-mediated regulation of growth cone microtubules. *Genes Dev.* 25, 1968–1981. doi: 10.1101/gad.17015911

Irrcher, I., Aleyasin, H., Seifert, E. L., Hewitt, S. J., Chhabra, S., Phillips, M., et al. (2010). Loss of the Parkinson's disease-linked gene DJ-1 perturbs mitochondrial dynamics. *Hum. Mol. Genet.* 19, 3734–3746. doi: 10.1093/hmg/ddq288

Ishida, Y., Migita, K., Izumi, Y., Nakao, K., Ida, H., Kawakami, A., et al. (2004). The role of IL-18 in the modulation of matrix metalloproteinases and migration of human natural killer (NK) cells. *FEBS Lett.* 569, 156–160. doi: 10.1016/j.febslet.2004.05.039

Itoh, Y., Takamura, A., Ito, N., Maru, Y., Sato, H., Suenaga, N., et al. (2001). Homophilic complex formation of MT1-MMP facilitates proMMP-2 activation on the cell surface and promotes tumor cell invasion. *EMBO J.* 20, 4782–4793. doi: 10.1093/emboj/20.17.4782

Jansen, T., and Daiber, A. (2012). Direct antioxidant properties of bilirubin and biliverdin. Is there a role for biliverdin reductase? *Front. Pharmacol.* 3:30. doi: 10.3389/fphar.2012.00030

Jansen, T., Hortmann, M., Oelze, M., Opitz, B., Steven, S., Schell, R., et al. (2010). Conversion of biliverdin to bilirubin by biliverdin reductase contributes to endothelial cell protection by heme oxygenase-1-evidence for direct and indirect antioxidant actions of bilirubin. *J. Mol. Cell Cardiol.* 49, 186–195. doi: 10.1016/j.yjmcc.2010.04.011

Kim, S. Y., and Park, S. C. (2012). Physiological antioxidative network of the bilirubin system in aging and age-related diseases. *Front. Pharmacol.* 3:45. doi: 10.3389/fphar.2012.00045

Kinoshita, A., Kinoshita, M., Akiyama, H., Tomimoto, H., Akiguchi, I., Kumar, S., et al. (1998). Identification of septins in neurofibrillary tangles in Alzheimer's disease. *Am. J. Pathol.* 153, 1551–1560. doi: 10.1016/S0002-9440(10)65743-4

Koh, P. O. (2008). Melatonin attenuates the focal cerebral ischemic injury by inhibiting the dissociation of pBad from 14-3-3. *J. Pineal Res.* 44, 101–106. doi: 10.1111/j.1600-079X.2007.00495.x

Korolainen, M. A., Nyman, T. A., Aittokallio, T., and Pirttilä, T. (2010). An update on clinical proteomics in Alzheimer's research. *J. Neurochem.* 112, 1386–1414. doi: 10.1111/j.1471-4159.2009.06558.x

Krapfenbauer, K., Engidawork, E., Cairns, N., Fountoulakis, M., and Lubec, G. (2003). Aberrant expression of peroxiredoxin subtypes in neurodegenerative disorders. *Brain Res.* 967, 152–160. doi: 10.1016/S0006-8993(02)04243-9

Lee, E. K., Lee, Y. S., Lee, H., Choi, C. Y., and Park, S. H. (2009a). 14-3-3epsilon protein increases matrix metalloproteinase-2 gene expression via p38 MAPK signaling in NIH3T3 fibroblast cells. *Exp. Mol. Med.* 41, 453–561. doi: 10.3858/emm.2009.41.7.050

Lee, H. P., Zhu, X., Casadesus, G., Nunomura, A., Smith, M. A., Lee, H., et al. (2010). Antioxidant approaches for the treatment of Alzheimer's disease. *Expert Rev. Neurother.* 10, 1201–1208. doi: 10.1586/ern.10.74

Lee, S. B., Ho, J. N., Yoon, S. H., Kang, G. Y., Hwang, S. G., and Um, H. D. (2009b). Peroxiredoxin 6 promotes lung cancer cell invasion by inducing urokinase-type plasminogen activator via p38 kinase, phosphoinositide 3-kinase, and Akt. *Mol. Cells* 28, 583–588. doi: 10.1007/s10059-009-0152-6

Lehti, K., Lohi, J., Valtanen, H., and Keski-Oja, J. (1998). Proteolytic processing of membrane-type-1 matrix metalloproteinase is associated with gelatinase A activation at the cell surface. *Biochem. J.* 334, 345–353.

Lerner-Marmarosh, N., Shen, J., Torno, M. D., Kravets, A., Hu, Z., and Maines, M. D. (2005). Human biliverdin reductase: a member of the insulin receptor substrate family with serine/threonine/tyrosine kinase activity. *Proc. Natl. Acad. Sci. U.S.A.* 102, 7109–7114. doi: 10.1073/pnas.0502173102

Li, X., Wang, Q. J., Pan, N., Lee, S., Zhao, Y., Chait, B. T., et al. (2011). Phosphorylation-dependent 14-3-3 binding to LRRK2 is impaired by common mutations of familial Parkinson's disease. *PLoS ONE* 6:e17153. doi: 10.1371/journal.pone.0017153

Liao, M. C., and Van Nostrand, W. E. (2010). Degradation of soluble and fibrillar amyloid beta-protein by matrix metalloproteinase (MT1-MMP) *in vitro*. *Biochemistry* 49, 1127–1136. doi: 10.1021/bi901994d

Mallat, Z., Corbaz, A., Scoazec, A., Besnard, S., Lesèche, G., Chvatchko, Y., et al. (2001). Expression of interleukin-18 in human atherosclerotic plaques and relation to plaque instability. *Circulation* 104, 1598–1603. doi: 10.1161/hc3901.096721

Merlo, S., and Sortino, M. A. (2012). Estrogen activates matrix metalloproteinases-2 and -9 to increase beta amyloid degradation. *Mol. Cell Neurosci.* 49, 423–429. doi: 10.1016/j.mcn.2012.02.005

Morales, I., Guzmán-Martínez, L., Cerda-Troncoso, C., Farías, G. A., and Maccioni, R. B. (2014). Neuroinflammation in the pathogenesis of Alzheimer's disease. A rational framework for the search of novel therapeutic approaches. *Front. Cell. Neurosci.* 8:112. doi: 10.3389/fncel.2014.00112

Mori, T., Wada, T., Suzuki, T., Kubota, Y., and Inagaki, N. (2007). Singar1, a novel RUN domain-containing protein, suppresses formation of surplus axons for neuronal polarity. *J. Biol. Chem.* 282, 19884–19893. doi: 10.1074/jbc.M700770200

Mostowy, S., and Cossart, P. (2012). Septins: the fourth component of the cytoskeleton. *Nat. Rev. Mol. Cell Biol.* 13, 183–194. doi: 10.1038/nrm3284

Neuman, T., Stephens, R. W., Salonen, E. M., Timmusk, T., and Vaheri, A. (1989). Induction of morphological differentiation of human neuroblastoma cells is accompanied by induction of tissue-type plasminogen activator. *J. Neurosci. Res.* 23, 274–281. doi: 10.1002/jnr.490230305

Newman, S. F., Sultana, R., Perluigi, M., Coccia, R., Cai, J., Pierce, W. M., et al. (2007). An increase in S-glutathionylated proteins in the Alzheimer's disease inferior parietal lobule, a proteomics approach. *J. Neurosci. Res.* 85, 1506–1514. doi: 10.1002/jnr.21275

Obermajer, N., Doljak, B., Jamnik, P., Fonoviæ, U. P., and Kos, J. (2009). Cathepsin X cleaves the C-terminal dipeptide of alpha- and gamma-enolase and impairs survival and neuritogenesis of neuronal cells. *Int. J. Biochem. Cell Biol.* 41, 1685–1596. doi: 10.1016/j.biocel.2009.02.019

Ojala, J., Alafuzoff, I., Herukka, S. K., van Groen, T., Tanila, H., and Pirttilä, T. (2009). Expression of interleukin-18 is increased in the brains of Alzheimer's disease patients. *Neurobiol. Aging* 30, 198–209. doi: 10.1016/j.neurobiolaging.2007.06.006

Ojala, J. O., Sutinen, E. M., Salminen, A., and Pirttilä, T. (2008). Interleukin-18 increases expression of kinases involved in tau phosphorylation in SH-SY5Y neuroblastoma cells. *J. Neuroimmunol.* 205, 86–93. doi: 10.1016/j.jneuroim.2008.09.012

Opazo, C., Huang, X., Cherny, R. A., Moir, R. D., Roher, A. E., White, A. R., et al. (2002). Metalloenzyme-like activity of Alzheimer's disease beta-amyloid. Cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic H(2)O(2). *J. Biol. Chem.* 277, 40302–40308. doi: 10.1074/jbc.M206428200

Owen, J. B., Di Domenico, F., Sultana, R., Perluigi, M., Cini, C., Pierce, W. M., et al. (2009). Proteomics-determined differences in the concanavalin-A-fractionated proteome of hippocampus and inferior parietal lobule in subjects with Alzheimer's disease and mild cognitive impairment: implications for progression of AD. *J. Proteome Res.* 8, 471–482. doi: 10.1021/pr800667a

Perry, G., Zhu, X., Babar, A. K., Siedlak, S. L., Yang, Q., Ito, G., et al. (2008). Leucine-rich repeat kinase 2 colocalizes with alpha-synuclein in Parkinson's disease, but not tau-containing deposits in tauopathies. *Neurodegener. Dis.* 5, 222–224. doi: 10.1159/000113708

Peterson, E. A., and Petty, E. M. (2010). Conquering the complex world of human septins: implications for health and disease. *Clin. Genet.* 77, 511–524. doi: 10.1111/j.1399-0004.2010.01392.x

Petratos, S., Li, Q. X., George, A. J., Hou, X., Kerr, M. L., Unabia, S. E., et al. (2008). The beta-amyloid protein of Alzheimer's disease increases neuronal CRMP-2 phosphorylation by a Rho-GTP mechanism. *Brain*, 131, 90–108. doi: 10.1093/brain/awm260

Petratos, S., Ozturk, E., Azari, M. F., Kenny, R., Lee, J. Y., Magee, K. A., et al. (2012). Limiting multiple sclerosis related axonopathy by blocking Nogo receptor and CRMP-2 phosphorylation. *Brain* 135, 1794–1818. doi: 10.1093/brain/aws100

Pissuti Damalio, J. C., Garcia, W., Alves Macêdo, J. N., de Almeida Marques, I., Andreu, J. M., Giraldo, R., et al. (2012). Self assembly of human septin 2 into amyloid filaments. *Biochimie* 94, 628–636. doi: 10.1016/j.biochi.2011.09.014

Pope, A. J., Karuppiah, K., and Cardounel, A. J. (2009). Role of the PRMT-DDAH-ADMA axis in the regulation of endothelial nitric oxide production. *Pharmacol. Res.* 60, 461–465. doi: 10.1016/j.phrs.2009.07.016

Power, J. H. T., Asad, S., Chataway, T. K., Chegini, F., Manavis, J., Temlett, J. A., et al. (2008). Peroxiredoxin 6 in human brain: molecular forms, cellular distribution and association with Alzheimer's disease pathology. *Acta Neuropathol.* 115, 611–622. doi: 10.1007/s00401-008-0373-3

Reed, T. T., Pierce, W. M. Jr., Turner, D. M., Markesbery, W. R., and Butterfield, D. A. (2009). Proteomic identification of nitrated brain proteins in early Alzheimer's disease inferior parietal lobule. *J. Cell Mol. Med.* 13, 2019–2029. doi: 10.1111/j.1582-4934.2008.00478.x

Ridnour, L. A., Dhanapal, S., Hoos, M., Wilson, J., Lee, J., Cheng, R. Y., et al. (2012). Nitric oxide-mediated regulation of β-amyloid clearance via alterations of MMP-9/TIMP-1. *J. Neurochem.* 123, 736–749. doi: 10.1111/jnc.12028

Rizzu, P., Hinkle, D. A., Zhukareva, V., Bonifati, V., Severijnen, L. A., Martinez, D., et al. (2004). DJ-1 colocalizes with tau inclusions: a link between parkinsonism and dementia. *Ann. Neurol.* 55, 113–118. doi: 10.1002/ana.10782

Selley, M. L. (2004). Homocysteine increases the production of asymmetric dimethylarginine in cultured neurons. *J. Neurosci. Res.* 77, 90–93. doi: 10.1002/jnr.20070

Sinniger, V., Merton, R. E., Fabregas, P., Felez, J., and Longstaff, C. (1999). Regulation of tissue plasminogen activator activity by cells. Domains responsible for binding and mechanism of stimulation. *J. Biol. Chem.* 274, 12414–12422. doi: 10.1074/jbc.274.18.12414

Sorokina, E. M., Feinstein, S. I., Zhou, S., and Fisher, A. B. (2011). Intracellular targeting of peroxiredoxin 6 to lysosomal organelles requires MAPK activity and binding to 14-3-3ε. *Am. J. Physiol. Cell Physiol.* 300, C1430–C1441. doi: 10.1152/ajpcell.00285.2010

Steinacker, P., Aitken, A., and Otto, M. (2011). 14-3-3 proteins in neurodegeneration. *Semin. Cell Dev. Biol.* 22, 696–704. doi: 10.1016/j.semcdb.2011.08.005

Sugama, S., and Conti, B. (2008). Interleukin-18 and stress. *Brain Res. Rev.* 58, 85–95. doi: 10.1016/j.brainresrev.2007.11.003

Sultana, R., Boyd-Kimball, D., Cai, J., Pierce, W. M., Klein, J. B., Merchant, M., et al. (2007). Proteomics analysis of the Alzheimer's disease hippocampal proteome. *J. Alzheimers Dis.* 11, 153–164.

Sutinen, E. M., Pirttilä, T., Anderson, G., Salminen, A., and Ojala, J. O. (2012). Pro-inflammatory interleukin-18 increases Alzheimer's disease-associated amyloid-β production in human neuron-like cells. *J. Neuroinflammation* 9, 199. doi: 10.1186/1742-2094-9-199

Swatton, J. E., Prabakaran, S., Karp, N. A., Lilley, K. S., and Bahn, S. (2004). Protein profiling of human postmortem brain using 2-dimensional fluorescence difference gel electrophoresis (2-D DIGE). *Mol. Psychiatry* 9, 128–143. doi: 10.1038/sj.mp.4001475

Toyo-oka, K., Shionoya, A., Gambello, M. J., Cardoso, C., Leventer, R., Ward, H. L., et al. (2003). 14-3-3epsilon is important for neuronal migration by binding to NUDEL: a molecular explanation for Miller-Dieker syndrome. *Nat. Genet.* 34, 274–285. doi: 10.1038/ng1169

Tucker, H. M., Kihiko, M., Caldwell, J. N., Wright, S., Kawarabayashi, T., Price, D., et al. (2000). The plasmin system is induced by and degrades amyloid-beta aggregates. *J. Neurosci.* 20, 3937–3946.

Uchida, Y., Ohshima, T., Yamashita, N., Ogawara, M., Sasaki, Y., Nakamura, F., et al. (2009). Semaphorin3A signaling mediated by Fyn-dependent tyrosine phosphorylation of collapsin response mediator protein 2 at tyrosine 32. *J. Biol. Chem.* 284, 27393–27401. doi: 10.1074/jbc.M109.000240

Unlü, M., Morgan, M. E., and Minden, J. S. (1997). Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* 18, 2071–2077. doi: 10.1002/elps.1150181133

von Bernhardi, R., and Eugenín, J. (2012). Alzheimer's disease: redox dysregulation as a common denominator for diverse pathogenic mechanisms. *Antioxid. Redox Signal.* 16, 974–1031. doi: 10.1089/ars.2011.4082

Wang, S., Hu, C.-P., Jiang, D.-J., Peng, J., Zhou, Z., Yuan, Q., et al. (2009). All-trans retinoic acid inhibits cobalt chloride-induced apoptosis in PC12 Cells: role of the dimethylarginine dimethylaminohydrolase/ asymmetric dimethylarginine pathway. *J. Neurosci. Res.* 87, 1938–1946. doi: 10.1002/jnr.21999

Wang, Z., Liu, J., Chen, S., Wang, Y., Cao, L., Zhang, Y., et al. (2011). DJ-1 modulates the expression of Cu/Zn-superoxide dismutase-1 through the Erk1/2-Elk1 pathway in neuroprotection. *Ann. Neurol.* 70, 591–599. doi: 10.1002/ana.22514

Wegiel, B., Gallo, D., Csizmadia, E., Roger, T., Kaczmarek, E., Harris, C., et al. (2011). Biliverdin inhibits Toll-like receptor-4 (TLR4) expression through nitric oxide-dependent nuclear translocation of biliverdin reductase. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18849–18854. doi: 10.1073/pnas.1108571108

Wegiel, B., and Otterbein, L. E. (2012). Go green: the anti-inflammatory effects of biliverdin reductase. *Front. Pharmacol.* 3:47. doi: 10.3389/fphar.2012.00047

Wendt, W., Zhu, X. R., Lübbert, H., and Stichel, C. C. (2007). Differential expression of cathepsin X in aging and pathological central nervous system of mice. *Exp. Neurol.* 204, 525–540. doi: 10.1016/j.expneurol.2007.01.007

Williamson, R., van Aalten, L., Mann, D. M., Platt, B., Plattner, F., Bedford, L., et al. (2011). CRMP2 hyperphosphorylation is characteristic of Alzheimer's disease and not a feature common to other neurodegenerative diseases. *J. Alzheimers Dis.* 27, 615–625. doi: 10.3233/JAD-2011-110617

Wu, J. S., Cheung, W. M., Tsai, Y. S., Chen, Y. T., Fong, W. H., Tsai, H. D., et al. (2009). Ligand-activated peroxisome proliferator-activated receptor-gamma protects against ischemic cerebral infarction and neuronal apoptosis by 14-3-3 epsilon upregulation. *Circulation* 119, 1124–1134. doi: 10.1161/CIRCULATIONAHA.108.812537

Yacoubian, T. A., Slone, S. R., Harrington, A. J., Hamamichi, S., Schieltz, J. M., Caldwell, K. A., et al. (2010). Differential neuroprotective effects of 14-3-3 proteins in models of Parkinson's disease. *Cell Death Dis.* 1, e2. doi: 10.1038/cddis.2009.4

Yaguchi, T., Nagata, T., Yang, D., and Nishizaki, T. (2010). Interleukin-18 regulates motor activity, anxiety and spatial learning without affecting synaptic plasticity. *Behav. Brain Res.* 206, 47–51. doi: 10.1016/j.bbr.2009.08.033

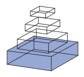
Yuan, Z., Agarwal-Mawal, A., and Paudel, H. K. (2004). 14-3-3 binds to and mediates phosphorylation of microtubule-associated tau protein by Ser9-phosphorylated glycogen synthase kinase 3beta in the brain. *J. Biol. Chem.* 279, 26105–26114. doi: 10.1074/jbc.M308298200

Zhou, Y., Zhang, W., Easton, R., Ray, J. W., Lampe, P., Jiang, Z., et al. (2002). Presenilin-1 protects against neuronal apoptosis caused by its interacting protein PAG. *Neurobiol. Dis.* 9, 126–138. doi: 10.1006/nbdi.2001.0472

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 20 May 2014; accepted: 16 July 2014; published online: 07 August 2014.*
*Citation: Sutinen EM, Korolainen MA, Häyrinen J, Alafuzoff I, Petratos S, Salminen A, Soininen H, Pirttilä T and Ojala JO (2014) Interleukin-18 alters protein expressions of neurodegenerative diseases-linked proteins in human SH-SY5Y neuron-like cells. Front. Cell. Neurosci. **8**:214. doi: 10.3389/fncel.2014.00214*
*This article was submitted to the journal Frontiers in Cellular Neuroscience.*
*Copyright © 2014 Sutinen, Korolainen, Häyrinen, Alafuzoff, Petratos, Salminen, Soininen, Pirttilä and Ojala. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*

ORIGINAL RESEARCH ARTICLE
published: 06 June 2014
doi: 10.3389/fncel.2014.00155



# The inflammatory molecules IL-1β and HMGB1 can rapidly enhance focal seizure generation in a brain slice model of temporal lobe epilepsy

***Angela Chiavegato[1], Emanuele Zurolo[2,3], Gabriele Losi[1], Eleonora Aronica[2,3] and Giorgio Carmignoto[1]****

*[1] Department of Biomedical Sciences and CNR Institute of Neuroscience, University of Padova, Padova, Italy*
*[2] Department of (Neuro)Pathology, Academic Medical Center, and Swammerdam Institute for Life Sciences, Center for Neuroscience, University of Amsterdam, Amsterdam, Netherlands*
*[3] SEIN – Stichting Epilepsie Instellingen Nederland, Heemstede, Netherlands*

***Edited by:***
*Silvia Alboni, University of Modena and Reggio Emilia, Italy*

***Reviewed by:***
*Rheinallt Parri, Aston University, UK*
*Giuseppe Biagini, University of Modena and Reggio Emilia, Italy*

****Correspondence:***
*Giorgio Carmignoto, Consiglio Nazionale delle Ricerche and Department of Biomedical Sciences, Neuroscience Institute, University of Padova, Viale Giuseppe Colombo 3, 35121 Padova, Italy*
*e-mail: giorgio.carmignoto@bio.unipd.it*

Epilepsy is a neurological disorder characterized by a hyperexcitable brain tissue and unpredictable seizures, i.e., aberrant firing discharges in large neuronal populations. It is well established that proinflammatory cytokines, in addition to their canonical involvement in the immune response, have a crucial role in the mechanism of seizure generation. The purpose of the present study was to investigate the role of interleukin-1β (IL-1β) and high mobility group B1 (HMGB1) in the generation of seizure-like discharges using two models of focal epilepsy in a rat entorhinal cortex slice preparation. Seizure like-discharges were evoked by either slice perfusion with low $Mg^{2+}$ and picrotoxin or with a double NMDA local stimulation in the presence of the proconvulsant 4-amino-pyridine. The effects of IL-1β or HMGB1 were evaluated by monitoring seizure discharge generation through laser scanning microscope imaging of $Ca^{2+}$ signals from neurons and astrocytes. In the picrotoxin model, we revealed that both cytokines increased the mean frequency of spontaneous ictal-like discharges, whereas only IL-1β reduced the latency and prolonged the duration of the first ictal-like event. In the second model, a single NMDA pulse, *per se* ineffective, became successful when it was performed after IL-β or HMGB1 local applications. These findings demonstrate that both IL-1β and HMGB1 can rapidly lower focal ictal event threshold and strengthen the possibility that targeting these inflammatory pathways may represent an effective therapeutic strategy to prevent seizures.

**Keywords: epileptogenesis, seizures, entorhinal cortex, calcium, proinflammatory cytokines, IL-1beta, HMGB1, astrocytes**

## INTRODUCTION

Epilepsy is a neurological disorder characterized by recurring, unprovoked seizures. The age-adjusted incidence of epilepsy ranges from 16 to 51 per 100,000 worldwide, with higher prevalence in developing countries (Banerjee et al., 2009; Peljto et al., 2014). Increasing evidence supports the involvement of inflammatory and immune processes in the etiopathogenesis of seizures (Vezzani and Granata, 2005; Vezzani et al., 2008, 2010). Inflammation induced by brain-damaging events such as trauma, stroke, infection, hyperthermia, and status epilepticus are associated with acute symptomatic seizures and a high risk of developing epilepsy (Pitkanen and Sutula, 2002; Bartfai et al., 2007). In particular, high levels of proinflammatory cytokines [e.g., interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNFα)], damage signals [high-mobility group box 1 (HMGB1), S100 beta] and downstream inflammatory mediators (e.g., prostaglandins, the complement system) have been measured in epileptogenic tissue from patients affected by epilepsy of various etiologies (Aronica and Crino, 2011; Vezzani et al., 2011). The major contributors to the synthesis of these inflammatory mediators are brain-resident cells such as activated microglia, astrocytes, and neurons (Devinsky et al., 2013), but also systemic invading leukocytes play an important role in epileptogenesis, particularly when the permeability of the blood-brain barrier is alterated (Fabene et al., 2008; Deprez et al., 2011).

Recently, two proinflammatory molecules were found to be proconvulsant in animal models of temporal lobe epilepsy (TLE): IL-1β and HMGB1 (Vezzani et al., 1999; Ravizza et al., 2008; Maroso et al., 2011). These molecules, applied *in vivo* before the induction of experimental TLE, were able to increase the time spent in seizures and reduce the onset time of the first seizure. The effects of HMGB1 and IL-1β are blocked by ifenprodil (Balosso et al., 2008; Maroso et al., 2010), a selective antagonist of NR2B-containing NMDA receptors (Yu et al., 1997).

Previous studies also reported that astrocytes can promote episodes of synchronous activity in the neuronal network (Fellin et al., 2004) and that this action may contribute to ictal discharge generation (Gomez-Gonzalo et al., 2010; Losi et al., 2010). Using two different focal seizure models in cortical slice preparations we investigate whether two pro-inflammatory cytokines, i.e., IL-1β and HMGB1, can affect neuronal excitability and favor the generation of epileptic activity. In the first model, slices

from the entorhinal cortex (EC) were perfused with picrotoxin in the virtual absence of extracellular $Mg^{2+}$. These conditions caused spontaneous epileptiform activities to arise from unpredictable foci (Demir et al., 1998). In the second model, slices were perfused with 0.5 mM $Mg^{2+}$ and 100 μM 4-aminopyridine (4-AP) before receiving local N-methyl-D-aspartate (NMDA) applications which trigger a focal ictal-like discharge (Gomez-Gonzalo et al., 2010; Losi et al., 2010). This latter model offers the unique opportunity to repetitively evoke an ictal-like discharge from the same restricted site and it thus represents a powerful approach to analyze the contribution of different molecules and signaling pathways to the generation of epileptiform events. By using fast laser-scanning microscope $Ca^{2+}$ imaging from neurons and astrocytes we monitored epileptiform network activities in these two models and found that local applications with both IL-1β and HMGB1 could rapidly lower the threshold for the initiation of focal ictal discharges.

## MATERIALS AND METHODS

### BRAIN SLICES AND LOADING

All experimental procedures were authorized by the Italian Ministry of Health; all efforts were made to minimize the number of animal used and their suffering. Coronal cortical-hippocampal slices were obtained from 13 to 17 days old Wistar rats as previously described (Fellin et al., 2004). Briefly, brain was removed and put into ice-cold cutting solution containing (in mM): 120 NaCl, 3.2 KCl, 1 $KH_2PO_4$, 26 $NaHCO_3$, 2 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 2 Na-pyruvate, and 0.6 ascorbic acid at pH 7.4 (with 5% $CO_2$/95% $O_2$). Slices were obtained by cutting with a Leica Vibratome VT1000S (Mannheim, Germany) in the presence of the NMDA receptor inhibitor kynurenic acid (2 mM). Slices were recovered for 15 min at 37°C and then loaded with the $Ca^{2+}$ sensitive dye Oregon Green 488 BAPTA-1 acetoxymethyl ester (OGB-1 AM, 20 μm; Invitrogen, Carlsbad, CA, U.S.A.) for 60 min at 37°C. Dye loading was performed in the cutting solution containing sulfinpyrazone (200 μM), pluronic acid (0.12%), and kynurenic acid (1 mM). After loading, slices were recovered and kept at room temperature in the presence of 200 μM sulfinpyrazone.

### CALCIUM IMAGING

Brain slices were continuosly perfused in a submerged chamber (Warner Instruments, Hamden, CT, USA) with a recording solution containing (in mM): 120 NaCl, 3.2 KCl, 1 $KH_2PO_4$, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose at pH 7.4 (with 5% $CO_2$/95% $O_2$) and $Ca^{2+}$ signal images (512 × 512 pixels) were acquired by a TCS-SP5-RS confocal microscope (Leica Microsystem, Germany) equipped with a 20× water/objective (NA, 1.0) and a CCD camera for differential interference contrast. Time frame acquisitions from 314 to 491 ms (with 6–7 line averaging) were used. The $Ca^{2+}$ responsiveness in neurons and astrocytes was determined on the basis of a threshold criterion. The onset was identified by the change in ΔF/F0 that should be more than two standard deviations over the average baseline and remained above this value in the successive frames for at least 2 s (two to six frames, depending on the frame acquisition rate). No background subtraction or other manipulations were applied to digitized $Ca^{2+}$ signal images, with the exception of difference images in **Figure 2** that were obtained by subtracting the pre-stimulation $Ca^{2+}$ image from the post-stimulation $Ca^{2+}$ image.

### SLICES MODELS OF EPILEPTIC ACTIVITY AND IL-1β/HMGB1 APPLICATIONS

At a cellular level interictal and ictal-seizure like events were identified as intense and synchronous discharges that involve large neuronal population (Gomez-Gonzalo et al., 2010; Gómez-Gonzalo et al., 2011). In $Ca^{2+}$ imaging of cortical slice the duration of the epileptic event was an important criterion for classifying interictal and ictal events. Interictal-like events lasted less than 3 s (D'Antuono et al., 2010), whereas ictal-like events were sustained for tens of seconds with a final pattern of highly synchronous activity that involved fundamentally all neurons in the recording field. In a first model, epileptiform activities were induced upon perfusion of cortical slice preparations with a recording solution containing the $GABA_A$ receptor inhibitor picrotoxin (50 μM, Sigma-Aldrich, Milan, Italy) in the virtual absence of $Mg^{2+}$. The ictal latency was evaluated by measuring the time between the onset of the picrotoxin perfusion and the first ictal-like event. In the second model, as previously described by Losi et al. (2010), focal ictal-like discharges were evoked by local NMDA applications in the presence of 4-aminopyridine (4-AP, 100 μM; Abcam, Cambridge, UK) and 0.5 mM $MgCl_2$. A pressure ejection unit (PDES, NPI Electronics, Germany) was used to apply pressure pulses (4–10 psi, 200–600 ms duration) to a pipette containing 1 mM N-methyl-D-aspartate (NMDA, Sigma-Aldrich) localized on layers V-VI of EC. Pulse pressure (or duration) was increased until a double NMDA pulse evoked an ictal-like event while a single NMDA pulse induced only a transient local $Ca^{2+}$ response. The parameters for successive stimulations remained unchanged over the entire recording experiment. The pipettes containing IL-1β (500 ng/ml rat recombinant IL-1β, Sigma-Aldrich) or HMGB1 (1 μM LPS-free HMGB1, HMGBiotech, Milan, Italy) were placed close to EC neurons and the inflammatory cytochines were locally applied by pressure pulses (2–5 psi for 200–600 ms) every 20 s for 15 min, just before picrotoxin or single NMDA pulses. Control experiments used the saline solution (1.2 M NaCl, 50 mM KCl, 10 mM $NaH_2PO_4$, 200 mM HEPES) in which the cytochines were dissolved.

A number of experiments were performed in the continuous presence of 2 μM tetrodotoxin (TTX, Abcam). In these experiments, repetitive single NMDA pulses (one every 2 min) were applied for 10 min before and 10 min after IL-1β, HMGB1 or saline pulse applications (one every 20 s) and both number of responsive neurons and amplitude of the $Ca^{2+}$ response were evaluated. Groups of TTX experiments were preceded by ictal-like activity in 4-AP to test if the epileptic activity could change the effects of the cytochines on the NMDA-mediated $Ca^{2+}$ response.

### DATA ANALYSIS

Data analysis of $Ca^{2+}$ signal was performed with LEICA LAS-AF (Leica), ORIGIN 7.5 (Microcal software, Northampton, MA, U.S.A.) and MATLAB (The MathWorks, Natick, MA, USA). $Ca^{2+}$signal changes from regions of interest were measured by $\Delta F/F_0$, where $F_0$ is the baseline fluorescence. In the picrotoxin

experiments we evaluated different neuronal parameters (latency and duration of the first ictal-like discharge event, interictal and ictal-like event frequency). In this group of experiments we applied the unpaired Student's *t*-test and compared the cytokine treated groups with the control-saline treated group. In the 4-AP/NMDA experiments we evaluated the number of responding cells and their $Ca^{2+}$ activity (maximal $\Delta F/F_0$ point) in response to a single NMDA pulse, both before and after cytokine applications. The Mann-Whitney non-parametric test on normalized values was used, with *p*-values $\leq 0.05$ taken as statistically significant. Data are shown as mean ± standard error of the mean (S.E.M.).

## RESULTS

### IL-1β AND HMGB1 FAVOR ICTAL-LIKE DISCHARGE GENERATION

#### *Picrotoxin/low $Mg^{2+}$ entorhinal cortex slice model*

The change in the cytosolic $Ca^{2+}$ signal is a useful tool to study seizure, ictal-like discharges in neuronal ensembles since it reflects faithfully the action potential bursts that characterize the epileptic discharges in individual neurons (Gomez-Gonzalo et al., 2010; Losi et al., 2010). To start investigating a possible role of inflammatory agents, such as IL-1β and HMGB1, in ictogenesis we loaded EC slice preparations from young rats with the $Ca^{2+}$ indicator OGB-1 and epileptiform activities were observed to arise spontaneosuly after a pro-

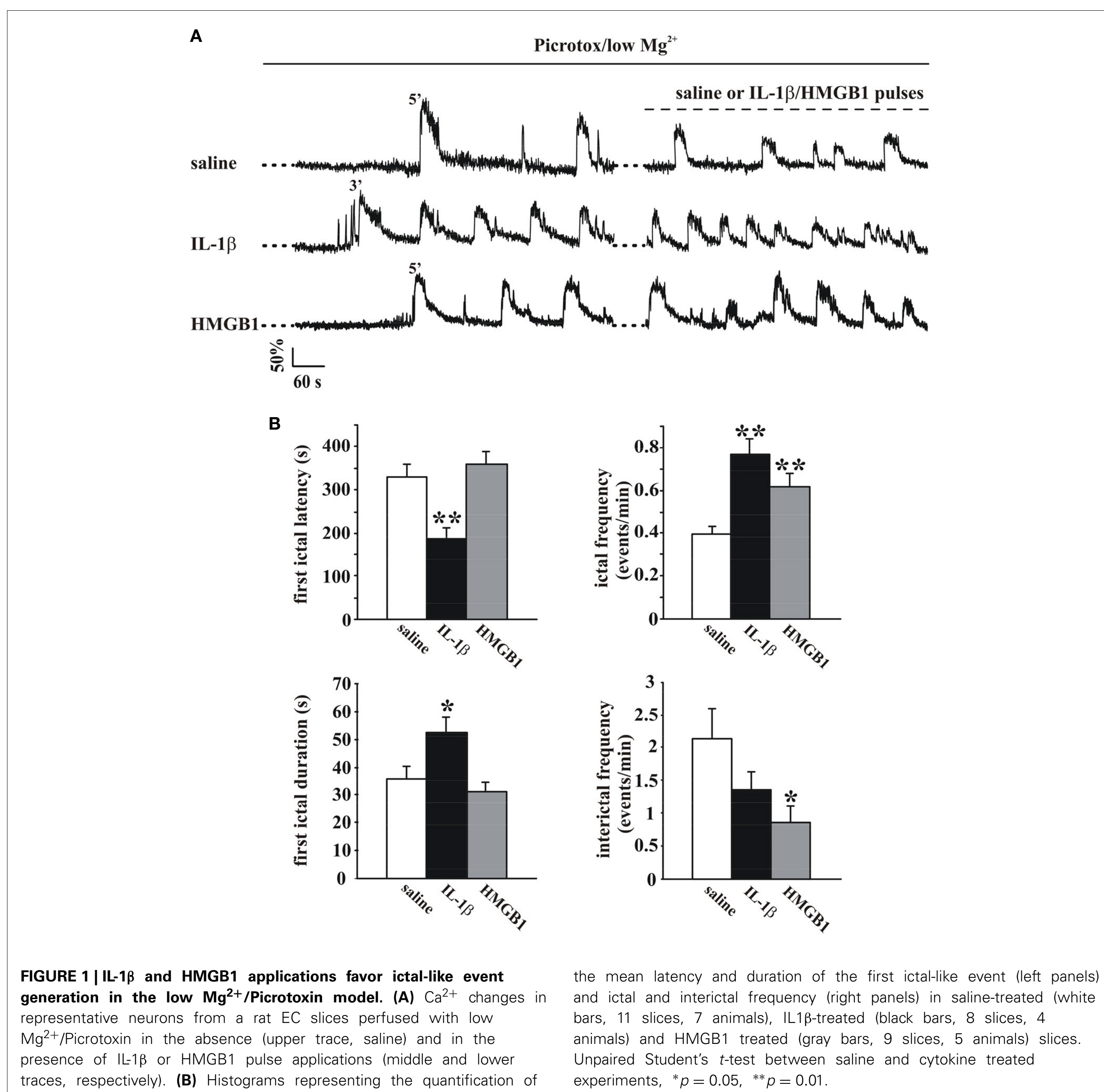



**FIGURE 1 | IL-1β and HMGB1 applications favor ictal-like event generation in the low $Mg^{2+}$/Picrotoxin model. (A)** $Ca^{2+}$ changes in representative neurons from a rat EC slices perfused with low $Mg^{2+}$/Picrotoxin in the absence (upper trace, saline) and in the presence of IL-1β or HMGB1 pulse applications (middle and lower traces, respectively). **(B)** Histograms representing the quantification of the mean latency and duration of the first ictal-like event (left panels) and ictal and interictal frequency (right panels) in saline-treated (white bars, 11 slices, 7 animals), IL1β-treated (black bars, 8 slices, 4 animals) and HMGB1 treated (gray bars, 9 slices, 5 animals) slices. Unpaired Student's *t*-test between saline and cytokine treated experiments, $^{*}p = 0.05$, $^{**}p = 0.01$.

longed slice perfusion with the $GABA_A$ receptor antagonist picrotoxin in low extracellular $Mg^{2+}$. We found that with respect to the onset of the picrotoxin/low $Mg^{2+}$ perfusion, the first ictal-like event occurred with a significantly shorter latency in slices pretreated with IL-1β pulses (one every 20 s for 15 min; see Materials and Methods; $p = 0.0008$, unpaired Student's $t$-test, $n = 8$) than in saline-pretreated slices (**Figures 1A,B**). Differently from IL-1β, HMGB1 affected neither the latency of the first ictal event nor the ictal-like duration ($n = 9$). However, both IL-1β and HMGB1 significantly increased the overall frequency of ictal-like events and reduced interictal events (**Figure 1B**).

### *Focal seizure model*

We next asked whether IL-1β and HMGB1 can also affect focal ictal generation. To this aim, we used an EC slice model in which focal ictal-like discharges were reproducibly generated at a restricted site by perfusing the slice with 100 μM 4-AP and 0.5 mM $Mg^{2+}$, and stimulating a small number of neurons with pressure pulses applied to an NMDA-containing glass pipette. As previously reported (Gomez-Gonzalo et al., 2010; Losi et al., 2010), in this model slices treated with a double, but not a single NMDA pulse triggered a focal ictal-like event. The differential contrast image (DIC) and the fluorescence images in **Figure 2A** show a representative field in EC layer V-VI, the NMDA- and

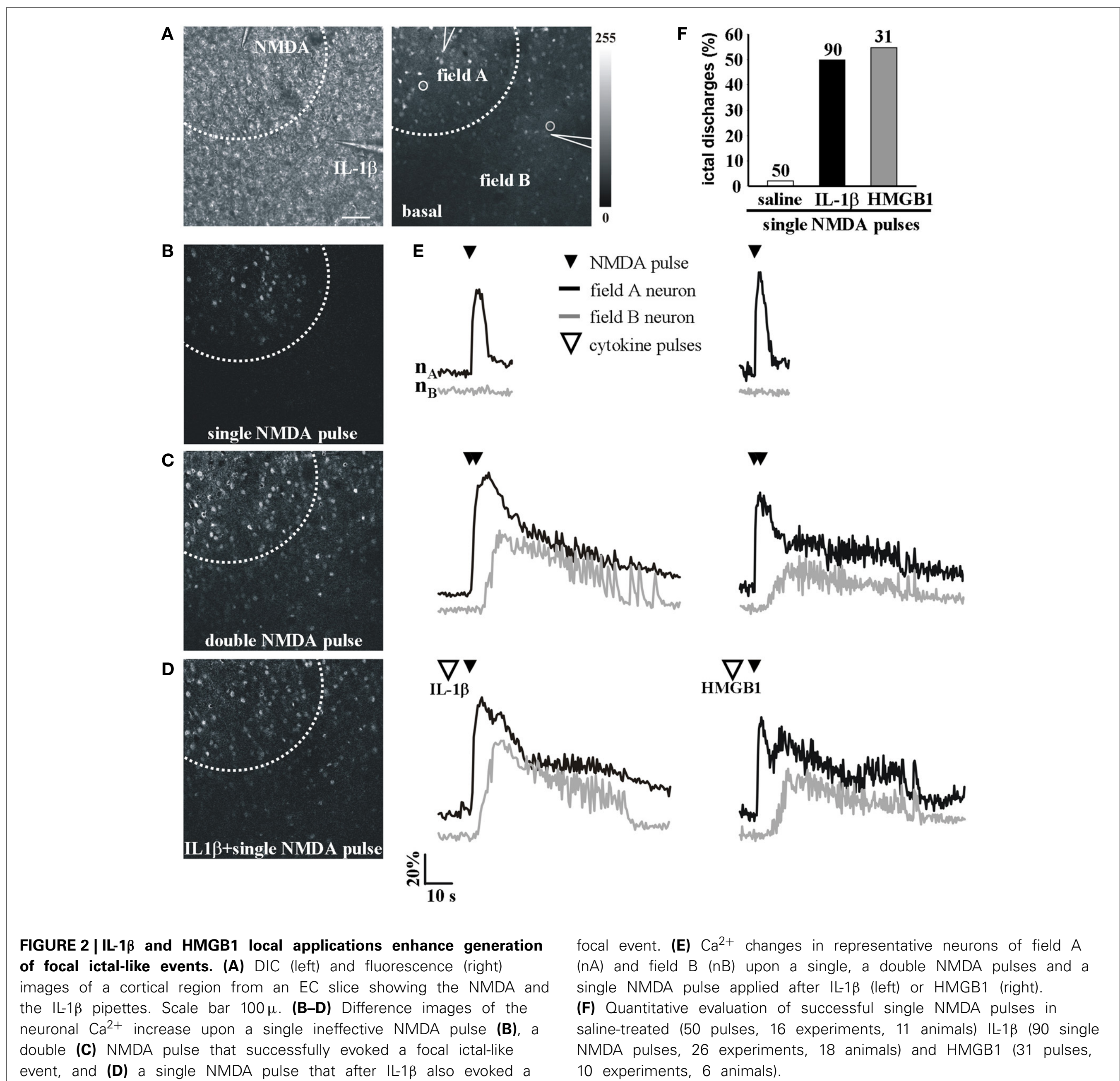



**FIGURE 2 | IL-1β and HMGB1 local applications enhance generation of focal ictal-like events. (A)** DIC (left) and fluorescence (right) images of a cortical region from an EC slice showing the NMDA and the IL-1β pipettes. Scale bar 100 μ. **(B–D)** Difference images of the neuronal $Ca^{2+}$ increase upon a single ineffective NMDA pulse **(B)**, a double **(C)** NMDA pulse that successfully evoked a focal ictal-like event, and **(D)** a single NMDA pulse that after IL-1β also evoked a focal event. **(E)** $Ca^{2+}$ changes in representative neurons of field A (nA) and field B (nB) upon a single, a double NMDA pulses and a single NMDA pulse applied after IL-1β (left) or HMGB1 (right). **(F)** Quantitative evaluation of successful single NMDA pulses in saline-treated (50 pulses, 16 experiments, 11 animals) IL-1β (90 single NMDA pulses, 26 experiments, 18 animals) and HMGB1 (31 pulses, 10 experiments, 6 animals).

the IL-1β-containing pipettes. As illustrated by the difference images generated by subtracting the fluorescence image captured at basal conditions to that obtained after the NMDA stimulation (**Figures 2B–D**), a single NMDA pulse induced only a transient $Ca^{2+}$ raise in a limited number of neurons close to the pipette tip, an area that we defined as the focal area (field A; **Figure 2B**). In contrast, a double NMDA pulse stimulation evoked a stronger activation of field A neurons as well as $Ca^{2+}$ elevations in the surrounding neurons (field B) with the typical pattern of an ictal-like discharge (**Figure 2E**). The ictal event evoked by a double NMDA pulse was highly reproducible while only 1 out of 50 single NMDA pulse performed in 16 slices generated an ictal event within 45 min of 4-AP perfusion. We found that if a single subthreshold NMDA stimulation (that was in general ineffective) was preceded by IL-1β or HMGB1 applications, a focal ictal-like event was evoked in 45 of 90 and 17 of 31 single pulse stimulations, respectively, suggesting that the cytokines can lower the threshold for ictal generation (**Figure 2F**). In a few IL-1β experiments (4 out of 26), we also noted that an ictal-like event was not generated, as usually, at the site of NMDA applications, but rather at the site where IL-1β was applied (**Figure 3**). According to the $Ca^{2+}$ signal change in these experiments, the focal ictal-like event initiated, indeed, in neurons from the IL-1β site and secondarily spread to neurons from the NMDA stimulation site (**Figure 3B**).

### AFTER IL-1β AND HMGB1 LOCAL APPLICATIONS NEURONS AND ASTROCYTES INCREASE THEIR RESPONSE TO NMDA

We then asked whether cytokines could lower the threshold for the generation of focal ictal-like discharges by enhancing the response of the epileptogenic network to NMDA stimulation. We measured the number of activated neurons and astrocytes as well as the amplitude of the $Ca^{2+}$ change in these cells in response to a single NMDA pulse that was preceded by either saline or IL-1β (or HMGB1) applications. Since in this latter case the single NMDA stimulation induced a focal ictal-like event, we restricted our analysis to the initial phase of the response to NMDA, i.e., the time interval between the NMDA pulse and the $Ca^{2+}$ rise in neurons surrounding the focus that marked the ictal discharge onset (dashed vertical lines in **Figure 4A**). As reported in the bar histogram of **Figure 4B**, both $Ca^{2+}$ elevation amplitude ($\Delta F/F0$) and the number of neurons and astrocytes activated by a single NMDA pulse were significantly increased after IL-1β and HMGB1 applications.

### IL-1β AND HMGB1 ACTION DEPENDS ON SYNAPTIC TRANSMISSION

IL-1β and HMGB1 can lower ictal threshold by enhancing either the direct response of neurons to NMDA or the synaptic transmission that follows NMDA receptor-mediated membrane depolarization. To clarify this issue, we performed experiments in the presence of 2 μM TTX that blocks synaptic transmission. In these experiments the amplitude of the $Ca^{2+}$ change and the number of neurons activated by five successive single NMDA pulses (applied every 2 min) were measured before and after saline, IL-1β or HMGB1 pulses (applied every 20 s). We found that when synaptic transmission was blocked by TTX, both IL-1β and HMGB1 failed to enhance the NMDA-mediated $Ca^{2+}$ response of neurons ($n = 6$ for both IL-1β and HMGB1 treated slices, Mann-Whitney test $p = 0.37886$ and $0.9362$, respectively). This observation suggests that to lower the threshold of ictal-like discharges the two cytokines do not act directly on the NMDA receptor activation. However, if TTX was applied to a brain slice that had already experienced ictal-like discharges, HMGB1 ($n = 7$), but

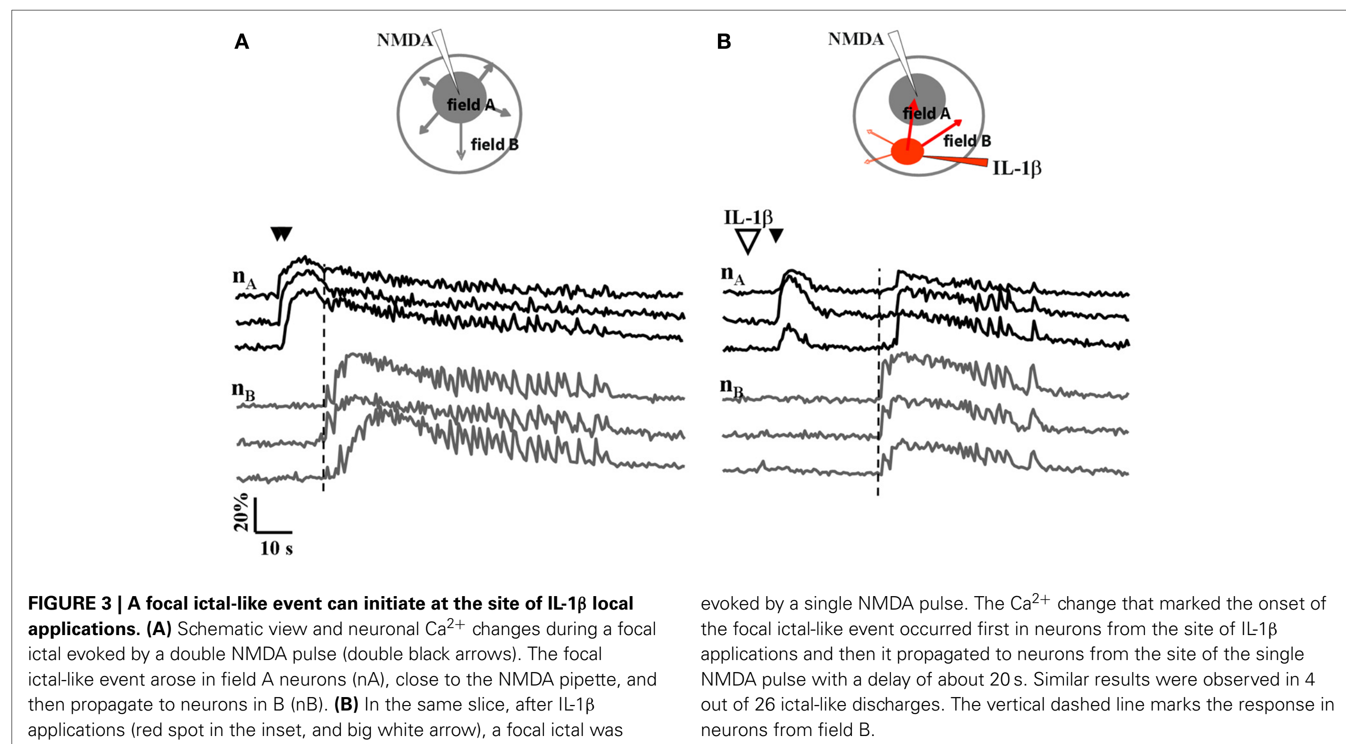



**FIGURE 3 | A focal ictal-like event can initiate at the site of IL-1β local applications. (A)** Schematic view and neuronal $Ca^{2+}$ changes during a focal ictal evoked by a double NMDA pulse (double black arrows). The focal ictal-like event arose in field A neurons (nA), close to the NMDA pipette, and then propagate to neurons in B (nB). **(B)** In the same slice, after IL-1β applications (red spot in the inset, and big white arrow), a focal ictal was evoked by a single NMDA pulse. The $Ca^{2+}$ change that marked the onset of the focal ictal-like event occurred first in neurons from the site of IL-1β applications and then it propagated to neurons from the site of the single NMDA pulse with a delay of about 20 s. Similar results were observed in 4 out of 26 ictal-like discharges. The vertical dashed line marks the response in neurons from field B.

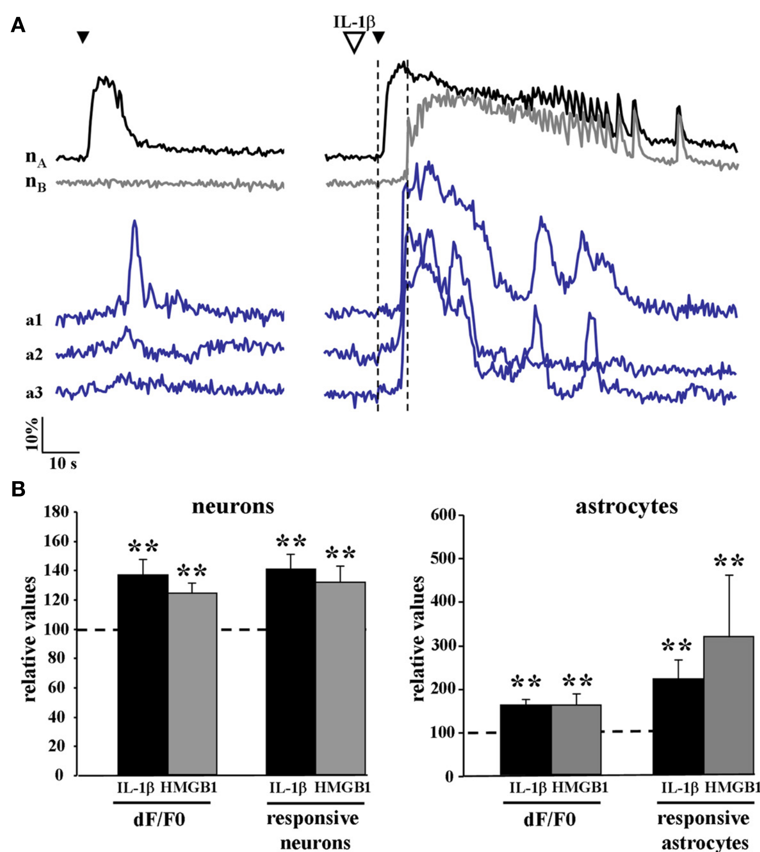



**FIGURE 4 | The $Ca^{2+}$ responsiveness in neurons and astrocytes to a single NMDA pulse was increased following IL-1β and HMGB1 applications. (A)** representative $Ca^{2+}$ changes in neurons and astrocytes evoked by a single NMDA pulse in the absence (left traces) and presence (right traces) of IL1β. The vertical dashed lines indicate the time interval between the NMDA pulse and the $Ca^{2+}$ rise in neurons surrounding the focus that marked the ictal discharge onset. **(B)** Bar histograms of neuron and astrocyte amplitude response to a single NMDA pulse applied after IL1β (black bars, 12 slices, 614 neurons and 356 astrocytes, 11 animals) or HMGB1 (gray bars, 7 slices, 351 neurons and 154 astrocytes, 5 animals). Mann-Whitney test, $^{**}p = 0.01$.

not IL-1β ($n = 6$), increased the responsiveness of neurons to a single NMDA pulse in terms of both of $Ca^{2+}$ elevation amplitude ($+11.7 \pm 3{,}6\%$, $p = 0.03$ with Mann-Whitney test) and number of activated neurons ($+30.2 \pm 10\%$, $p = 0.0021$ with Mann-Whitney test) (**Figure 5**). These latter results raised the hypothesis HMGB1 can directly act on the NMDA receptor, but this event needs a sustained epileptic activity.

## DISCUSSION

We here provide evidence that both IL-1β and HMGB1 can rapidly enhance the generation of epileptiform activities in two different EC slice models of focal ictal-like discharges. In the picrotoxin-low $Mg^{2+}$ model, both agents applied locally to EC slices, increased the frequency of spontaneous ictal-like events, while in the 4-AP model of focally evoked ictal-like events they decreased ictal-like discharge threshold. In this latter model, $Ca^{2+}$ imaging experiments revealed that the NMDA pulse applied to IL-1β- and HMGB1-treated slices evoked a larger activation of both neurons and astrocytes with respect to saline-treated slices. IL-1β or HMGB1 may lower ictal threshold by increasing the sensitivity of neurons to NMDA thus causing a larger recruitment of neurons into the initial episode of NMDA receptor-mediated excitation in a local circuit. These observations are compatible with the view that in a hyperexcitable brain network a focal seizure-like discharge can initiate when an episode of hyperactivity involves a critical mass of neurons.

Different observations in both experimental models and human TLE (for review, see Vezzani and Friedman, 2011) suggest an important role of inflammatory signals in epileptogenesis. The proconvulsant effect of IL-1β and HMGB1 was, indeed, previously reported in an *in vivo* mouse model in which IL-1β and HMGB1 enhanced seizure activity through a mechanism that involved the phosphorylation of the NMDA receptor subunit NR2B (Balosso et al., 2008). Other data obtained from cultured hippocampal neurons support the role of IL-1β in the modulation of NMDA channels through phosphorylation by Src kinases (Viviani et al., 2003). Our experiments in 4-AP/0.5 mM $Mg^{2+}$ confirmed the capability of both cytokines to increase the responsiveness of neurons to NMDA receptor activation. However, after IL-1β applications in the presence of TTX, we could observe neither an increased amplitude of the neuronal $Ca^{2+}$ response to NMDA nor an increased number of responsive neurons,

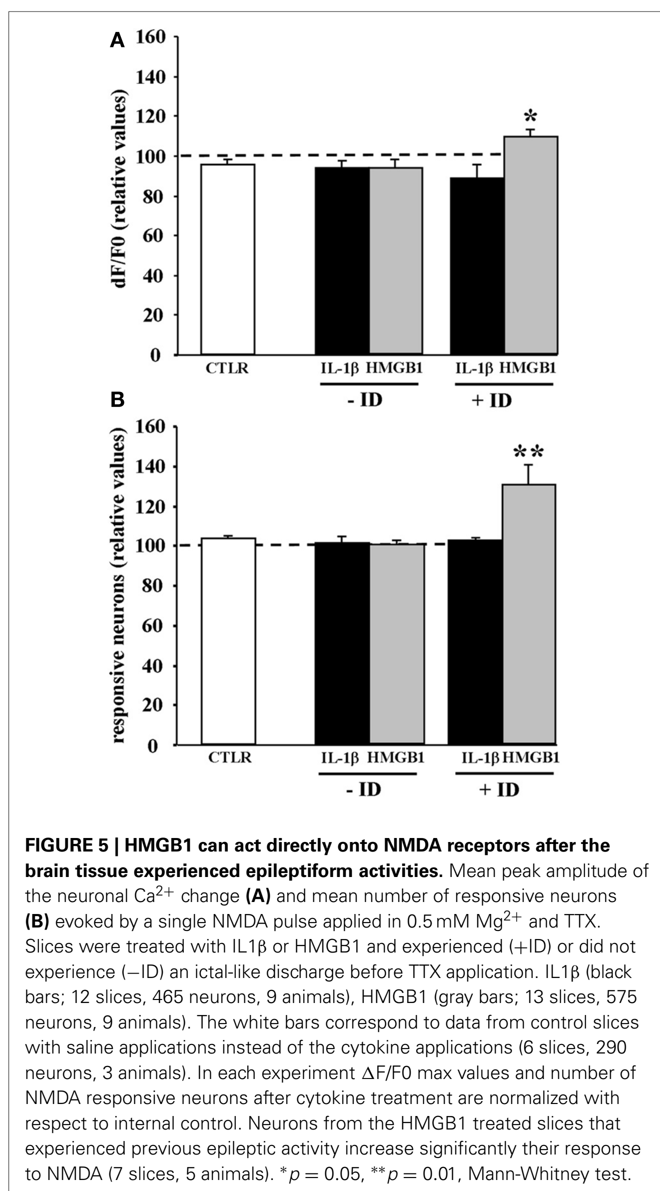



**FIGURE 5 | HMGB1 can act directly onto NMDA receptors after the brain tissue experienced epileptiform activities.** Mean peak amplitude of the neuronal $Ca^{2+}$ change **(A)** and mean number of responsive neurons **(B)** evoked by a single NMDA pulse applied in 0.5 mM $Mg^{2+}$ and TTX. Slices were treated with IL1β or HMGB1 and experienced (+ID) or did not experience (−ID) an ictal-like discharge before TTX application. IL1β (black bars; 12 slices, 465 neurons, 9 animals), HMGB1 (gray bars; 13 slices, 575 neurons, 9 animals). The white bars correspond to data from control slices with saline applications instead of the cytokine applications (6 slices, 290 neurons, 3 animals). In each experiment ΔF/F0 max values and number of NMDA responsive neurons after cytokine treatment are normalized with respect to internal control. Neurons from the HMGB1 treated slices that experienced previous epileptic activity increase significantly their response to NMDA (7 slices, 5 animals). $^{*}p = 0.05$, $^{**}p = 0.01$, Mann-Whitney test.

suggesting an indirect IL-1β action on the NMDA receptor. While we can not exclude a contribution of IL-1β-induced phosphorylation in the proconvulsant effect of IL-1β, other mechanisms, such as activation of presynaptic transient receptor potential vanilloid channels (Rossi et al., 2012), may be also involved in the increased NMDA response.

Interestingly, we occasionally observed that after the initial response of neurons to the NMDA stimulation at the focus, an ictal-like event initiated at the site of IL-1β applications and then spread to other regions including the NMDA application site. This observation suggests that cells that had a direct contact with IL-1β may be more sensitive to NMDA receptor activation.

## HMGB1 ENHANCES NEURONAL RESPONSIVENESS TO NMDA RECEPTOR ONLY AFTER THE TISSUE HAVE EXPERIENCED REPETITIVE ICTAL-LIKE EVENTS

When synaptic transmission was blocked by TTX, both IL-1β and HMGB1 failed to enhance the NMDA-mediated $Ca^{2+}$ response of neurons, suggesting that to lower the threshold of ictal-like events the two cytokines do not act directly on NMDA receptors. However, HMGB1, but not IL-1β, directly increased the $Ca^{2+}$ response to NMDA applications only if the brain tissue experienced an epileptic event prior to NMDA challenges. Note that in the picrotoxin/low $Mg^{2+}$ model HMGB1 significantly increased the mean frequency of ictal-like events, but, differently from IL-1β, it had no effect on the latency of the first epileptic event. We advance the hypothesis that epileptiform activity exerts a priming effect on the tissue to become sensitive to the HMGB1 action. Consistent with this hypothesis, an upregulation of TLR4 expression, i.e., the primary HMGB1 receptor, has been described in both neurons and astrocytes in human cortical malformations (Zurolo et al., 2011) as well as after seizures in animal models (Maroso et al., 2010). It should be noted that since the time scale of our experiments can hardly be consistent with a *de novo* synthesis of this receptor, an alternative mechanism may involve an accelerated mobilization of pre-synthesized receptor from the Golgi to the membrane (Saitoh and Miyake, 2009; McGettrick and O'Neill, 2010). Also to be considered is that rapid changes of the redox state of HMGB1, that may occur during epileptiform activities, are critical for NMDA receptor phosphorylation by this inflammatory agent (Balosso et al., 2014).

In a previous study, we showed that cultured astrocytes can release HMGB1 following IL-1β stimulation (Zurolo et al., 2011). Also noteworthy is that astrocytes, microglia and neurons (expressing TLR4) may respond to HMGB1 stimulation with a production of several pro-epileptogenic inflammatory mediators (Andersson et al., 2005; Kim et al., 2006; Pedrazzi et al., 2007) providing a positive feedback loop that can amplify neuronal excitability.

Since we are investigating the action of two inflammatory agents, on a cautionary note we have to consider the inflammatory components that are potentially induced by slice cutting procedures. It is known that microglia are quickly activated during these procedures and may contribute to the inflammatory status of the slices. Microglia may also actively participate in the modulation of excitatory neurotransmission by recruiting astrocytes via ATP release (Pascual et al., 2012). Nevertheless, our model can not be considered a model of neuroinflammation and this has to be taken into account in evaluating the action of IL-1β and HMGB1 that we reported here.

## ASTROCYTE CALCIUM ELEVATION

In an excitatory loop with neurons, astrocytes have been previously shown to promote neuronal synchronization in local circuits (Fellin et al., 2004) and through this action to enhance the generation of focal ictal-like events in EC slice preparations (Gomez-Gonzalo et al., 2010). A selective inhibition of $Ca^{2+}$ signals in astrocytes increased focal ictal-like threshold, whereas a selective activation of $Ca^{2+}$ increases in astrocytes enhanced ictal generation. In the present study, with respect to controls we observed a significant higher response of astrocytes to a single NMDA pulse applied in IL1β- or HMGB1-pretreated slices. Moreover, blocking synaptic transmission with TTX prevented $Ca^{2+}$ elevations in astrocytes. This evidence suggests that the $Ca^{2+}$ elevation in astrocytes depends on the synaptic activity and

that the proconvulsant effects of IL1β and HMGB1 may reflect a regulation of the neuron-glia communication.

Astrocytes have been, indeed, shown to express a large variety of metabotropic and ionotropic glutamate receptors (Schipke et al., 2001; Lalo et al., 2006; Verkhratsky and Kirchhoff, 2007; D'Antoni et al., 2008; Lundborg et al., 2011). Furthermore, an increasing number of studies provide evidence that the release of gliotransmitters, such as glutamate, ATP or D-serine, can modulate basal synaptic transmission (Di Castro et al., 2011; Panatier et al., 2011) and short- and long-term changes of synaptic strenght in both *in vitro* (Pascual et al., 2005; Serrano et al., 2006; Henneberger et al., 2010; Navarrete and Araque, 2010; Min and Nevian, 2012) and *in vivo* models (Takata et al., 2011; Navarrete et al., 2012; Chen et al., 2013).

By secreting and sensing a large variety of cytokines and chemokines astrocytes may provide a fundamental contribution in the control of the inflammatory status of the brain and through this mechanism to contribute to the generation of epileptiform activities (Aronica et al., 2012). Here we propose that the proconvulsant action of the inflammatory molecules IL-1β and HMGB1 involves also an amplification of neuron-astrocyte reciprocal signaling in local circuits which favors neuronal synchronization and ultimately leads to a decreased threshold for focal ictal-like events.

In conclusion, although the precise underlying cellular mechanism needs to be investigated, our findings demonstrate that both IL-1β and HMGB1 can rapidly affect neuronal excitability and under proepileptic conditions lower the threshold of focal ictal-like discharges. Our findings raise the possibility that targeting these inflammatory pathways may represent an effective therapeutic strategy to prevent seizures.

## ACKNOWLEDGMENTS

We thank for support: European Union HEALTH-F2-2007-202167, Telethon Italy (GGP10138B; GGP12265), Italian Institute of Technology (IIT), CNR Aging Project, FIRB RBAP11X42L, and Cariparo Foundation to Giorgio Carmignoto; National Epilepsy Fund to Eleonora Aronica (NEF09-5).

## REFERENCES

Andersson, A. K., Ronnback, L., and Hansson, E. (2005). Lactate induces tumour necrosis factor-alpha, interleukin-6 and interleukin-1beta release in microglial- and astroglial-enriched primary cultures. *J. Neurochem.* 93, 1327–1333. doi: 10.1111/j.1471-4159.2005.03132.x

Aronica, E., and Crino, P. B. (2011). Inflammation in epilepsy: clinical observations. *Epilepsia* 52(Suppl. 3), 26–32. doi: 10.1111/j.1528-1167.2011.03033.x

Aronica, E., Ravizza, T., Zurolo, E., and Vezzani, A. (2012). Astrocyte immune responses in epilepsy. *Glia* 60, 1258–1268. doi: 10.1002/glia.22312

Balosso, S., Liu, J., Bianchi, M., and Vezzani, A. M. (2014). Disulfide-containing high mobility group box-1 promotes N-methyl-D-aspartate receptor function and excitotoxicity by activating toll-like-receptor 4-dependent signalling in hippocampal neurons. *Antioxid. Redox Signal.* doi: 10.1089/ars.2013.5349. [Epub ahead of print].

Balosso, S., Maroso, M., Sanchez-Alavez, M., Ravizza, T., Frasca, A., Bartfai, T., et al. (2008). A novel non-transcriptional pathway mediates the proconvulsive effects of interleukin-1beta. *Brain* 131, 3256–3265. doi: 10.1093/brain/awn271

Banerjee, P. N., Filippi, D., and Hauser, W. A. (2009). The descriptive epidemiology of epilepsy – a review. *Epilepsy Res.* 85, 31–45. doi: 10.1016/j.eplepsyres.2009.03.003

Bartfai, T., Sanchez-Alavez, M., Andell-Jonsson, S., Schultzberg, M., Vezzani, A., Danielsson, E., et al. (2007). Interleukin-1 system in CNS stress: seizures, fever, and neurotrauma. *Ann. N.Y. Acad. Sci.* 1113, 173–177. doi: 10.1196/annals.1391.022

Chen, J., Tan, Z., Zeng, L., Zhang, X., He, Y., Gao, W., et al. (2013). Heterosynaptic long-term depression mediated by ATP released from astrocytes. *Glia* 61, 178–191. doi: 10.1002/glia.22425

D'Antuono, M., Kohling, R., Ricalzone, S., Gotman, J., Biagini, G., and Avoli, M. (2010). Antiepileptic drugs abolish ictal but not interictal epileptiform discharges *in vitro*. *Epilepsia* 51, 423–431. doi: 10.1111/j.1528-1167.2009.02273.x

D'Antoni, S., Berretta, A., Bonaccorso, C. M., Bruno, V., Aronica, E., Nicoletti, F., et al. (2008). Metabotropic glutamate receptors in glial cells. *Neurochem. Res.* 33, 2436–2443. doi: 10.1007/s11064-008-9694-9

Demir, R., Haberly, L. B., and Jackson, M. B. (1998). Voltage imaging of epileptiform activity in slices from rat piriform cortex: onset and propagation. *J. Neurophysiol.* 80, 2727–2742.

Deprez, F., Zattoni, M., Mura, M. L., Frei, K., and Fritschy, J. M. (2011). Adoptive transfer of T lymphocytes in immunodeficient mice influences epileptogenesis and neurodegeneration in a model of temporal lobe epilepsy. *Neurobiol. Dis.* 44, 174–184. doi: 10.1016/j.nbd.2011.06.011

Devinsky, O., Vezzani, A. M., Najjar, S., De Lanerolle, N. C., and Rogawski, M. A. (2013). Glia and epilepsy: excitability and inflammation. *Trends Neurosci.* 36, 174–184. doi: 10.1016/j.tins.2012.11.008

Di Castro, M. A., Chuquet, J., Liaudet, N., Bhaukaurully, K., Santello, M., Bouvier, D., et al. (2011). Local $Ca^{2+}$ detection and modulation of synaptic release by astrocytes. *Nat. Neurosci.* 14, 1276–1284. doi: 10.1038/nn.2929

Fabene, P. F., Navarro Mora, G., Martinello, M., Rossi, B., Merigo, F., Ottoboni, L., et al. (2008). A role for leuukocyte-endothelial adhesion mechanisms in epilepsy. *Nat. Med.* 14, 1377–1383. doi: 10.1038/nm.1878

Fellin, T., Pascual, O., Gobbo, S., Pozzan, T., Haydon, P. G., and Carmignoto, G. (2004). Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. *Neuron* 43, 729–743. doi: 10.1016/j.neuron.2004.08.011

Gómez-Gonzalo, M., Losi, G., Brondi, M., Uva, L., Sato, S. S., De Curtis, M., et al. (2011). Ictal but not interictal epileptic discharges activate astrocyte endfeet and elicit cerebral arteriole responses. *Front. Cell. Neurosci.* 5:8. doi: 10.3389/fncel.2011.00008

Gomez-Gonzalo, M., Losi, G., Chiavegato, A., Zonta, M., Cammarota, M., Brondi, M., et al. (2010). An excitatory loop with astrocytes contributes to drive neurons to seizure threshold. *PLoS Biol.* 8:e1000352. doi: 10.1371/journal.pbio.1000352

Henneberger, C., Papouin, T., Oliet, S. H., and Rusakov, D. A. (2010). Long-term potentiation depends on release of D-serine from astrocytes. *Nature* 463, 232–236. doi: 10.1038/nature08673

Kim, J. B., Choi, J. S., Nam, K., Lee, M., Park, J. S., and Lee, J. K. (2006). Enhanced transfection of primary cortical cultures using arginine-grafted PAMAM dendrimer, PAMAM-Arg. *J. Control. Release* 114, 110–117. doi: 10.1016/j.jconrel.2006.05.011

Lalo, U., Pankratov, Y., Kirchhoff, F., North, R. A., and Verkhratsky, A. (2006). NMDA receptors mediate neuron-to-glia signaling in mouse cortical astrocytes. *J. Neurosci.* 26, 2673–2683. doi: 10.1523/JNEUROSCI.4689-05.2006

Losi, G., Cammarota, M., Chiavegato, A., Gomez-Gonzalo, M., and Carmignoto, G. (2010). A new experimental model of focal seizures in the entorhinal cortex. *Epilepsia* 51, 1493–1502. doi: 10.1111/j.1528-1167.2009.02472.x

Lundborg, C., Westerlund, A., Bjorklund, U., Biber, B., and Hansson, E. (2011). Ifenprodil restores GDNF-evoked Ca(2+) signalling and Na(+)/K(+)-ATPase expression in inflammation-pretreated astrocytes. *J. Neurochem.* 119, 686–696. doi: 10.1111/j.1471-4159.2011.07465.x

Maroso, M., Balosso, S., Ravizza, T., Liu, J., Aronica, E., Iyer, A. M., et al. (2010). Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. *Nat. Med.* 16, 413–419. doi: 10.1038/nm.2127

Maroso, M., Balosso, S., Ravizza, T., Liu, J., Bianchi, M. E., and Vezzani, A. (2011). Interleukin-1 type 1 receptor/toll-like receptor signalling in epilepsy: the importance of IL-1beta and high-mobility group box 1. *J. Intern. Med.* 270, 319–326. doi: 10.1111/j.1365-2796.2011.02431.x

McGettrick, A. F., and O'Neill, L. A. (2010). Localisation and trafficking of toll-like receptors: an important mode of regulation. *Curr. Opin. Immunol.* 22, 20–27. doi: 10.1016/j.coi.2009.12.002

Min, R., and Nevian, T. (2012). Astrocyte signaling controls spike timing-dependent depression at neocortical synapses. *Nat. Neurosci.* 15, 746–753. doi: 10.1038/nn.3075

Navarrete, M., and Araque, A. (2010). Endocannabinoids potentiate synaptic transmission through stimulation of astrocytes. *Neuron* 68, 113–126. doi: 10.1016/j.neuron.2010.08.043

Navarrete, M., Perea, G., Fernandez de Sevilla, D., Gómez-Gonzalo, M., Núñez, A., Martín, E. D., et al. (2012). Astrocytes mediate *in vivo* cholinergic-induced synaptic plasticity. *PLoS Biol.* 10:e1001259. doi: 10.1371/journal.pbio.1001259

Panatier, A., Vallée, J., Haber, M., Murai, K. K., Lacaille, J. C., and Robitaille, R. (2011). Astrocytes are endogenous regulators of basal transmission at central synapses. *Cell* 146, 785–798. doi: 10.1016/j.cell.2011.07.022

Pascual, O., Ben Achour, S., Rostaing, P., Triller, A., and Bessis, A. (2012). Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. *Proc. Natl. Acad. Sci. U.S.A.* 109, E197–E205. doi: 10.1073/pnas.1111098109

Pascual, O., Casper, K. B., Kubera, C., Zhang, J., Revilla-Sanchez, R., Sul, J. Y., et al. (2005). Astrocytic purinergic signaling coordinates synaptic networks. *Science* 310, 113–116. doi: 10.1126/science.1116916

Pedrazzi, M., Patrone, M., Passalacqua, M., Ranzato, E., Colamassaro, D., Sparatore, B., et al. (2007). Selective proinflammatory activation of astrocytes by high-mobility group box 1 protein signaling. *J. Immunol.* 179, 8525–8532. doi: 10.4049/jimmunol.179.12.8525

Peljto, A. L., Barker-Cummings, C., Vasoli, V. M., Leibson, C. L., Hauser, W. A., Buchhalter, J. R., et al. (2014). Familial risk of epilepsy: a population-based study. *Brain* 137, 795–805. doi: 10.1093/brain/awt368

Pitkanen, A., and Sutula, T. P. (2002). Is epilepsy a progressive disorder? Prospects for new therapeutic approaches in temporal-lobe epilepsy. *Lancet Neurol.* 1, 173–181. doi: 10.1016/S1474-4422(02)00073-X

Ravizza, T., Gagliardi, B., Noe, F., Boer, K., Aronica, E., and Vezzani, A. (2008). Innate and adaptive immunity during epileptogenesis and spontaneous seizures: evidence from experimental models and human temporal lobe epilepsy. *Neurobiol. Dis.* 29, 142–160. doi: 10.1016/j.nbd.2007.08.012

Rossi, S., Furlan, R., De Chiara, V., Motta, C., Studer, V., Mori, F., et al. (2012). Interleukin-1β causes synaptic hyperexcitability in multiple sclerosis. *Ann. Neurol.* 71, 76–83. doi: 10.1002/ana.22512

Saitoh, S., and Miyake, K. (2009). Regulatory molecules required for nucleotide-sensing toll-like receptors. *Immunol. Rev.* 227, 32–43. doi: 10.1111/j.1600-065X.2008.00729.x

Schipke, C. G., Ohlemeyer, C., Matyash, M., Nolte, C., Kettenmann, H., and Kirchhoff, F. (2001). Astrocytes of the mouse neocortex express functional N-methyl-D-aspartate receptors. *FASEB J.* 15, 1270–1272. doi: 10.1096/fj.00-0439fje

Serrano, A., Haddjeri, N., Lacaille, J. C., and Robitaille, R. (2006). GABAergic network activation of glial cells underlies hippocampal heterosynaptic depression. *J. Neurosci.* 26, 5370–5382. doi: 10.1523/JNEUROSCI.5255-05.2006

Takata, N., Mishima, T., Hisatsune, C., Nagai, T., Ebisui, E., Mikoshiba, K., et al. (2011). Astrocyte calcium signaling transforms cholinergic modulation to cortical plasticity *in vivo*. *J. Neurosci.* 31, 18155–18165. doi: 10.1523/JNEUROSCI.5289-11.2011

Verkhratsky, A., and Kirchhoff, F. (2007). NMDA Receptors in glia. *Neuroscientist* 13, 28–37. doi: 10.1177/1073858406294270

Vezzani, A., Balosso, S., Maroso, M., Zardoni, D., Noe, F., and Ravizza, T. (2010). ICE/caspase 1 inhibitors and IL-1beta receptor antagonists as potential therapeutics in epilepsy. *Curr. Opin. Investig. Drugs* 11, 43–50.

Vezzani, A., Balosso, S., and Ravizza, T. (2008). The role of cytokines in the pathophysiology of epilepsy. *Brain Behav. Immun.* 22, 797–803. doi: 10.1016/j.bbi.2008.03.009

Vezzani, A., Conti, M., De Luigi, A., Ravizza, T., Moneta, D., Marchesi, F., et al. (1999). Interleukin-1beta immunoreactivity and microglia are enhanced in the rat hippocampus by focal kainate application: functional evidence for enhancement of electrographic seizures. *J. Neurosci.* 19, 5054–5065.

Vezzani, A., French, J., Bartfai, T., and Baram, T. Z. (2011). The role of inflammation in epilepsy. *Nat. Rev. Neurol.* 7, 31–40. doi: 10.1038/nrneurol.2010.178

Vezzani, A., and Friedman, A. (2011). Brain inflammation as a biomarker in epilepsy. *Biomark. Med.* 5, 607–614. doi: 10.2217/bmm.11.61

Vezzani, A., and Granata, T. (2005). Brain inflammation in epilepsy: experimental and clinical evidence. *Epilepsia* 46, 1724–1743. doi: 10.1111/j.1528-1167.2005.00298.x

Viviani, B., Bartesaghi, S., Gardoni, F., Vezzani, A., Behrens, M. M., Bartfai, T., et al. (2003). Interleukin-1beta enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. *J. Neurosci.* 23, 8692–8700.

Yu, X. M., Askalan, R., Keil, G. J., and Salter, M. W. (1997). NMDA channel regulation by channel-associated protein tyrosin kinase Src. *Science* 31, 674–678. doi: 10.1126/science.275.5300.674

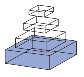
Zurolo, E., Iyer, A., Maroso, M., Carbonell, C., Anink, J. J., Ravizza, T., et al. (2011). Activation of Toll-like receptor, RAGE and HMGB1 signalling in malformations of cortical development. *Brain* 134, 1015–1032. doi: 10.1093/brain/awr032

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 11 March 2014; accepted: 16 May 2014; published online: 06 June 2014.*
*Citation: Chiavegato A, Zurolo E, Losi G, Aronica E and Carmignoto G (2014) The inflammatory molecules IL-1β and HMGB1 can rapidly enhance focal seizure generation in a brain slice model of temporal lobe epilepsy. Front. Cell. Neurosci.* ***8****:155. doi: 10.3389/fncel.2014.00155*
*This article was submitted to the journal Frontiers in Cellular Neuroscience.*
*Copyright © 2014 Chiavegato, Zurolo, Losi, Aronica and Carmignoto. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 14 August 2014
doi: 10.3389/fncel.2014.00234



# Increased astrocyte expression of IL-6 or CCL2 in transgenic mice alters levels of hippocampal and cerebellar proteins

**Donna L. Gruol[1]*, Khanh Vo[1] and Jennifer G. Bray[2]**

[1] Molecular and Cellular Neuroscience Department, The Scripps Research Institute, La Jolla, CA, USA
[2] Department of Biology, University of Wisconsin-Stevens Point, Stevens Point, WI, USA

***Edited by:***
*Silvia Alboni, University of Modena and Reggio Emilia, Italy*

***Reviewed by:***
*Laurens Bosman, Erasmus MC, Netherlands*
*Hailan Yao, Roskamp Institute, USA*

****Correspondence:***
*Donna L. Gruol, Molecular and Cellular Neuroscience Department, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA*
*e-mail: gruol@scripps.edu*

Emerging research has identified that neuroimmune factors are produced by cells of the central nervous system (CNS) and play critical roles as regulators of CNS function, directors of neurodevelopment and responders to pathological processes. A wide range of neuroimmune factors are produced by CNS cells, primarily the glial cells, but the role of specific neuroimmune factors and their glial cell sources in CNS biology and pathology have yet to be fully elucidated. We have used transgenic mice that express elevated levels of a specific neuroimmune factor, the cytokine IL-6 or the chemokine CCL2, through genetic modification of astrocyte expression to identify targets of astrocyte produced IL-6 or CCL2 at the protein level. We found that in non-transgenic mice constitutive expression of IL-6 and CCL2 occurs in the two CNS regions studied, the hippocampus and cerebellum, as measured by ELISA. In the CCL2 transgenic mice elevated levels of CCL2 were evident in the hippocampus and cerebellum, whereas in the IL-6 transgenic mice, elevated levels of IL-6 were only evident in the cerebellum. Western blot analysis of the cellular and synaptic proteins in the hippocampus and cerebellum of the transgenic mice showed that the elevated levels of CCL2 or IL-6 resulted in alterations in the levels of specific proteins and that these actions differed for the two neuroimmune factors and for the two brain regions. These results are consistent with cell specific profiles of action for IL-6 and CCL2, actions that may be an important aspect of their respective roles in CNS physiology and pathophysiology.

**Keywords: neuroimmune, western blot, synapse, signal transduction, GFAP, STAT3**

## INTRODUCTION

Immune factors that play a critical role in the peripheral immune system, such as cytokines and chemokines, are now known to be produced within the CNS by cells of the CNS and to play important roles in normal CNS functions as well as pathological states (Gruol and Nelson, 1997; Glabinski and Ransohoff, 1999; Bajetto et al., 2001; Banisadr et al., 2005; Deverman and Patterson, 2009; Spooren et al., 2011; Erta et al., 2012; Arisi, 2014). Immune factors produced by CNS cells are referred to as neuroimmune factors to distinguish their origin from immune factors that are produced by cells of the peripheral immune system trafficketing through the CNS. The primary source of neuroimmune factors in the CNS is glial cells (astrocytes and microglia) (Kettenmann et al., 2011; Smith et al., 2012; Jensen et al., 2013; Choi et al., 2014). Depending on conditions, some neurons also produce neuroimmune factors (Tsakiri et al., 2008; Wei et al., 2013). Production of neuroimmune factors in the CNS is generally low, but increases significantly during adverse conditions such as injury and disease, when neuroimmune factors are thought to play important protective and/or repair roles. However, if the production of neuroimmune factors becomes dysregulated, the elevated levels may promote pathological processes rather than ameliorate the negative effects of adverse conditions.

CNS cells produce multiple neuroimmune factors, particularly during adverse conditions. The complexity of this situation makes it difficult to distinguish effects of individual neuroimmune factors and to identify target sites of action, information that is basic to an understanding of physiological and pathological roles and the development of new therapeutic strategies. Moreover, the cell source of neuroimmune factors under physiological or pathological conditions can be questionable, as multiple CNS cell types are capable of producing neuroimmune factors. To gain a clearer understanding of the actions and potential roles of neuroimmune factors in the CNS, we have focused on identifying the targets of individual neuroimmune factors when the initial source is CNS astrocytes. Astrocytes are closely associated with neurons and synapses and are known to participate in processes that are essential for normal CNS function, such as regulating synaptic transmission (Halassa et al., 2007; Bernardinelli et al., 2014) and neurodevelopment (Corty and Freeman, 2013). Neurons express receptors for neuroimmune factors, consistent with a role for these factors in astrocyte-neuron interactions. Moreover, astrocytes also express receptors for the same neuroimmune factors that they produce, providing a mechanism for feedback control of astrocyte function, and indirectly astrocyte regulation of neuronal function.

Two neuroimmune factors, the cytokine IL-6 and the chemokine CCL2, are particularly relevant to a number of pathological conditions. Elevated levels of these neuroimmune factors in the CNS parenchyma or cerebrospinal fluid (CSF) have been reported for a number of CNS disease states, and in particular, disease states associated with altered cognitive function. For example, elevated levels of IL-6 occur in the CNS or CSF of patients with clinical depression (Miller and O'Callaghan, 2005; Jones and Thomsen, 2013), active epilepsy (Billiau et al., 2007), Alzheimer's disease (Brosseron et al., 2014), HIV infection (Gallo et al., 1989) and inflammatory neurological disorders (Wullschleger et al., 2013). Similarly, elevated levels of CCL2 occur in the CNS or CSF in HIV infection (Cinque et al., 1998; Kelder et al., 1998), multiple sclerosis (Conductier et al., 2010), Alzheimer's disease (Galimberti et al., 2006; Westin et al., 2012), epilepsy (Wu et al., 2008), and psychiatric disorders (Stuart and Baune, 2014). Although it is unknown whether IL-6 and CCL2 contribute to the cause and/or consequences of disease states and symptoms, correlative evidence indicates a potential role in the etiology of some disease states. For example, increased levels of IL-6 were observed in the CNS of depressed patients and correlated with the severity of symptoms (Lindqvist et al., 2009). IL-6 levels were significantly higher in CSF of systemic lupus erythematosus (SLE) patients that showed psychiatric manifestations compared with SLE patients that did not show psychiatric manifestations (Hirohata and Miyamoto, 1990; Hirohata et al., 2009). IL-6 levels were significantly higher in the CSF of patients with cerebrovascular disease with dementia compared with patients with cerebrovascular disease without dementia (Wada-Isoe et al., 2004). Elevated levels of IL-6 were found in the CNS and CSF of autistic patients and correlated with increased levels IL-6 expression by astrocytes (Vargas et al., 2005; Wei et al., 2011). Similarly, increased levels of CCL2 were observed in the CSF of HIV-infected individuals and correlated with the level of viral load and severity of dementia (Kelder et al., 1998). In older Alzheimer's patients, increased levels of CCL2 in the CSF correlated with cognitive deficits (Galimberti et al., 2006). CSF levels of CCL2 increase significantly with the age of patients with and without neuropsychiatric disease, suggesting that CCL2 plays an important role in the detrimental effects of aging on the CNS (Blasko et al., 2006). Experimental evidence also indicates a role of IL-6 in aging, and in particular, the negative effects of aging on cognitive function (Godbout and Johnson, 2004; Lekander et al., 2011; Burton and Johnson, 2012).

In addition to a role in pathological conditions, expression of IL-6 and CCL2 at low levels in the normal CNS suggest physiological roles for these neuroimmune factors. Studies in experimental animals support such a role for IL-6, but information on CCL2 is lacking. For example, the highest level of mRNA for both IL-6 and IL-6 receptors in the CNS occurs in the hippocampus, suggesting an important physiological role for IL-6 in the hippocampus (Gadient and Otten, 1994a,b; Gruol and Nelson, 1997). Consistent with this possibility, recent studies showed that astrocyte produced IL-6 provides homeostatic control of synaptic function in hippocampal neuronal circuits involved in memory and learning, essential cognitive functions that are disrupted in Alzheimer's disease and other neurologic disorders (Balschun et al., 2004; Baier et al., 2009; del Rey et al., 2013). In these studies, animals implanted with electrodes and subjected to a stimulation paradigm that induced long-term potentiation (LTP) in the hippocampus showed dramatically upregulated IL-6 gene expression (Jankowsky et al., 2000; Balschun et al., 2004; del Rey et al., 2013). LTP is a form of synaptic plasticity characterized by an enhancement in the strength of synaptic transmission that is thought to be a key cellular mechanism underlying memory and learning. Studies using combined *in situ* hybridization and immunohistochemistry identified astrocytes as the source of the elevated levels of IL-6 in the LTP experiments (Jankowsky et al., 2000). Consistent with these studies, IL-6 gene expression was shown to be upregulated in behavioral experiments involving a hippocampal-dependent learning task (i.e., a spatial learning task) (del Rey et al., 2013). Behavioral studies of IL-6 deficient mice are also consistent with a physiological role for IL-6 in hippocampal function. For example, IL-6 deficient mice showed impaired memory in a behavioral test of hippocampal dependent memory (i.e., the Morris water maze), a result consistent with a regulatory role for IL-6 in memory management (Baier et al., 2009).

Studies involving exogenous application of IL-6 or CCL2 also support the idea that IL-6 and CCL2 can regulate neuronal function and behavior, actions that could play a role in the physiological or pathophysiological consequences of IL-6 or CCL2 expression in the CNS. For example, our studies of cultured hippocampus and cerebellum show that acute or chronic exposure to IL-6 or CCL2 can alter the physiological properties of neurons including neuronal excitability, synaptic transmission and glutamate receptor function (e.g., Qiu et al., 1998; Nelson et al., 2002, 2004; Gruol and Nelson, 2005; van Gassen et al., 2005). Similarly, studies of hippocampal slices acutely isolated from animals and studied *in vitro* showed that exogenous application of IL-6 reduced LTP (Li et al., 1997; Tancredi et al., 2000), while exogenous application of CCL2 to hippocampal slices increased excitability and synaptic transmission (Zhou et al., 2011). Exogenous application of CCL2 also increased neuronal excitability in striatal slices from mice (Guyon et al., 2009). In parallel studies, intranigral injections of CCL2 in mice increased locomotor activity, a result consistent with the excitatory actions of CCL2 in the slice studies (Guyon et al., 2009). Increasing the CNS levels of IL-6 also altered the behavior of mice as assessed by several different behavioral tests. For example, increasing levels of IL-6 in the CNS of mice by the use of the adenovirus expression system resulted in impaired cognitive ability and altered synaptic function (Wei et al., 2012).

Thus, there is a growing body of knowledge that supports physiological and/or pathological roles for IL-6 and CCL2 in the CNS. However, the exact role of these neuroimmune factors and mechanisms underlying their CNS actions are yet to be elucidated. Our goal in the current study was to determine if specific cellular and synaptic proteins are targets of IL-6 or CCL2 action, effects that could contribute to the mechanisms underlying the physiological or pathological actions of these neuroimmune factors. For these studies, we took advantage of two transgenic mouse models that express elevated levels of IL-6 or CCL2 in the CNS. In the transgenic mouse models, the elevated levels of IL-6 or CCL2 were accomplished by gene manipulation

of astrocyte expression. Astrocytes are the most abundant cell type in the CNS (Heneka et al., 2010) and a primary producer of IL-6 and CCL2 in the normal CNS and during pathological conditions (Farina et al., 2007; Qin and Benveniste, 2012; Jensen et al., 2013; Choi et al., 2014). Expression of IL-6 or CCL2 in the astrocytes of the respective transgenic mice is under control of the promoter for GFAP, an astrocyte specific structural protein. Thus, the elevated production of these two neuroimmune factors is likely to involve similar if not identical pathways, and, at least initially, result in a similar spatial distribution of the secreted peptide. Moreover, the restricted expression in astrocytes enables identification of the initial source of the elevated levels of IL-6 or CCL2 in the transgenic CNS.

Studies of mice from the IL-6 and CCL2 transgenic lines indicate that the elevated astrocyte expression of the respective neuroimmune factor has neurological effects, although most effects were not prominent until later in the lifespan and differ for the two transgenic lines. The IL-6 transgenic (tg) mice and their non-transgenic (non-tg) littermates used in our study are heterozygote, low expressor mice from the 167 line (Campbell et al., 1993). Several studies have described the neurologic deficits of these mice (Chiang et al., 1994; Heyser et al., 1997; Boztug et al., 2002; Vallieres et al., 2002; Samland et al., 2003; Nelson et al., 2012). The IL-6 tg mice progressively develop tremor and ataxia by 6 months of age, indicative of cerebellar dysfunction, and infrequently, seizures (Campbell et al., 1993). In tests of a hippocampal-dependent behavior, avoidance learning, the IL-6 tg mice show progressive deficits that were not prominent until 12 months of age. Thus, at 3 months of age there was no difference in the ability of the IL-6 tg and non-tg mice to learn the avoidance response, whereas at 6 months of age and older the IL-6 tg mice progressively exhibited more errors in learning than the non-tg mice (Heyser et al., 1997). Functional alterations at earlier ages (at 2 months of age) were also demonstrated by increased susceptibility of the IL-6 tg mice to kainic acid and NMDA induced seizures (Samland et al., 2003) and enhanced synaptic transmission in the IL-6 tg hippocampus studied *in vitro* (Nelson et al., 2012). Interestingly, in transgenic mice that express elevated levels of IL-6 through neuronal expression, including expression in hippocampal pyramidal neurons and cerebellar Purkinje neurons, astrogliosis but no neurological deficits were observed (Fattori et al., 1995), suggesting that the source of IL-6 is an important factor in the neuronal consequences of IL-6 actions in the CNS.

Characterization of the CCL2-tg mice is more limited than for the IL-6 tg mice. However, in the heterozygote CCL2-tg mice used in our studies, no overt CNS pathology or neurological impairments were observed up to 7 months of age (Huang et al., 2002, 2005). At older ages, neurological impairments such as difficulty with righting reflex and limb weakness were observed (Huang et al., 2005). Behavioral tests at 2–3 months of age showed no significant difference between the CCL2-tg and CCL2 non-tg animals in rotarod performance, a test of cerebellar function, or in a behavior test of cued and contextual fear conditioning, which involves hippocampal and amygdala function (Bray et al., 2013). However, *in vitro* studies of hippocampal synaptic function at 2–3 months of age showed increased excitability in CA1 pyramidal neurons in CCL2-tg hippocampus, indicative of neuroadaptive changes due to the enhanced astrocyte expression of CCL2 (Bray et al., 2013).

IL-6 and CCL2 produce their biological effects by acting at specific membrane receptors. The IL-6 receptor is a transmembrane receptor that lacks a signal transduction element. Instead, IL-6 receptors utilize a common transmembrane signaling receptor referred to as gp130 (glycoprotein 130). When activated by IL-6, the IL-6 receptor associates with gp130 and induces dimerization with a second gp130 (Taga and Kishimoto, 1997). The gp130/gp130 homodimer can then activate JAK, STAT3, and/or MAPK signaling cascades, which leads to gene expression and other downstream actions. gp130 is widely distributed throughout the CNS, but IL-6 receptor distribution is more restricted. IL-6 can also produce biological effects by trans-signaling, which results from shedding of the membrane receptor to form a soluble receptor or alternative splicing of IL-6 receptor mRNA (Rose-John et al., 2006). The soluble receptor after binding IL-6 can interact with gp130 in IL-6 receptor expressing cells and in cells that normally do not express IL-6 receptor but do express gp130. Studies suggest that trans-signaling plays a central role in pathological actions of IL-6 in the CNS (Rose-John et al., 2006; Burton et al., 2011; Campbell et al., 2014). In contrast to IL-6, receptors for chemokines including CCL2 are G-protein coupled receptors (GPCRs) linked to Gi/Go. A variety of signal transduction pathways are activated by chemokine receptors including MAPK. These signal transduction pathways can ultimately lead to altered gene expression and downstream changes in protein levels. Therefore, modulation of protein levels could be one mechanism through which IL-6 and CCL2 participate in homeostatic control of neuronal and glial function, engage in protection and/or repair, and/or take part in pathological processes.

To identify targets (direct or indirect) of astrocyte produced IL-6 and CCL2 that are involved in the CNS actions of these neuroimmune factors, we compared protein levels in the hippocampus and cerebellum of IL-6 tg and CCL2-tg mice and their non-tg littermates. Both CNS regions play key roles in behavior. The hippocampus plays a key role in memory and learning, while the cerebellum plays a key role in coordinated movement. Results show that the levels of specific cellular and synaptic proteins are altered by elevated astrocyte expression of IL-6 and/or CCL2 and that these actions differed for the two neuroimmune factors in an age and CNS region dependent manner. These results are consistent with cell specific profiles of action for IL-6 and CCL2, actions that may be an important aspect of their respective roles in CNS physiology and pathophysiology.

## MATERIALS AND METHODS

### ANIMALS

All animal procedures were performed in accordance with the Scripps Research Institute and the National Institutes of Health Guideline for the Care and Use of Laboratory Animals. Animal facilities and experimental protocols were in accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care. Heterozygote IL-6 or CCL2 (previously known as monocyte chemoattractant protein-1 or MCP-1) transgenic mice and their non-transgenic littermates (as controls) were used for all studies. The IL-6 line was obtained from Dr. Ian Campbell

of the University of Sydney and the CCL2 line was obtained from Dr. Richard Ransohoff of the Cleveland Clinic Foundation. Methods for the generation of the transgenic lines were previously published. Briefly, for the IL-6 line, full-length murine IL-6 cDNA was modified and inserted into the glial fibrillary acidic protein (GFAP) gene. The hybrid (transgene) DNA was subsequently microinjected into fertilized eggs of (C57BL/6J × SJL) $F_1$ hybrid mice (Campbell et al., 1993). Transgene expression in astrocytes was documented by *in situ* hybridization studies and expression of the lacZ reporter gene as assessed by immunohistochemical detection of β-gal (Campbell et al., 1993; Vallieres et al., 2002). For the CCL2 line, the murine CCL2 gene was placed under control of the huGFAP promoter and a purified GFAP-CCL2 fusion gene fragment was injected into fertilized eggs of SWXJ ($H\text{-}21^{q,s}$) mice (Huang et al., 2002). For both lines, transgenic offspring were identified by analysis of tail DNA using standard protocols. Cut tail tips from individual animals were obtained at weaning (21–28 days postnatal). Tail DNA was extracted using the Mouse Tail Quick Extraction Kit (Pioneer Inc., San Diego CA). Mice positive for the IL-6 or CCL2 transgene were identified by PCR. Samples were prepared for PCR using the HotStart Taq Master Mix with Loading Dye (Pioneer). Both lines are congenic and have been maintained for several years by breeding heterozygous transgenic mice with wildtype C57BL/6J mice.

**PROTEIN ASSAYS**

Protein samples for ELISA or Western blot assays were prepared from hippocampus and cerebellum of transgenic and non-transgenic mice using standard protocols. To obtain the tissue, mice were anesthetized with isoflurane and decapitated. The brains were rapidly removed and immersed in chilled artificial cerebral spinal fluid (ACSF). The ACSF composition was 130.0 mM NaCl, 3.5 mM KCl, 1.25 mM $NaH_2PO_4$, 24.0 mM $NaHCO_3$, 0.2 mM $CaCl_2$, 5.0 mM $MgSO_4$, and 10.0 mM glucose (all chemicals from Sigma-Aldrich, St. Louis, MO). The ACSF was maintained on ice and was bubbled continuously with 95% $O_2$/5% $CO_2$ to provide oxygen and to stabilize pH at 7.2–7.4. The hippocampus and cerebellum were dissected from the brain, snap frozen on dry ice and stored at −80°C until use. In some studies, instead of immediately freezing the hippocampus, hippocampal slices were prepared using a protocol previously described (Nelson et al., 2011). Briefly, after cooling the brain in ACSF, the left and right hippocampi were removed from the brain and cut into 400 μm slices using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Surrey, UK). Approximately six slices were obtained from each hippocampus. The slices were placed in two gas-fluid interface chambers maintained at ∼33°C and were continuously superfused with oxygenated ACSF at rate of 0.55 ml/min to retain viability. After 2 h incubation, slices were pooled according to chamber, snap frozen and stored at −80°C.

Proteins were extracted from all tissue samples by sonication in cold lysis buffer containing 50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% NP-40, a Complete Protease Inhibitor Cocktail Tablet (Roche Diagnostics, Mannheim, Germany), and a cocktail of phosphatase inhibitors ($Na^+$ pyrophosphate, β-glycerophosphate, NaF, $Na^+$ orthovanadate; all from Sigma-Aldrich). The samples were incubated on ice for 30 min, centrifuged at 13,860 g for 30 min at 4°C, and the supernatants were collected. Protein concentration in the supernatants was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). Aliquots were stored at −80°C.

IL-6 and/or CCL2 levels in hippocampal and cerebellar protein samples were determined by ELISA using the Mouse IL-6 ELISA Ready-SET-Go! Kit or the Mouse CCL2 ELISA Ready-SET-Go! Kit, respectively (eBioscience, Inc., San Diego, CA) following manufacturer's instructions. Levels of other proteins were determined by Western blot following previously published protocols (Nelson et al., 2012). Briefly, equal amounts of hippocampal or cerebellar protein samples were subjected to SDS-PAGE using 4–12% Novex NuPAGE Bis-Tris gels (Invitrogen Life Technologies, Grand Island, NY). Transgenic and non-transgenic protein samples were run on the same gel. Samples were run in duplicate. Proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA) and uniform transfer assessed by Ponceau S staining (Pierce, Rockford, IL). Membranes were washed and blocked in a 5% casein solution (Pierce). The membranes were incubated in primary antibody overnight (4°C), washed, and then incubated (room temperature) in secondary antibody coupled to horseradish peroxidase (HRP). Protein bands were visualized by chemiluminescence and quantified by densitometry measurements using NIH Image software.[1] Membranes were stripped and reprobed for β-actin. To adjust for possible loading errors, the density of each band was normalized to the density of the band for β-actin in the same lane. Normalized data from transgenic mice were then normalized to the average normalized value for non-transgenic mice run on the same gel. In some studies to enable comparisons, hippocampal and cerebellar samples were run on the same ELISA and/or Western blot. Also, in some studies CCL2 and IL-6 samples were run on the same ELISA and/or Western blot. Data were combined according to mouse line, genotype and age of the animal and reported as mean ± SEM.

The following antibodies were used for Western blot studies: a monoclonal antibody to β-actin (#AC-15, 1:5000; Sigma, St. Louis, Missouri); a monoclonal antibody to GFAP (#MAB360; 1:10,000; Millipore); a monoclonal antibody raised against neuron specific enolase (#MAB314; 1:5000; Millipore); a mouse monoclonal antibody to glutamine synthetase (Glu syn) raised against a recombinant fragment corresponding to amino acids 274–374 of human glutamine synthetase (ab64613; 1:1000; abcam, Cambridge, MA); a purified rabbit polyclonal antibody raised against a peptide mapping at the carboxy terminus of C/EBP beta (C-19; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA); a rabbit polyclonal antibody raised against a synthetic peptide to the C-terminus of rat GAD 65/67 (#AB1511; 1:1000; Millipore); a purified rabbit antibody to synapsin 1 (#51-5200; 1:5,000; Invitrogen; Syn 1); a purified rabbit polyclonal antibody raised against a synthetic peptide of the rat GluA1 subunit of the AMPA receptor conjugated to keyhole limpet hemocyanin (KLH; a protein carrier) with a cysteine added (#07-660; 1:500; Millipore); a purified goat polyclonal antibody raised against a

[1] http://rsb.info.nih.gov/nih-image/

peptide corresponding to an amino acid mapping the C-terminus of the human GluN1 subunit of the NMDA receptor (sc-1467; 1:500; Santa Cruz Biotechnology); a rabbit polyclonal antibody raised against p44/p42 MAPK (#61-7400; 1:5000, Zymed, Carlsbad, CA, USA); a purified rabbit polyclonal antibody raised against a synthetic phospho-peptide (KLH-coupled) corresponding to residues around Thr202/Tyr204 of human p44/p42 MAPK (#9101; 1:500; Cell Signaling Technologies, Danvers, MA; pp44/42 MAPK); a purified rabbit polyclonal antibody raised against a synthetic peptide (KLH-coupled) corresponding to the sequence of mouse STAT3 (AB#9132; 1:1000; Cell Signaling Technologies), a purified rabbit polyclonal antibody raised against a synthetic phospho-peptide (KLH-coupled) corresponding to the residues surrounding Tyr705 of mouse STAT3 (AB#9131; 1:1000; Cell Signaling Technologies).

### STATISTICAL ANALYSES

Statistical analyses of differences were performed using the unpaired *t*-test or ANOVA. Statistical significance was set at $p < 0.05$. For most studies, statistical comparisons were made between transgenic (IL-6 tg or CCL2-tg) vs. non-transgenic (IL-6 non-tg or CCL2 non-tg) values for hippocampus or cerebellum within the same age group and mouse line. For the IL-6 line results were obtained from: (a) mice 1–2 months of age: 25 IL-6 tg and 31 non-tg hippocampi (52 Western blots) and 9 IL-6 tg and 12 non-tg cerebella (73 Western blots); (b) mice 3–5 months of age: 42 IL-6 tg and 41 non-tg hippocampi (69 Western blots) and 9 IL-6 tg and 10 non-tg cerebella (17 Western blots); (c) mice 12 months of age: 10 IL-6 tg and 9 non-tg hippocampi (41 Western blots) and 11 IL-6 tg and 8 non-tg cerebella (21 Western blots). For the CCL2 line results were obtained from: (a) mice 1–2 months of age: 11 CCL2-tg and 11 non-tg hippocampi (34 Western blots) and 8 CCL2-tg and 9 non-tg cerebella (7 Western blots); (b) mice 3–5 months of age: 30 CCL2-tg and 23 non-tg hippocampi (56 Western blots) and 25 CCL2-tg and 26 non-tg cerebella (15 Western blots); and (c) mice 7–9 months of age: 26 CCL2-tg and 26 non-tg hippocampi (45 Western blots).

## RESULTS

### IL-6 AND CCL2 EXPRESSION

Levels of IL-6 and CCL2 in hippocampus and cerebellum of transgenic and non-transgenic mice at different ages were determined by ELISA. Results are shown in **Figure 1**. For the IL-6 line, low levels of IL-6 were observed in the hippocampus with no apparent genotypic difference (**Figure 1A**). IL-6 levels in the cerebellum were higher than in the hippocampus and a significant genotypic difference was observed (IL-6 tg > non-tg; **Figure 1A**). No prominent age-dependent differences were observed for levels of IL-6 in IL-6 tg and non-tg hippocampus and cerebellum. In contrast, levels of CCL2 were prominent in the CCL2-tg hippocampus and higher in the hippocampus than in the cerebellum. CCL2 levels showed a significant genotypic difference (CCL2-tg > non-tg) in both CNS regions (**Figure 1B**). No prominent age-dependent differences were observed for levels of CCL2 in CCL2-tg and non-tg hippocampus. CCL2 levels were also measured in the IL-6 tg and non-tg hippocampus and cerebellum and were comparable to that observed in the CCL2 non-tg hippocampus and cerebellum (**Figure 1C**). Similarly, IL-6 levels were measured in the CCL2-tg and non-tg hippocampus and cerebellum and were comparable to that observed in the IL-6 non-tg hippocampus and cerebellum (data not shown).

To determine if astrocytes constitutively secrete IL-6 or CCL2, an *in vitro* experimental protocol was used. In these studies, hippocampal slices were prepared from transgenic and non-transgenic mice and IL-6 or CCL2 levels were measured in the tissue after an *in vitro* incubation period. The rationale for these studies is that if the astrocytes in the CCL2-tg or IL-6 tg hippocampus constitutively secrete CCL2 or IL-6, respectively, tissue levels would decline during the incubation period, provided that the rate of synthesis did not match release. To identify such a change, tissue levels in the slices after incubation were compared to levels in hippocampal tissue that was directly snap frozen after removal from the animal. The protocol for the preparation and maintenance of the slices followed a standard protocol used for neurophysiological studies of hippocampal slices *in vitro* (e.g., Nelson et al., 2012). The slices were incubated for 2 h under physiological conditions with constant superfusion of oxygenated ACSF to maintain viability and remove secreted protein from the slices. The slices were then snap frozen for later protein assay. Results from ELISA measurements are shown in **Figures 1D,E**. Hippocampal samples prepared from tissue directly snap frozen after removal from the animal were measured in the same ELISAs. Levels of IL-6 in slices were generally higher than in the animal samples (**Figure 1D**), whereas the opposite was the case for CCL2 (**Figure 1E**). The most dramatic effect was a prominent reduction in the level of CCL2 in CCL2-tg hippocampal slices compared with hippocampal samples directly obtained from CCL2-tg animals. This result is consistent with constitutive astrocyte secretion of CCL2 (**Figure 1E**). Although constitutive secretion of IL-6 from astrocytes may have occurred, the higher levels of IL-6 in the hippocampal slices confounded assessment of secretion. The higher levels of IL-6 in the slices may have resulted from increased production, perhaps induced by the experimental manipulations. Alternatively, the higher levels could reflect reduced secretion, perhaps because IL-6 secretion from astrocytes requires a specific trigger.

Taken together, results from these studies show prominent differences in the levels of astrocyte produced IL-6 or CCL2 in the respective transgenic mice both within and across CNS regions. They also show that *in vitro* incubation differentially alters the levels of IL-6 and CCL2 in hippocampal tissue. These differences indicate that astrocyte production and/or trafficking varies for these two neuroimmune factors. In addition, these results show that increased expression of IL-6 does not trigger increased expression of CCL2 in the two CNS regions studied.

### HOUSEKEEPING PROTEINS

To assess global effects of upregulated astrocyte expression of IL-6 or CCL2 in the hippocampus and cerebellum, several housekeeping proteins were measured by Western blot in tissue obtained from transgenic and non-transgenic mice at different ages. These proteins included β-actin, a cytoskeletal protein expressed in all cells, GFAP, an astrocyte specific cytosketal protein, glutamine synthetase, a protein important in astrocyte

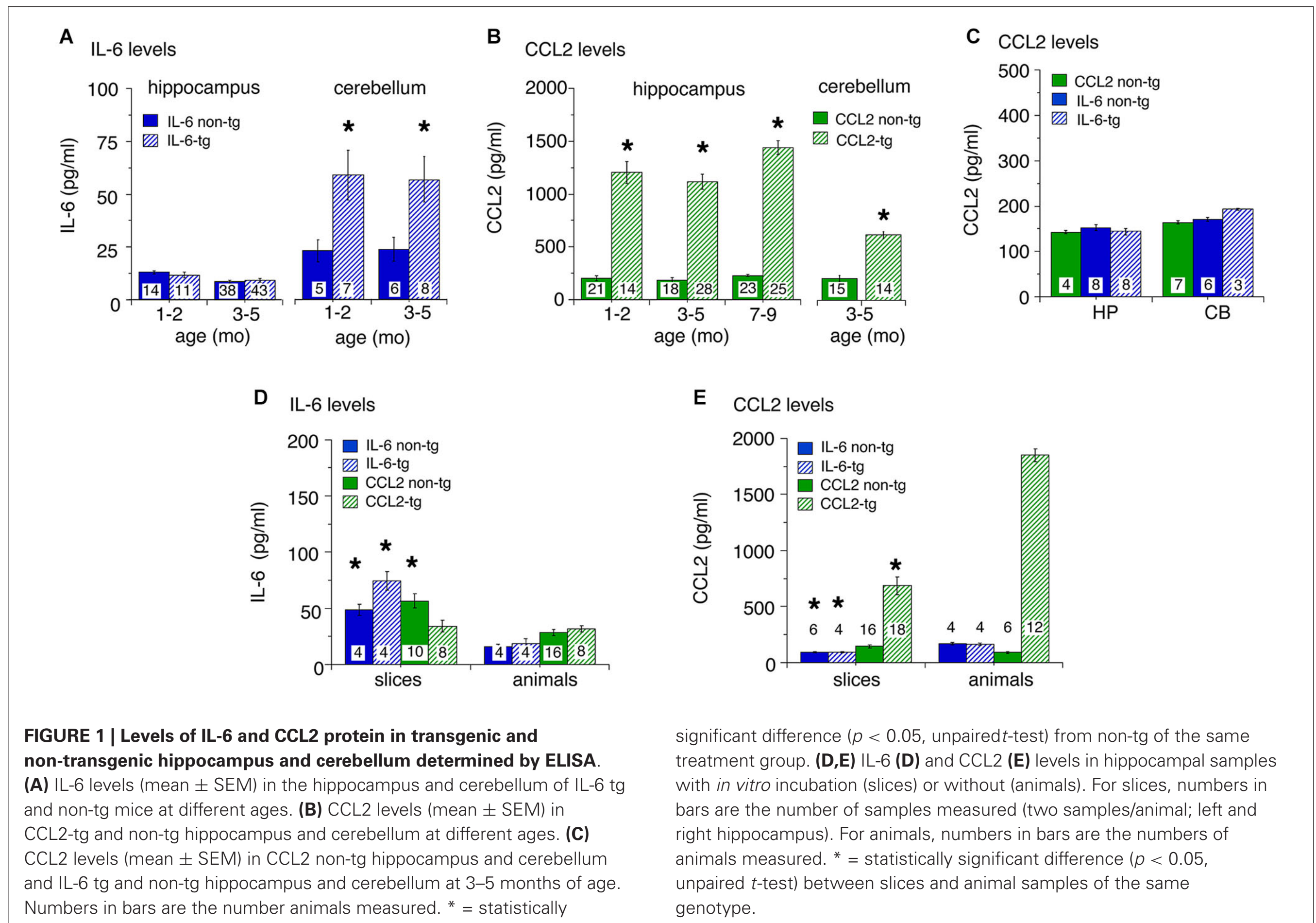



**FIGURE 1 | Levels of IL-6 and CCL2 protein in transgenic and non-transgenic hippocampus and cerebellum determined by ELISA.** **(A)** IL-6 levels (mean ± SEM) in the hippocampus and cerebellum of IL-6 tg and non-tg mice at different ages. **(B)** CCL2 levels (mean ± SEM) in CCL2-tg and non-tg hippocampus and cerebellum at different ages. **(C)** CCL2 levels (mean ± SEM) in CCL2 non-tg hippocampus and cerebellum and IL-6 tg and non-tg hippocampus and cerebellum at 3–5 months of age. Numbers in bars are the number animals measured. * = statistically significant difference ($p < 0.05$, unpaired *t*-test) from non-tg of the same treatment group. **(D,E)** IL-6 **(D)** and CCL2 **(E)** levels in hippocampal samples with *in vitro* incubation (slices) or without (animals). For slices, numbers in bars are the number of samples measured (two samples/animal; left and right hippocampus). For animals, numbers in bars are the numbers of animals measured. * = statistically significant difference ($p < 0.05$, unpaired *t*-test) between slices and animal samples of the same genotype.

trafficking of glutamate, and neuron specific enolase, a specific neuronal protein. Results are shown in **Figures 2**, **3**. In the IL-6 line, a significant genotypic difference (IL-6 tg > non-tg) was observed for GFAP levels in the hippocampus and cerebellum at all ages studied (**Figure 2A**). There was no significant genotypic difference in the levels of β-actin, glutamine synthetase and enolase in hippocampus at all ages studied. However, age-dependent genotypic differences were observed in the cerebellum. A prominent increase in the level of glutamine synthetase was observed at 1–2 months of age in the IL-6 tg cerebellum with a smaller increase at 3–5 months of age. A significant decrease in the level of enolase was also observed in the IL-6 tg cerebellum at 1–2 months of age. In addition, β-actin levels showed an increase in the IL-6 tg cerebellum at 3–5 months of age. No genotypic differences were observed in the IL-6 cerebellum at other ages studied. In contrast, in the CCL2 line, there were no genotypic differences in the levels of GFAP, β-actin, glutamine synthetase and enolase in the hippocampus or cerebellum at the ages studied (**Figure 3A**).

Taken together, these results show that increased expression of IL-6 in the IL-6 tg hippocampus and cerebellum, although not detected by ELISA in the hippocampus, has a pronounced effect on the expression of some hippocampal and cerebellar housekeeping proteins such as GFAP. Increased expression of CCL2, which can be detected by ELISA, has no apparent effect on levels of the same proteins. In addition, the absence of increased levels of GFAP in the CCL2-tg hippocampus and cerebellum indicates that CCL2 does not induce upregulated levels of IL-6 in these CNS regions of the CCL2-tg mice.

### SIGNAL TRANSDUCTION PROTEINS

The level of signal transduction proteins p42/44 MAPK, STAT3 and C/EBPβ were also measured by Western blot in the hippocampus and cerebellum of the transgenic and non-transgenic mice (**Figures 2B**, **3B**). All three signal transduction proteins are known to be important regulators of normal neuronal and glial development and function (Sterneck and Johnson, 1998; Taubenfeld et al., 2001; Thomas and Huganir, 2004; Paquin et al., 2005; Ejarque-Ortiz et al., 2007; Dziennis and Alkayed, 2008; Kfoury and Kapatos, 2009; Cheng et al., 2013; Nicolas et al., 2013). For example, in the hippocampus, p42/44 MAPK and STAT3 play central roles in synaptic plasticity involved in memory and learning (Sweatt, 2001; Nicolas et al., 2012). Importantly, STAT3 and p42/44 MAPK are key players in IL-6 signal transduction and p42/44 MAPK is also involved in CCL2 signal transduction.

No significant genotypic differences in the levels of p42/44 MAPK were observed in the IL-6 hippocampus or cerebellum at the ages studied (**Figure 2B**). In contrast, a significant increase

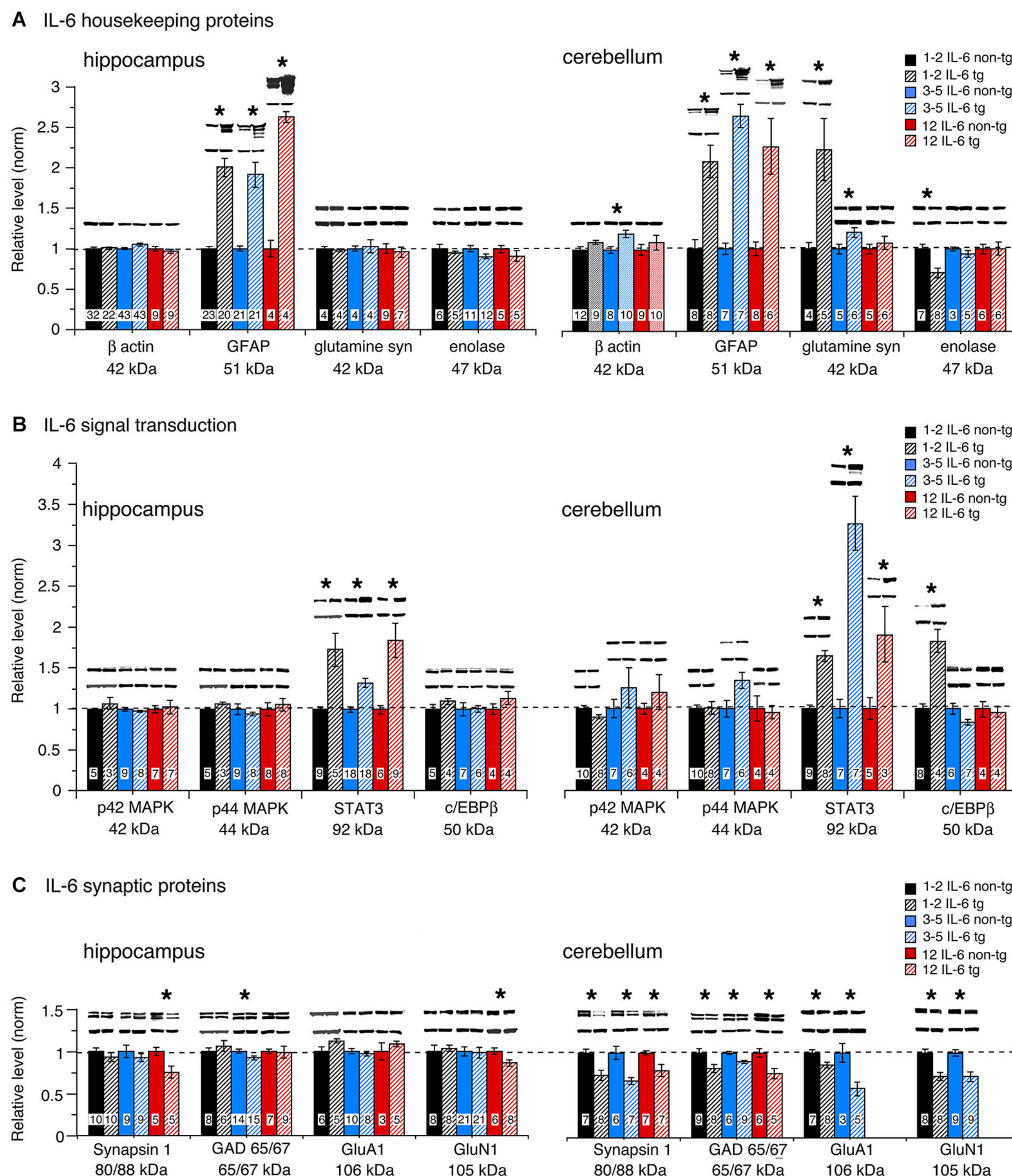



**FIGURE 2 | Protein levels in hippocampus and cerebellum of IL-6 tg and non-tg mice at different ages determined by Western blot. (A)** Mean (±SEM) values for levels of housekeeping proteins. **(B)** Mean (±SEM) values for levels of signal transduction proteins. **(C)** Mean (±SEM) values for levels of synaptic proteins. Representative Western blots are shown above the respective bar. Top blot is the protein indicated for the graph; bottom blot is β actin in the same lane. Numbers in bars are the number of animals measured. * = statistically significant difference between IL-6 tg and non-tg for the age group.

in STAT3 levels was observed in the IL-6 tg hippocampus and cerebellum at all ages studied (**Figure 2B**). C/EBPβ levels were prominently increased in the IL-6 tg cerebellum at 1–2 months of age, but not at other ages or in the IL-6 tg hippocampus (**Figure 2B**). No genotypic differences were observed in the hippocampus and cerebellum of the CCL2 line for p42/44 MAPK, STAT3 or C/EBPβ (**Figure 3B**).

Levels of the active form (i.e., phosphorylated form) of STAT3 (pSTAT3) were also examined in hippocampus and cerebellum from IL-6 tg and non-tg mice (3–5 months of age). Results showed that pSTAT3 levels were prominently increased in the IL-6 tg hippocampus (normalized values—tg/non-tg: IL-6 tg = 4.0 ± 0.9, $n = 7$; IL-6 non-tg = 1.0 ± 0.2, $n = 7$) and cerebellum (normalized values—tg/non-tg: IL-6 tg = 297 ± 102, $n = 9$; IL-6 non-tg = 1.0 ± 0.2, $n = 7$). Levels of the active form (i.e., phosphorylated form) of p42/44 MAPK (pp42/44 MAPK) were examined in hippocampus from IL-6 tg and non-tg mice (3–5 months of age) and in hippocampus from CCL2-tg and

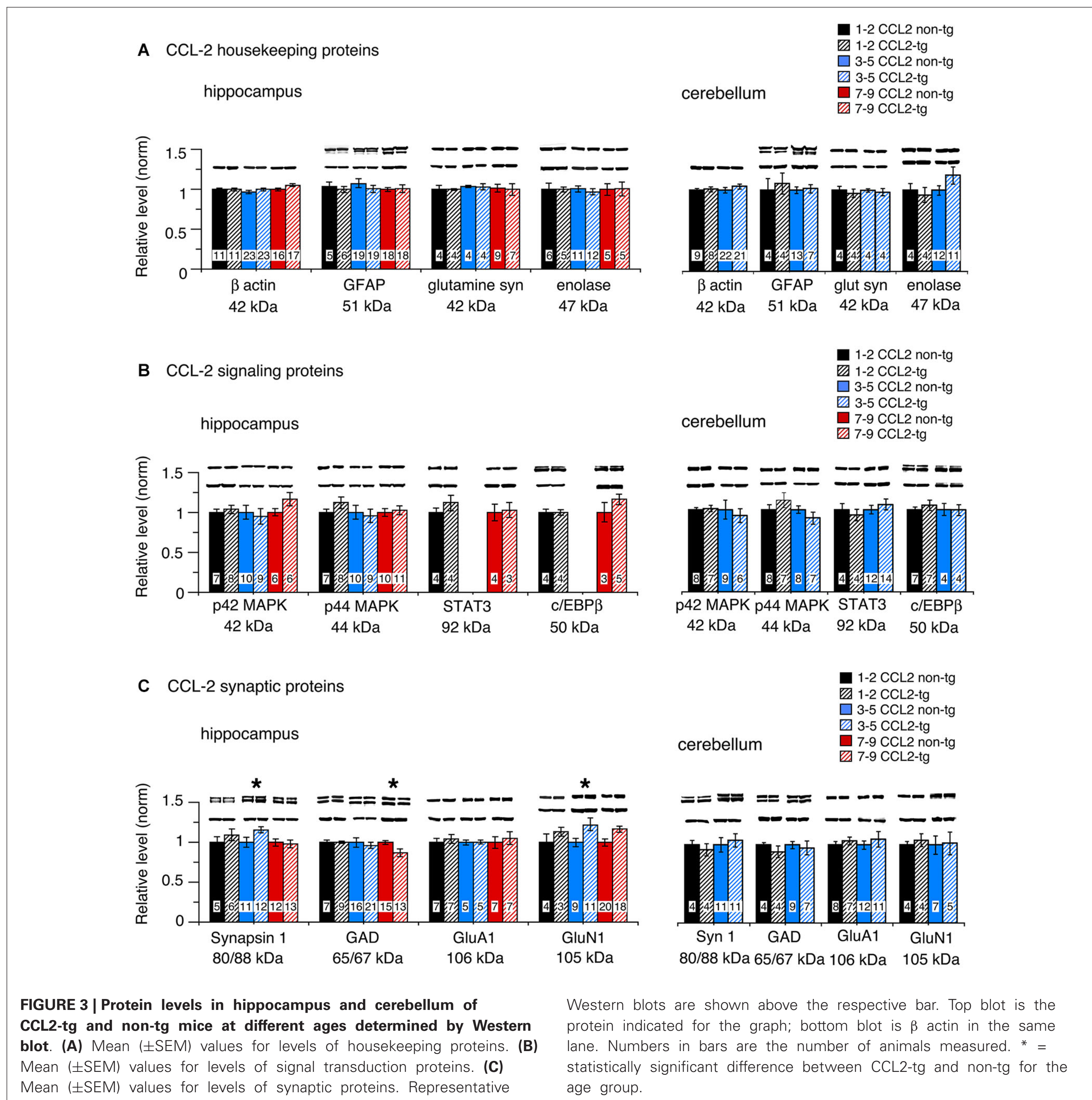



**FIGURE 3 | Protein levels in hippocampus and cerebellum of CCL2-tg and non-tg mice at different ages determined by Western blot**. **(A)** Mean (±SEM) values for levels of housekeeping proteins. **(B)** Mean (±SEM) values for levels of signal transduction proteins. **(C)** Mean (±SEM) values for levels of synaptic proteins. Representative Western blots are shown above the respective bar. Top blot is the protein indicated for the graph; bottom blot is β actin in the same lane. Numbers in bars are the number of animals measured. * = statistically significant difference between CCL2-tg and non-tg for the age group.

non-tg mice (3–9 months of age). The IL-6 tg hippocampus showed a 30% increase in pp42/44 MAPK relative to the non-tg hippocampus (normalized values—tg/non-tg: pp42 MAPK, IL-6 tg = 1.3 ± 0.1, $n$ = 9; IL-6 non-tg = 1.0 ± 0.1, $n$ = 8; pp44 MAPK, IL-6 tg = 1.3 ± 0.1, $n$ = 9; IL-6 non-tg = 1.0 ± 0.1, $n$ = 8). The CCL2-tg hippocampus showed a 30% decrease in pp44 MAPK but no change in pp42 MAPK relative to the non-tg hippocampus (normalized values—tg/non-tg: pp42 MAPK, CCL2-tg = 1.0 ± 0.1, $n$ = 17; CCL2 non-tg = 1.0 ± 0.05, $n$ = 19; pp44 MAPK, CCL2-tg = 0.7 ± 0.1, $n$ = 10; CCL2 non-tg = 1.0 ± 0.1, $n$ = 13).

## SYNAPTIC PROTEINS

Our previous studies showed that upregulated astrocyte expression of IL-6 or CCL2 in the respective transgenic mice produced neuroadaptive changes that altered synaptic function (Nelson et al., 2011, 2012; Bray et al., 2013). To determine if neuroadaptive changes occurred at the level of synaptic protein expression, we examined pre- and post-synaptic proteins by Western blot in hippocampus and cerebellum from transgenic and non-transgenic mice. Two presynaptic proteins were examined, Syn 1, a synaptic vesicle protein involved in transmitter release, and GAD65/67, the synthetic enzyme for the inhibitory transmitter

GABA. Two postsynaptic proteins were also examined, GluA1, a subunit of the AMPA receptor, and GluN1, a subunit of the NMDA receptor. Both glutamate receptors, GluA1 and GluN1, are important mediators of excitatory synaptic transmission in hippocampal and cerebellar circuits.

Results show both age and region-dependent genotypic differences, the most pronounced effects occurring in the IL-6 tg cerebellum. In the IL-6 line, there was a small (∼7%) but significant decrease in GAD65/67 in the IL-6 tg hippocampus at 3–5 months of age and a significant decrease in the levels of Syn 1 and GluN1 at 12 months of age (**Figure 2C**). Levels of Syn 1, GAD65/67, GluA1 and GluN1 were all significantly reduced in the IL-6 tg cerebellum at all ages studied. In the CCL2-tg hippocampus, levels of Syn 1 and GluN1 were significantly increased at 3–5 months of age, whereas the level of GAD 65/67 was significantly decreased at 7–9 months of age (**Figure 3C**). There was no significant genotypic difference in levels of Syn 1, GAD65/67, GluA1 and GluN1 in the CCL2-tg cerebellum at all ages studied (**Figure 3C**).

## DISCUSSION

In the current study we measured the relative levels of cellular and synaptic proteins expressed in the hippocampus and cerebellum of transgenic mice that are genetically modified to produce elevated levels of IL-6 or CCL2 in CNS astrocytes. The goal of these studies was to identify CNS targets of the astrocyte produced IL-6 or CCL2, as evidenced by a change in the level of protein expression. Measurements were made at several ages in the lifespan of the animals, to reveal changes that could reflect developmental sensitivity and/or duration of exposure. Results show several changes in the level of neuronal and glial proteins in the hippocampus and cerebellum of the IL-6 tg mice but relatively few in the CCL2-tg mice (**Table 1**). Some changes were age and/or region specific. The ability of elevated levels of IL-6 or CCL2 to produce such changes could play a role in neurological, psychiatric or other disorders that show persistently elevated levels of IL-6 or CCL2 in the CNS. Moreover, IL-6 or CCL2 regulation of these targets could play a role in the normal physiological function of these CNS regions.

ELISA studies confirmed the presence of elevated levels of IL-6 and CCL2 in the hippocampus and/or cerebellum of the respective transgenic mice at all ages studied. Elevated levels were observed at the youngest age studied, 1–2 months of age, with no apparent age-dependent difference in the expression pattern at older ages. Animals at 1–2 months of age are considered to be adolescent/young adults, whereas the older ages (3–5 and 7–12) studied are considered to be adult ages. Astrocytes normally start to produce GFAP about 1 week postnatal with expression continuing throughout the lifetime of the animal. CNS expression of mRNA for IL-6 and CCL2 also begins during the postnatal period in normal animals (Gadient and Otten, 1994a; Pousset, 1994). CNS expression of IL-6 mRNA in the IL-6 transgenic mice was evident at 1 week postnatal and reached a maximum at 3 months of age (Chiang et al., 1994). GFAP mRNA expression, which is regulated by IL-6, and GFAP protein were also evident at 1 week postnatal but an increased level of GFAP in the IL-6 tg CNS was not prominent until about 1 month postnatal (Chiang et al., 1994). These results suggest that the consequences of elevated expression of IL-6 in the CNS are not prominent until about 1 month of age. CCL2 expression in CCL2-tg mice has not been studied at early ages but it is likely that the time course of expression is similar to that for IL-6, because the elevated levels of expression for both IL-6 and CCL2 is linked to GFAP expression in astrocytes (Brenner et al., 1994).

Elevated levels of IL-6 were not observed in the CCL2-tg hippocampus or cerebellum, indicating that elevated levels of CCL2 did not induce production of IL-6 in these CNS regions. Similarly, elevated levels of CCL2 were not observed in the IL-6 tg hippocampus and cerebellum, suggesting that elevated levels of IL-6 did not induce production of CCL2. These results are consistent with *in vitro* studies of cultured astrocytes involving exogenous application of IL-6 or CCL2 (Oh et al., 1999; Semple et al., 2010) and suggest that glial activation was not prominent in the transgenic mice. In both the transgenic and non-transgenic hippocampus and cerebellum, levels of CCL2 were considerably higher than levels of IL-6. This difference was also observed in CSF samples from human control subjects, the most common site for measurement of neuroimmune factors in humans. For example, IL-6 levels were reported to be ∼1 pg/ml in the human CSF (Lindqvist et al., 2009) compared with ∼840 pg/ml for CCL2 in human CSF (Janelidze et al., 2013).

Elevated levels of CCL2 were observed in the hippocampus and cerebellum of the CCL2-tg mice, but elevated levels of IL-6 were only observed in the cerebellum in the IL-6 tg mice. Moreover, IL-6 levels were higher in the cerebellum than in the hippocampus of the IL-6 tg mice, whereas in the CCL2 tg mice, CCL2 levels were higher in the hippocampus than the cerebellum. These regional difference may relate to differences in the production of these two neuroimmune factors by the Bergman glia, which are present in the cerebellum but not in the hippocampus. The Bergman glia express the highest level of IL-6 transgene in the IL-6 transgenic CNS (Campbell et al., 1993). The high levels of IL-6 in the IL-6 tg cerebellum may contribute to the damage and cell loss observed in this brain region at older ages, and consequently the ataxia characteristic of the older IL-6 tg mice (Campbell et al., 1993). A neurotoxic effect of high concentrations of IL-6 is consistent with our previous studies in a culture model, which showed that neurotoxicity was produced when cultured cerebellar granule neurons were exposed chronically to high concentrations of IL-6 in the culture media (e.g., 5–10 ng/ml) (Conroy et al., 2004).

Previous studies of rat brain showed higher levels IL-6 mRNA in the hippocampus than in the cerebellum (Gadient and Otten, 1994a), whereas our ELISA studies of non-tg mice showed higher levels of IL-6 protein in the cerebellum than in the hippocampus. Thus, there appears to be a mismatch between mRNA and protein levels. Because both cerebellar and hippocampal samples were run on the same ELISA, it is unlikely that technical difficulties account for this difference. Assuming that this apparent mismatch is not due to an across species comparison, it is likely that brain region specific post-translational events (e.g., post-translational regulation) are responsible for the mismatch between mRNA and protein levels observed in our studies, as has been shown for other proteins in other systems (Shebl et al., 2010; Khositseth et al., 2011; Vogel and Marcotte, 2012).

**Table 1 | Changes in protein expression in hippocampus and cerebellum of IL-6 and CCL2 transgenic mice.**

| | IL-6 | | | | | | CCL2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Hippocampus | | | Cerebellum | | | Hippocampus | | | Cerebellum | | |
| **Protein** | **1–2 months** | **3–5 months** | **12 months** | **1–2 months** | **3–5 months** | **12 months** | **1–2 months** | **3–5 months** | **7–9 months** | **1–2 months** | **3–5 months** | **7–9 months** |
| β-actin | none | none | none | none | ▲ | none | none | none | none | none | none | nd |
| GFAP | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | none | none | none | none | none | nd |
| Glu syn | none | none | none | ▲ | ▲ | none | none | none | none | none | none | nd |
| Enolase | none | none | none | ▼ | none | none | none | none | none | none | none | nd |
| p42 MAPK | none | none | none | none | none | none | none | none | none | none | none | nd |
| p44 MAPK | none | none | none | none | none | none | none | none | none | none | none | nd |
| STAT3 | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | none | nd | none | none | none | nd |
| C/EBPβ | none | none | none | ▲ | none | none | none | nd | none | none | none | nd |
| Synapsin 1 | none | none | ▼ | ▼ | ▼ | ▼ | none | none | none | none | none | nd |
| GAD65/67 | none | ▼ | none | ▼ | ▼ | ▼ | none | none | ▼ | none | none | nd |
| GluA1 | none | none | none | ▼ | ▼ | nd | none | none | none | none | none | nd |
| GluN1 | none | none | ▼ | ▼ | ▼ | nd | none | ▲ | none | none | none | nd |

** for each group, tg is compared to non-tg; ▲ = tg > non-tg; ▼ = tg < non-tg; none = no significant difference between tg and non-tg; nd = not determined.*

Although elevated levels of IL-6 were not observed in the IL-6 tg hippocampus, evidence for increased expression of IL-6 was provided by our Western blot results showing elevated levels GFAP, STAT3 and pSTAT3 in both hippocampus and cerebellum. GFAP levels are known to be regulated by IL-6 through a signal transduction pathway involving STAT3 (Heinrich et al., 1998; Herrmann et al., 2008; Shu et al., 2011). Therefore, the increased levels of GFAP, STAT3 and pSTAT3 provide evidence of increased production of IL-6. The differences in the ability to detect elevated levels of IL-6 vs. CCL2 in the respective transgenic tissue suggests that astrocyte trafficking differs for IL-6 and CCL2 and imply that IL-6 is likely to be released after production, whereas CCL2 can be released and/or stored. Studies of astrocytes cultured from the CNS of IL-6 tg or CCL2-tg mice demonstrated the ability of astrocytes to secrete IL-6 (Campbell et al., 1993) or CCL2 (Huang et al., 2002), respectively. For example, IL-6 bioactivity in the supernatant of cultured astrocytes obtained from the CNS of IL-6 tg mice was approximately 150 pg/ml compared with 5 pg/ml for IL-6 non-tg astrocytes (Campbell et al., 1993). CCL2 levels in the supernatant of cultured astrocytes obtained from the CNS of CCL2-tg mice was approximately 3500 pg/ml compared with 1 pg/ml for astrocytes from the CCL2 non-tg mice (Huang et al., 2002).

Differences in the levels of cellular and synaptic protein measured by Western blot between the IL-6 tg and IL-6 non-tg mice were observed for both the hippocampus and cerebellum. However, the cerebellum showed more prominent effects of IL-6 than the hippocampus, presumably due to the higher level of expression of IL-6 in the cerebellum. Differences in cell types that comprise the two regions could also be a contributing factor. The proteins most affected by the elevated levels of IL-6 in both CNS regions were GFAP, STAT3 and pSTAT3, which were all increased. GFAP is restricted to astrocytes, whereas STAT3 is expressed by both neurons and glia, as are IL-6 receptors (Schöbitz et al., 1992; Planas et al., 1997; Nelson et al., 1999; Murata et al., 2000). Because our studies used whole hippocampus and cerebellum, we were unable to identify the cell types or subcellular compartments that showed the increased STAT3 and pSTAT3 levels in these CNS regions. In previous studies of the IL-6 tg cerebellum, pSTAT3 was detected primarily in the nucleus of glial cells (Sanz et al., 2008), suggesting that glial cells are the primary cell type responding to the chronic expression of IL-6 in the IL-6 tg cerebellum. However, the primary site of neuronal effects may be at non-nuclear sites. Both STAT3 and pSTAT3 have been shown to be localized to synaptic sites in the hippocampus and cortex (Murata et al., 2000) and, independent of nuclear localization, to play a role in long-term depression, a form of synaptic plasticity essential for normal CNS function (Nicolas et al., 2012). LTP, another form of synaptic plasticity, has been shown to be regulated by IL-6 in the hippocampus and to involve glial release of the IL-6, which presumably acts at neuronal IL-6 receptors (Li et al., 1997; Jankowsky et al., 2000; Tancredi et al., 2000; Balschun et al., 2004). IL-6 signaling through IL-6 receptors involving both STAT3 and/or p42/44 MAPK has been demonstrated in neuronal cells (Schumann et al., 1999; Park et al., 2012; Fang et al., 2013). Thus, the elevated levels of STAT3 and/or pSTAT3 could occur primarily at synaptic or other subcellular sites in neurons in the IL-6 tg mice, a possibility that will require further investigation.

In addition to GFAP, another glial protein was increased in the IL-6 tg cerebellum, glutamine synthetase. This protein was not elevated in the IL-6 tg hippocampus and was only prominently elevated at 1–2 months of age in the IL-6 tg cerebellum. Glutamine synthetase plays a central role in the trafficking of glutamate, the primary excitatory transmitter in the CNS and a precursor for the inhibitory transmitter GABA (Mathews and Diamond, 2003; Albrecht et al., 2010; Tani et al., 2014). Glutamate that is released at excitatory synapses is taken up by astrocytes, converted to glutamine by glutamine synthetase and the glutamine is then released to the environment for neuronal uptake and processing. Glutamine synthetase is an important player in the neuroprotective role of astrocytes against glutamate toxicity (Zou et al., 2010; Zhang et al., 2011; Tani et al., 2014). An increased level of extracellular glutamate, which could lead to glutamate toxicity, induces an upregulation of glutamine synthetase (Lehmann et al., 2009). Thus, the increased levels of glutamine synthetase in the IL-6 tg cerebellum may play a protective role at early ages. Neuronal toxicity is known to occur in the IL-6 transgenic hippocampus and cerebellum, but does not become prominent until older ages (Heyser et al., 1997).

The transcription factor C/EBPβ was also elevated in the cerebellum and not the hippocampus of the IL-6 tg mice and only at 1–2 month of age. C/EBPβ is expressed prominently in neurons but also by glial in the CNS (Ejarque-Ortiz et al., 2007; Kfoury and Kapatos, 2009; Peña-Altamira et al., 2014). C/EBPβ regulates a host of neuronal genes (Kfoury and Kapatos, 2009) and its upregulation may be a contributing factor to the lower level of enolase in the IL-6 tg cerebellum at 1–2 months of age. In addition to enolase, several other neuronal proteins were reduced in the cerebellum at 1–2 months of age including Syn 1, GAD 65/67, GluA1 and GluN1, which are all synaptic proteins. However, the levels of these proteins were reduced at older ages, whereas the reduced level of enolase and increased level of C/EBPβ only occurred at 1–2 months of age. Whether or not the correlation between levels of enolase and C/EBPβ has a causative aspect will require further study. With respect to the decreased levels of synaptic proteins in the cerebellum, it seems unlikely that this decrease reflects neuronal loss, at least at 1–2 months of age when neuronal death is not prominent in the IL-6 tg cerebellum. Moreover, at older ages housekeeping and signal transduction proteins did not show a decrease, as would be expected if prominent cell loss occurred in the IL-6 tg cerebellum. One possible explanation is that the decreased levels of synaptic proteins reflect reduced axon or synapse formation that occurred during cerebellar development. Our previous studies using a culture model system showed that the level of α-internexin, a major neurofilament expressed in axons of cerebellar granule neurons (Chien et al., 1996), was reduced in granule neuron cultures exposed to elevated levels of IL-6 during development (Conroy et al., 2004). α-internexin has been proposed to play a role in neuronal maturation and axon stability (Chien et al., 1996) and in the development and organization of postsynaptic densities (Suzuki et al., 1997). Further studies will be necessary to determine if the changes in protein levels observed in the current study correlate with changes in cell structure or cell number.

In the IL-6 tg hippocampus, there were no genotypic differences for the levels of β-actin, glutamine synthetase, p42/44 MAPK, and C/EBPβ Based on the high abundance of these proteins in the hippocampus, it seems unlikely that the elevated levels of IL-6 resulted in prominent cell death, at least at the younger ages studied. This interpretation is consistent with studies using fluoro-jade staining for degenerating neurons in 3–4 month old IL-6 tg and non-tg hippocampus, which revealed no evidence of cell toxicity (Vallieres et al., 2002). Gross histological analysis of cresyl violet and TUNEL stained IL-6 tg and non-tg hippocampus also revealed no evidence of prominent cell loss at 2–6 months of age (Samland et al., 2003). The levels of Syn 1 and GluN1 were reduced in the IL-6 tg hippocampus but only at the oldest age studied (12 months of age). Decreased levels of Syn 1 in the IL-6 tg hippocampus at 12 months of age was also observed in a previous study (Heyser et al., 1997). The reduction in Syn 1 and GluN1 at the older age could reflect synaptic damage and/or neuronal loss, changes that could be important contributors to the deficits observed in studies of hippocampal-dependent behavior (Heyser et al., 1997).

In contrast to the IL-6 tg mice, in the CCL2-tg mice there was little evidence of genotypic differences in the level of cellular proteins for the hippocampus and no genotypic differences for the cerebellum. In the CCL2 hippocampus, a modest but significant increase in the levels of Syn 1 and GluR1 was observed at 3–5 months of age, a genotypic difference that was opposite to the reduced levels of these proteins observed in the IL-6 hippocampus at 12 months of age. However, a small but significant decrease in GAD65/67 was observed in both the CCL2-tg and IL-6 tg hippocampus, although for different age groups. Thus, IL-6 and CCL2 appear to directly or indirectly target some of the same synaptic proteins, effects that could have bearings relative to conditions where both neuroimmune factors are elevated in the CNS. There was no evidence of cell loss in the CCL2-tg mice, as evidenced by the relative lack of genotypic differences.

Taken together, results from our studies show specific profiles of action of IL-6 and CCL2 in the respective transgenic mice, with the most pronounced effects occurring in the cerebellum of the IL-6 tg mice. The pronounced changes in the cerebellum may be an important factor in the ataxia characteristic of the IL-6 tg mice. Although CCL2 did not appear to prominently affect the level of protein expression in the CCL2-tg mice, our previous studies showed that the persistently elevated levels of CCL2 in the hippocampus of the CCL2-tg mice produced neuroadaptive changes that altered aspects of synaptic function and sensitivity to alcohol (Nelson et al., 2011; Bray et al., 2013). Synaptic function was also altered in our studies of the hippocampus of IL-6 tg mice (Nelson et al., 2011), but the effects differed from that observed in the CCL2-tg mice. Thus, in the CCL2-tg hippocampus, somatic excitability was altered (increased), whereas in the IL-6 tg hippocampus, the dendritic synaptic response was altered (increased). The mechanisms mediating these neuroadaptive effects on synaptic function remain to be resolved but may involve some of the changes in protein expression observed in the current studies.

## AUTHOR CONTRIBUTIONS

Donna L. Gruol and Jennifer G. Bray designed the studies and wrote the paper. Donna L. Gruol, Khanh Vo and Jennifer G. Bray managed the mouse colony. Jennifer G. Bray performed the dissections. Donna L. Gruol and Khanh Vo carried out the biochemical analyses.

## ACKNOWLEDGMENTS

We thank Christine Hao, Stephanie Chow, Alana Puro and Katie Dutchman for excellent technical support. Supported by NIAAA Grant AA019261. Jennifer G. Bray was supported by a National Research Service Award, FAA020441.

## REFERENCES

Albrecht, J., Sidoryk-Wegrzynowicz, M., Zielinska, M., and Aschner, M. (2010). Roles of glutamine in neurotransmission. *Neuron Glia Biol.* 6, 263–276. doi: 10.1017/S1740925X11000093

Arisi, G. M. (2014). Nervous and immune systems signals and connections: cytokines in hippocampus physiology and pathology. *Epilepsy Behav.* doi: 10.1016/j.yebeh.2014.01.017. [Epub ahead of print].

Baier, P. C., May, U., Scheller, J., Rose-John, S., and Schiffelholz, T. (2009). Impaired hippocampus-dependent and -independent learning in IL-6 deficient mice. *Behav. Brain Res.* 200, 192–196. doi: 10.1016/j.bbr.2009.01.013

Bajetto, A., Bonavia, R., Barbero, S., Florio, T., and Schettini, G. (2001). Chemokines and their receptors in the central nervous system. *Front. Neuroendocrinol.* 22, 147–184. doi: 10.1006/frne.2001.0214

Balschun, D., Wetzel, W., Del Rey, A., Pitossi, F., Schneider, H., Zuschratter, W., et al. (2004). Interleukin-6: a cytokine to forget. *FASEB J.* 18, 1788–1790. doi: 10.1096/fj.04-1625fje

Banisadr, G., Rostene, W., Kitabgi, P., and Parsadaniantz, S. M. (2005). Chemokines and brain functions. *Curr. Drug Targets Inflamm. Allergy* 4, 387–399. doi: 10.2174/1568010054022097

Bernardinelli, Y., Muller, D., and Nikonenko, I. (2014). Astrocyte-synapse structural plasticity. *Neural Plast.* 2014:232105. doi: 10.1155/2014/232105

Billiau, A. D., Witters, P., Ceulemans, B., Kasran, A., Wouters, C., and Lagae, L. (2007). Intravenous immunoglobulins in refractory childhood-onset epilepsy: effects on seizure frequency, EEG activity and cerebrospinal fluid cytokine profile. *Epilepsia* 48, 1739–1749. doi: 10.1111/j.1528-1167.2007.01134.x

Blasko, I., Lederer, W., Oberbauer, H., Walch, T., Kemmler, G., Hinterhuber, H., et al. (2006). Measurement of thirteen biological markers in CSF of patients with Alzheimer's disease and other dementias. *Dement. Geriatr. Cogn. Disord.* 21, 9–15. doi: 10.1159/000089137

Boztug, K., Carson, M. J., Pham-Mitchell, N., Asensio, V. C., Demartino, J., and Campbell, I. L. (2002). Leukocyte infiltration, but not neurodegeneration, in the CNS of transgenic mice with astrocyte production of the CXC chemokine ligand 10. *J. Immunol.* 169, 1505–1515. doi: 10.4049/jimmunol.169.3.1505

Bray, J. G., Reyes, K. C., Roberts, A. J., Ransohoff, R., and Gruol, D. L. (2013). Synaptic plasticity in the hippocampus shows resistance to acute ethanol exposure in transgenic mice with astrocyte-targeted enhanced CCL2 expression. *Neuropharmacology* 67, 115–125. doi: 10.1016/j.neuropharm.2012.11.007

Brenner, M., Kisseberth, W. C., Su, Y., Besnard, F., and Messing, A. (1994). GFAP promoter directs astrocyte-specific expression in transgenic mice. *J. Neurosci.* 14, 1030–1037.

Brosseron, F., Krauthausen, M., Kummer, M., and Heneka, M. T. (2014). Body fluid cytokine levels in mild cognitive impairment and Alzheimer's disease: a comparative overview. *Mol. Neurobiol.* doi: 10.1007/s12035-014-8657-1. [Epub ahead of print].

Burton, M. D., and Johnson, R. W. (2012). Interleukin-6 trans-signaling in the senescent mouse brain is involved in infection-related deficits in contextual fear conditioning. *Brain Behav. Immun.* 26, 732–738. doi: 10.1016/j.bbi.2011.10.008

Burton, M. D., Sparkman, N. L., and Johnson, R. W. (2011). Inhibition of interleukin-6 trans-signaling in the brain facilitates recovery from lipopolysaccharide-induced sickness behavior. *J. Neuroinflammation* 8:54. doi: 10.1186/1742-2094-8-54

Campbell, I. L., Abraham, C. R., Masliah, E., Kemper, P., Inglis, J. D., Oldstone, M. B., et al. (1993). Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. *Proc. Natl. Acad. Sci. U S A* 90, 10061–10065. doi: 10.1073/pnas.90.21.10061

Campbell, I. L., Erta, M., Lim, S. L., Frausto, R., May, U., Rose-John, S., et al. (2014). Trans-signaling is a dominant mechanism for the pathogenic actions of interleukin-6 in the brain. *J. Neurosci.* 34, 2503–2513. doi: 10.1523/JNEUROSCI.2830-13.2014

Cheng, P., Alberts, I., and Li, X. (2013). The role of ERK1/2 in the regulation of proliferation and differentiation of astrocytes in developing brain. *Int. J. Dev. Neurosci.* 31, 783–789. doi: 10.1016/j.ijdevneu.2013.09.008

Chiang, C.-S., Stalder, A., Samimi, A., and Campbell, I. L. (1994). Reactive gliosis as a consequence of interleukin-6 expression in the brain: studies in transgenic mice. *Dev. Neurosci.* 16, 212–221. doi: 10.1159/000112109

Chien, C. L., Mason, C. A., and Liem, R. K. (1996). alpha-Internexin is the only neuronal intermediate filament expressed in developing cerebellar granule neurons. *J. Neurobiol.* 29, 304–318. doi: 10.1002/(sici)1097-4695(199603)29:3<304::aid-neu3>3.0.co;2-d

Choi, S. S., Lee, H. J., Lim, I., Satoh, J., and Kim, S. U. (2014). Human astrocytes: secretome profiles of cytokines and chemokines. *PLoS One* 9:e92325. doi: 10.1371/journal.pone.0092325

Cinque, P., Vago, L., Mengozzi, M., Torri, V., Ceresa, D., Vicenzi, E., et al. (1998). Elevated cerebrospinal fluid levels of monocyte chemotactic protein-1 correlate with HIV-1 encephalitis and local viral replication. *AIDS* 12, 1327–1332. doi: 10.1097/00002030-199811000-00014

Conductier, G., Blondeau, N., Guyon, A., Nahon, J. L., and Rovere, C. (2010). The role of monocyte chemoattractant protein MCP1/CCL2 in neuroinflammatory diseases. *J. Neuroimmunol.* 224, 93–100. doi: 10.1016/j.jneuroim.2010.05.010

Conroy, S. M., Nguyen, V., Quina, L. A., Blakely-Gonzales, P., Ur, C., Netzeband, J. G., et al. (2004). Interleukin-6 produces neuronal loss in developing cerebellar granule neuron cultures. *J. Neuroimmunol.* 155, 43–54. doi: 10.1016/j.jneuroim.2004.06.014

Corty, M. M., and Freeman, M. R. (2013). Cell biology in neuroscience: architects in neural circuit design: glia control neuron numbers and connectivity. *J. Cell Biol.* 203, 395–405. doi: 10.1083/jcb.201306099

del Rey, A., Balschun, D., Wetzel, W., Randolf, A., and Besedovsky, H. O. (2013). A cytokine network involving brain-borne IL-1beta, IL-1ra, IL-18, IL-6 and TNFalpha operates during long-term potentiation and learning. *Brain Behav. Immun.* 33, 15–23. doi: 10.1016/j.bbi.2013.05.011

Deverman, B. E., and Patterson, P. H. (2009). Cytokines and CNS development. *Neuron* 64, 61–78. doi: 10.1016/j.neuron.2009.09.002

Dziennis, S., and Alkayed, N. J. (2008). Role of signal transducer and activator of transcription 3 in neuronal survival and regeneration. *Rev. Neurosci.* 19, 341–361. doi: 10.1515/REVNEURO.2008.19.4-5.341

Ejarque-Ortiz, A., Medina, M. G., Tusell, J. M., Perez-Gonzalez, A. P., Serratosa, J., and Saura, J. (2007). Upregulation of CCAAT/enhancer binding protein beta in activated astrocytes and microglia. *Glia* 55, 178–188. doi: 10.1002/glia.20446

Erta, M., Quintana, A., and Hidalgo, J. (2012). Interleukin-6, a major cytokine in the central nervous system. *Int. J. Biol. Sci.* 8, 1254–1266. doi: 10.7150/ijbs.4679

Fang, X. X., Jiang, X. L., Han, X. H., Peng, Y. P., and Qiu, Y. H. (2013). Neuroprotection of interleukin-6 against NMDA-induced neurotoxicity is mediated by JAK/STAT3, MAPK/ERK and PI3K/AKT signaling pathways. *Cell. Mol. Neurobiol.* 33, 241–251. doi: 10.1007/s10571-012-9891-6

Farina, C., Aloisi, F., and Meinl, E. (2007). Astrocytes are active players in cerebral innate immunity. *Trends Immunol.* 28, 138–145. doi: 10.1016/j.it.2007.01.005

Fattori, E., Lazzaro, D., Musiani, P., Modesti, A., Alonzi, T., and Ciliberto, G. (1995). IL-6 expression in neurons of transgenic mice causes reactive astrocytosis and increase in ramified microglial cells but no neuronal damage. *Eur. J. Neurosci.* 7, 2441–2449. doi: 10.1111/j.1460-9568.1995.tb01042.x

Gadient, R. A., and Otten, U. (1994a). Expression of interleukin-6 (IL-6) and interleukin-6 receptor (IL-6R) mRNAs in rat brain during postnatal development. *Brain Res.* 637, 10–14. doi: 10.1016/0006-8993(94)91211-4

Gadient, R. A., and Otten, U. (1994b). Identification of interleukin-6 (IL-6)-expressing neurons in the cerebellum and hippocampus of normal adult rats. *Neurosci. Lett.* 182, 243–246. doi: 10.1016/0304-3940(94)90807-9

Galimberti, D., Schoonenboom, N., Scheltens, P., Fenoglio, C., Bouwman, F., Venturelli, E., et al. (2006). Intrathecal chemokine synthesis in mild cognitive impairment and Alzheimer disease. *Arch. Neurol.* 63, 538–543. doi: 10.1001/archneur.63.4.538

Gallo, P., Frei, K., Rordorf, C., Lazdins, J., Tavolato, B., and Fontana, A. (1989). Human immunodeficiency type 1 (HIV-1) infection of the central nervous system: an evaluation of cytokines in cerebrospinal fluid. *J. Neuroimmunol.* 23, 109–116. doi: 10.1016/0165-5728(89)90029-5

Glabinski, A. R., and Ransohoff, R. M. (1999). Chemokines and chemokine receptors in CNS pathology. *J. Neurovirol.* 5, 3–12. doi: 10.3109/13550289909029740

Godbout, J. P., and Johnson, R. W. (2004). Interleukin-6 in the aging brain. *J. Neuroimmunol.* 147, 141–144. doi: 10.1016/j.jneuroim.2003.10.031

Gruol, D. L., and Nelson, T. E. (1997). Physiological and pathological roles of interleukin-6 in the central nervous system. *Mol. Neurobiol.* 15, 307–339. doi: 10.1007/bf02740665

Gruol, D. L., and Nelson, T. E. (2005). Purkinje neuron physiology is altered by the inflammatory factor interleukin-6. *Cerebellum* 4, 198–205. doi: 10.1080/14734220500199987

Guyon, A., Skrzydelski, D., De Giry, I., Rovere, C., Conductier, G., Trocello, J. M., et al. (2009). Long term exposure to the chemokine CCL2 activates the nigrostriatal dopamine system: a novel mechanism for the control of dopamine release. *Neuroscience* 162, 1072–1080. doi: 10.1016/j.neuroscience.2009.05.048

Halassa, M. M., Fellin, T., and Haydon, P. G. (2007). The tripartite synapse: roles for gliotransmission in health and disease. *Trends Mol. Med.* 13, 54–63. doi: 10.1016/j.molmed.2006.12.005

Heinrich, P. C., Behrmann, I., Muller-Newen, G., Schaper, F., and Graeve, L. (1998). Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem. J.* 334, 297–314.

Heneka, M. T., Rodriguez, J. J., and Verkhratsky, A. (2010). Neuroglia in neurodegeneration. *Brain Res. Rev.* 63, 189–211. doi: 10.1016/j.brainresrev.2009.11.004

Herrmann, J. E., Imura, T., Song, B., Qi, J., Ao, Y., Nguyen, T. K., et al. (2008). STAT3 is a critical regulator of astrogliosis and scar formation after spinal

cord injury. *J. Neurosci.* 28, 7231–7243. doi: 10.1523/JNEUROSCI.1709-08.2008

Heyser, C. J., Masliah, E., Samimi, A., Campbell, I. L., and Gold, L. H. (1997). Progressive decline in avoidance learning paralleled by inflammatory neurodegeneration in transgenic mice expressing interleukin 6 in the brain. *Proc. Natl. Acad. Sci. U S A* 94, 1500–1505. doi: 10.1073/pnas.94.4.1500

Hirohata, S., Kanai, Y., Mitsuo, A., Tokano, Y., Hashimoto, H., and NPSLE Research Subcommittee. (2009). Accuracy of cerebrospinal fluid IL-6 testing for diagnosis of lupus psychosis. A multicenter retrospective study. *Clin. Rheumatol.* 28, 1319–1323. doi: 10.1007/s10067-009-1226-8

Hirohata, S., and Miyamoto, T. (1990). Elevated levels of interleukin-6 in cerebrospinal fluid from patients with systemic lupus erythematosus and central nervous system involvement. *Arthritis Rheum.* 33, 644–649. doi: 10.1002/art.1780330506

Huang, D., Tani, M., Wang, J., Han, Y., He, T. T., Weaver, J., et al. (2002). Pertussis toxin-induced reversible encephalopathy dependent on monocyte chemoattractant protein-1 overexpression in mice. *J. Neurosci.* 22, 10633–10642.

Huang, D., Wujek, J., Kidd, G., He, T. T., Cardona, A., Sasse, M. E., et al. (2005). Chronic expression of monocyte chemoattractant protein-1 in the central nervous system causes delayed encephalopathy and impaired microglial function in mice. *FASEB J.* 19, 761–772. doi: 10.1096/fj.04-3104com

Janelidze, S., Ventorp, F., Erhardt, S., Hansson, O., Minthon, L., Flax, J., et al. (2013). Altered chemokine levels in the cerebrospinal fluid and plasma of suicide attempters. *Psychoneuroendocrinology* 38, 853–862. doi: 10.1016/j.psyneuen.2012.09.010

Jankowsky, J. L., Derrick, B. E., and Patterson, P. H. (2000). Cytokine responses to LTP induction in the rat hippocampus: a comparison of in vitro and in vivo techniques. *Learn. Mem.* 7, 400–412. doi: 10.1101/lm.32600

Jensen, C. J., Massie, A., and De Keyser, J. (2013). Immune players in the CNS: the astrocyte. *J. Neuroimmune Pharmacol.* 8, 824–839. doi: 10.1007/s11481-013-9480-6

Jones, K. A., and Thomsen, C. (2013). The role of the innate immune system in psychiatric disorders. *Mol. Cell. Neurosci.* 53, 52–62. doi: 10.1016/j.mcn.2012.10.002

Kelder, W., McArthur, J. C., Nance-Sproson, T., Mcclernon, D., and Griffin, D. E. (1998). Beta-chemokines MCP-1 and RANTES are selectively increased in cerebrospinal fluid of patients with human immunodeficiency virus-associated dementia. *Ann. Neurol.* 44, 831–835. doi: 10.1002/ana.410440521

Kettenmann, H., Hanisch, U. K., Noda, M., and Verkhratsky, A. (2011). Physiology of microglia. *Physiol. Rev.* 91, 461–553. doi: 10.1152/physrev.00011.2010

Kfoury, N., and Kapatos, G. (2009). Identification of neuronal target genes for CCAAT/enhancer binding proteins. *Mol. Cell. Neurosci.* 40, 313–327. doi: 10.1016/j.mcn.2008.11.004

Khositseth, S., Pisitkun, T., Slentz, D. H., Wang, G., Hoffert, J. D., Knepper, M. A., et al. (2011). Quantitative protein and mRNA profiling shows selective post-transcriptional control of protein expression by vasopressin in kidney cells. *Mol. Cell. Proteomics* 10:M110.004036. doi: 10.1074/mcp.m110.004036

Lehmann, C., Bette, S., and Engele, J. (2009). High extracellular glutamate modulates expression of glutamate transporters and glutamine synthetase in cultured astrocytes. *Brain Res.* 1297, 1–8. doi: 10.1016/j.brainres.2009.08.070

Lekander, M., Von Essen, J., Schultzberg, M., Andreasson, A. N., Garlind, A., Hansson, L. O., et al. (2011). Cytokines and memory across the mature life span of women. *Scand. J. Psychol.* 52, 229–235. doi: 10.1111/j.1467-9450.2010.00865.x

Li, A. J., Katafuchi, T., Oda, S., Hori, T., and Oomura, Y. (1997). Interleukin-6 inhibits long-term potentiation in rat hippocampal slices. *Brain Res.* 748, 30–38. doi: 10.1016/s0006-8993(96)01283-8

Lindqvist, D., Janelidze, S., Hagell, P., Erhardt, S., Samuelsson, M., Minthon, L., et al. (2009). Interleukin-6 is elevated in the cerebrospinal fluid of suicide attempters and related to symptom severity. *Biol. Psychiatry* 66, 287–292. doi: 10.1016/j.biopsych.2009.01.030

Mathews, G. C., and Diamond, J. S. (2003). Neuronal glutamate uptake contributes to GABA synthesis and inhibitory synaptic strength. *J. Neurosci.* 23, 2040–2048.

Miller, D. B., and O'Callaghan, J. P. (2005). Depression, cytokines and glial function. *Metabolism* 54, 33–38. doi: 10.1016/j.metabol.2005.01.011

Murata, S., Usuda, N., Okano, A., Kobayashi, S., and Suzuki, T. (2000). Occurrence of a transcription factor, signal transducer and activators of transcription 3 (Stat3), in the postsynaptic density of the rat brain. *Brain Res. Mol. Brain Res.* 78, 80–90. doi: 10.1016/s0169-328x(00)00077-2

Nelson, T. E., Campbell, I. L., and Gruol, D. L. (1999). Altered physiology of Purkinje neurons in cerebellar slices from transgenic mice with chronic central nervous system expression of interleukin-6. *Neuroscience* 89, 127–136. doi: 10.1016/S0306-4522(98)00316-9

Nelson, T. E., Hao, C., Manos, J., Ransohoff, R. M., and Gruol, D. L. (2011). Altered hippocampal synaptic transmission in transgenic mice with astrocyte-targeted enhanced CCL2 expression. *Brain Behav. Immun.* 25(Suppl. 1), S106–S119. doi: 10.1016/j.bbi.2011.02.013

Nelson, T. E., Netzeband, J. G., and Gruol, D. L. (2004). Chronic interleukin-6 exposure alters metabotropic glutamate receptor-activated calcium signalling in cerebellar Purkinje neurons. *Eur. J. Neurosci.* 20, 2387–2400. doi: 10.1111/j.1460-9568.2004.03706.x

Nelson, T. E., Olde Engberink, A., Hernandez, R., Puro, A., Huitron-Resendiz, S., Hao, C., et al. (2012). Altered synaptic transmission in the hippocampus of transgenic mice with enhanced central nervous systems expression of interleukin-6. *Brain Behav. Immun.* 26, 959–971. doi: 10.1016/j.bbi.2012.05.005

Nelson, T. E., Ur, C. L., and Gruol, D. L. (2002). Chronic interleukin-6 exposure alters electrophysiological properties and calcium signaling in developing cerebellar Purkinje neurons in culture. *J. Neurophysiol.* 88, 475–486.

Nicolas, C. S., Amici, M., Bortolotto, Z. A., Doherty, A., Csaba, Z., Fafouri, A., et al. (2013). The role of JAK-STAT signaling within the CNS. *JAKSTAT* 2:e22925. doi: 10.4161/jkst.22925

Nicolas, C. S., Peineau, S., Amici, M., Csaba, Z., Fafouri, A., Javalet, C., et al. (2012). The Jak/STAT pathway is involved in synaptic plasticity. *Neuron* 73, 374–390. doi: 10.1016/j.neuron.2011.11.024

Oh, J. W., Schwiebert, L. M., and Benveniste, E. N. (1999). Cytokine regulation of CC and CXC chemokine expression by human astrocytes. *J. Neurovirol.* 5, 82–94. doi: 10.3109/13550289909029749

Paquin, A., Barnabe-Heider, F., Kageyama, R., and Miller, F. D. (2005). CCAAT/enhancer-binding protein phosphorylation biases cortical precursors to generate neurons rather than astrocytes in vivo. *J. Neurosci.* 25, 10747–10758. doi: 10.1523/jneurosci.2662-05.2005

Park, K. W., Nozell, S. E., and Benveniste, E. N. (2012). Protective role of STAT3 in NMDA and glutamate-induced neuronal death: negative regulatory effect of SOCS3. *PLoS One* 7:e50874. doi: 10.1371/journal.pone.0050874

Peña-Altamira, E., Polazzi, E., Moretto, E., Lauriola, M., and Monti, B. (2014). The transcription factor CCAAT enhancer-binding protein beta protects rat cerebellar granule neurons from apoptosis through its transcription-activating isoforms. *Eur. J. Neurosci.* 39, 176–185. doi: 10.1111/ejn.12407

Planas, A. M., Berruezo, M., Justicia, C., Barron, S., and Ferrer, I. (1997). Stat3 is present in the developing and adult rat cerebellum and participates in the formation of transcription complexes binding DNA at the sis-inducible element. *J. Neurochem.* 68, 1345–1351. doi: 10.1046/j.1471-4159.1997.68041345.x

Pousset, F. (1994). Development expression of cytokine genes in the cortex and hippocampus of the rat central nervous system. *Brain Res. Dev. Brain Res.* 81, 143–146. doi: 10.1016/0165-3806(94)90078-7

Qin, H., and Benveniste, E. N. (2012). ELISA methodology to quantify astrocyte production of cytokines/chemokines in vitro. *Methods Mol. Biol.* 814, 235–249. doi: 10.1007/978-1-61779-452-0_16

Qiu, Z., Sweeney, D. D., Netzeband, J. G., and Gruol, D. L. (1998). Chronic interleukin-6 alters NMDA receptor-mediated membrane responses and enhances neurotoxicity in developing CNS neurons. *J. Neurosci.* 18, 10445–10456.

Rose-John, S., Scheller, J., Elson, G., and Jones, S. A. (2006). Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. *J. Leukoc. Biol.* 80, 227–236. doi: 10.1189/jlb.1105674

Samland, H., Huitron-Resendiz, S., Masliah, E., Criado, J., Henriksen, S. J., and Campbell, I. L. (2003). Profound increase in sensitivity to glutamatergic- but not cholinergic agonist-induced seizures in transgenic mice with astrocyte production of IL-6. *J. Neurosci. Res.* 73, 176–187. doi: 10.1002/jnr.10635

Sanz, E., Hofer, M. J., Unzeta, M., and Campbell, I. L. (2008). Minimal role for STAT1 in interleukin-6 signaling and actions in the murine brain. *Glia* 56, 190–199. doi: 10.1002/glia.20602

Schöbitz, B., Voorhuis, D. A. M., and De Kloet, E. R. (1992). Localization of interleukin 6 mRNA and interleukin 6 receptor mRNA in rat brain. *Neurosci. Lett.* 136, 189–192. doi: 10.1016/0304-3940(92)90046-A

Schumann, G., Huell, M., Machein, U., Hocke, G., and Fiebich, B. L. (1999). Interleukin-6 activates signal transducer and activator of transcription and mitogen-activated protein kinase signal transduction pathways and induces de novo protein synthesis in human neuronal cells. *J. Neurochem.* 73, 2009–2017. doi: 10.1046/j.1471-4159.1999.02009.x

Semple, B. D., Frugier, T., and Morganti-Kossmann, M. C. (2010). CCL2 modulates cytokine production in cultured mouse astrocytes. *J. Neuroinflammation* 7:67. doi: 10.1186/1742-2094-7-67

Shebl, F. M., Pinto, L. A., Garcia-Pineres, A., Lempicki, R., Williams, M., Harro, C., et al. (2010). Comparison of mRNA and protein measures of cytokines following vaccination with human papillomavirus-16 L1 virus-like particles. *Cancer Epidemiol. Biomarkers Prev.* 19, 978–981. doi: 10.1158/1055-9965.epi-10-0064

Shu, M., Zhou, Y., Zhu, W., Wu, S., Zheng, X., and Yan, G. (2011). Activation of a pro-survival pathway IL-6/JAK2/STAT3 contributes to glial fibrillary acidic protein induction during the cholera toxin-induced differentiation of C6 malignant glioma cells. *Mol. Oncol.* 5, 265–272. doi: 10.1016/j.molonc.2011.03.003

Smith, J. A., Das, A., Ray, S. K., and Banik, N. L. (2012). Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. *Brain Res. Bull.* 87, 10–20. doi: 10.1016/j.brainresbull.2011.10.004

Spooren, A., Kolmus, K., Laureys, G., Clinckers, R., De Keyser, J., Haegeman, G., et al. (2011). Interleukin-6, a mental cytokine. *Brain Res. Rev.* 67, 157–183. doi: 10.1016/j.brainresrev.2011.01.002

Sterneck, E., and Johnson, P. F. (1998). CCAAT/enhancer binding protein beta is a neuronal transcriptional regulator activated by nerve growth factor receptor signaling. *J. Neurochem.* 70, 2424–2433. doi: 10.1046/j.1471-4159.1998.70062424.x

Stuart, M. J., and Baune, B. T. (2014). Chemokines and chemokine receptors in mood disorders, schizophrenia and cognitive impairment: a systematic review of biomarker studies. *Neurosci. Biobehav. Rev.* 42C, 93–115. doi: 10.1016/j.neubiorev.2014.02.001

Suzuki, T., Mitake, S., Okumura-Noji, K., Shimizu, H., Tada, T., and Fujii, T. (1997). Excitable membranes and synaptic transmission: postsynaptic mechanisms. Localization of alpha-internexin in the postsynaptic density of the rat brain. *Brain Res.* 765, 74–80. doi: 10.1016/s0006-8993(97)00492-7

Sweatt, J. D. (2001). The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. *J. Neurochem.* 76, 1–10. doi: 10.1046/j.1471-4159.2001.00054.x

Taga, T., and Kishimoto, T. (1997). Gp130 and the interleukin-6 family of cytokines. *Annu. Rev. Immunol.* 15, 797–819. doi: 10.1146/annurev.immunol.15.1.797

Tancredi, V., D'antuono, M., Cafe, C., Giovedi, S., Bue, M. C., D'arcangelo, G., et al. (2000). The inhibitory effects of interleukin-6 on synaptic plasticity in the rat hippocampus are associated with an inhibition of mitogen-activated protein kinase ERK. *J. Neurochem.* 75, 634–643. doi: 10.1046/j.1471-4159.2000.0750634.x

Tani, H., Dulla, C. G., Farzampour, Z., Taylor-Weiner, A., Huguenard, J. R., and Reimer, R. J. (2014). A local glutamate-glutamine cycle sustains synaptic excitatory transmitter release. *Neuron* 81, 888–900. doi: 10.1016/j.neuron.2013.12.026

Taubenfeld, S. M., Wiig, K. A., Monti, B., Dolan, B., Pollonini, G., and Alberini, C. M. (2001). Fornix-dependent induction of hippocampal CCAAT enhancer-binding protein [beta] and [delta] co-localizes with phosphorylated cAMP response element-binding protein and accompanies long-term memory consolidation. *J. Neurosci.* 21, 84–91.

Thomas, G. M., and Huganir, R. L. (2004). MAPK cascade signalling and synaptic plasticity. *Nat. Rev. Neurosci.* 5, 173–183. doi: 10.1038/nrn1346

Tsakiri, N., Kimber, I., Rothwell, N. J., and Pinteaux, E. (2008). Mechanisms of interleukin-6 synthesis and release induced by interleukin-1 and cell depolarisation in neurones. *Mol. Cell. Neurosci.* 37, 110–118. doi: 10.1016/j.mcn.2007.09.001

Vallieres, L., Campbell, I. L., Gage, F. H., and Sawchenko, P. E. (2002). Reduced hippocampal neurogenesis in adult transgenic mice with chronic astrocytic production of interleukin-6. *J. Neurosci.* 22, 486–492.

van Gassen, K. L., Netzeband, J. G., De Graan, P. N., and Gruol, D. L. (2005). The chemokine CCL2 modulates Ca2+ dynamics and electrophysiological properties of cultured cerebellar Purkinje neurons. *Eur. J. Neurosci.* 21, 2949–2957. doi: 10.1111/j.1460-9568.2005.04113.x

Vargas, D. L., Nascimbene, C., Krishnan, C., Zimmerman, A. W., and Pardo, C. A. (2005). Neuroglial activation and neuroinflammation in the brain of patients with autism. *Ann. Neurol.* 57, 67–81. doi: 10.1002/ana.20315

Vogel, C., and Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat. Rev. Genet.* 13, 227–232. doi: 10.1038/nrg3185

Wada-Isoe, K., Wakutani, Y., Urakami, K., and Nakashima, K. (2004). Elevated interleukin-6 levels in cerebrospinal fluid of vascular dementia patients. *Acta Neurol. Scand.* 110, 124–127. doi: 10.1111/j.1600-0404.2004.00286.x

Wei, H., Chadman, K. K., Mccloskey, D. P., Sheikh, A. M., Malik, M., Brown, W. T., et al. (2012). Brain IL-6 elevation causes neuronal circuitry imbalances and mediates autism-like behaviors. *Biochim. Biophys. Acta* 1822, 831–842. doi: 10.1016/j.bbadis.2012.01.011

Wei, X. H., Na, X. D., Liao, G. J., Chen, Q. Y., Cui, Y., Chen, F. Y., et al. (2013). The up-regulation of IL-6 in DRG and spinal dorsal horn contributes to neuropathic pain following L5 ventral root transection. *Exp. Neurol.* 241, 159–168. doi: 10.1016/j.expneurol.2012.12.007

Wei, H., Zou, H., Sheikh, A. M., Malik, M., Dobkin, C., Brown, W. T., et al. (2011). IL-6 is increased in the cerebellum of autistic brain and alters neural cell adhesion, migration and synaptic formation. *J. Neuroinflammation* 8:52. doi: 10.1186/1742-2094-8-52

Westin, K., Buchhave, P., Nielsen, H., Minthon, L., Janciauskiene, S., and Hansson, O. (2012). CCL2 is associated with a faster rate of cognitive decline during early stages of Alzheimer's disease. *PLoS One* 7:e30525. doi: 10.1371/journal.pone.0030525

Wu, Y., Wang, X., Mo, X., Xi, Z., Xiao, F., Li, J., et al. (2008). Expression of monocyte chemoattractant protein-1 in brain tissue of patients with intractable epilepsy. *Clin. Neuropathol.* 27, 55–63. doi: 10.5414/npp27055

Wullschleger, A., Kapina, V., Molnarfi, N., Courvoisier, D. S., Seebach, J. D., Santiago-Raber, M. L., et al. (2013). Cerebrospinal fluid interleukin-6 in central nervous system inflammatory diseases. *PLoS One* 8:e72399. doi: 10.1371/journal.pone.0072399

Zhang, W., Miao, Y., Zhou, S., Jiang, J., Luo, Q., and Qiu, Y. (2011). Neuroprotective effects of ischemic postconditioning on global brain ischemia in rats through upregulation of hippocampal glutamine synthetase. *J. Clin. Neurosci.* 18, 685–689. doi: 10.1016/j.jocn.2010.08.027

Zhou, Y., Tang, H., Liu, J., Dong, J., and Xiong, H. (2011). Chemokine CCL2 modulation of neuronal excitability and synaptic transmission in rat hippocampal slices. *J. Neurochem.* 116, 406–414. doi: 10.1111/j.1471-4159.2010.07121.x

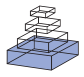
Zou, J., Wang, Y. X., Dou, F. F., Lu, H. Z., Ma, Z. W., Lu, P. H., et al. (2010). Glutamine synthetase down-regulation reduces astrocyte protection against glutamate excitotoxicity to neurons. *Neurochem. Int.* 56, 577–584. doi: 10.1016/j.neuint.2009.12.021

**Conflict of Interest Statement**: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 02 May 2014; accepted: 25 July 2014; published online: 14 August 2014.*
*Citation: Gruol DL, Vo K and Bray JG (2014) Increased astrocyte expression of IL-6 or CCL2 in transgenic mice alters levels of hippocampal and cerebellar proteins. Front. Cell. Neurosci.* ***8****:234. doi: 10.3389/fncel.2014.00234*
*This article was submitted to the journal Frontiers in Cellular Neuroscience.*
*Copyright © 2014 Gruol, Vo and Bray. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 06 March 2014
doi: 10.3389/fncel.2014.00065



# CXCL12 chemokine and its receptors as major players in the interactions between immune and nervous systems

**Alice Guyon***

*Institut de Pharmacologie Moléculaire et Cellulaire, UMR 7275 Centre National de la Recherche Scientifique/Université Nice Sophia Antipolis, Valbonne, France*

**Edited by:**
*Laura Maggi, Sapienza, Italy*

**Reviewed by:**
*Silvia Di Angelantonio, University La Sapienza, Italy*
*Richard Miller, Northwestern University, USA*

***Correspondence:**
*Alice Guyon, Institut de Pharmacologie Moléculaire et Cellulaire, UMR 7275 Centre National de la Recherche Scientifique/ Université Nice Sophia Antipolis, 660 Route des Lucioles, 06560 Valbonne, France*
*e-mail: alice.guyon@ipmc.cnrs.fr*

The chemokine CXCL12/stromal cell-derived factor 1 alpha has first been described in the immune system where it functions include chemotaxis for lymphocytes and macrophages, migration of hematopoietic cells from fetal liver to bone marrow and the formation of large blood vessels. Among other chemokines, CXCL12 has recently attracted much attention in the brain as it has been shown that it can be produced not only by glial cells but also by neurons. In addition, its receptors CXCR4 and CXCR7, which are belonging to the G protein-coupled receptors family, are abundantly expressed in diverse brain area, CXCR4 being a major co-receptor for human immunodeficiency virus 1 entry. This chemokine system has been shown to play important roles in brain plasticity processes occurring during development but also in the physiology of the brain in normal and pathological conditions. For example, in neurons, CXCR4 stimulation has been shown regulate the synaptic release of glutamate and γ-aminobutyric acid (GABA). It can also act post-synaptically by activating a G protein activated inward rectifier $K^+$ (GIRK), a voltage-gated K channel Kv2.1 associated to neuronal survival, and by increasing high voltage activated $Ca^{2+}$ currents. In addition, it has been recently evidenced that there are several cross-talks between the CXCL12/CXCR4–7 system and other neurotransmitter systems in the brain (such as GABA, glutamate, opioids, and cannabinoids). Overall, this chemokine system could be one of the key players of the neuro-immune interface that participates in shaping the brain in response to changes in the environment.

**Keywords: chemokine, CXCR4, CXCR7, CXCL12/SDF-1, glutamate, GABA**

## INTRODUCTION

The pathways by which nervous and immune systems interact to modulate plasticity in response to changes in the environment are still a matter of debate. It has been shown that many immune cells express receptors to neurotransmitters such as dopamine (DA), serotonin, or acetylcholine (Franco et al., 2007). Neurotransmitters released by nerve terminals in the blood or in lymphoid organs could by this way influence immune cells. Conversely, cytokines/chemokines and their receptors that were first described in the immune system have been recently found in the brain, in glial cells, and neurons themselves. Indeed, following inflammation or infection, cytokines are released in the blood. Besides their effect on the immune system, cytokines can also act in the brain to modulate our behaviors, inducing, for example, anorexia upon inflammation when produced in large amount, but cytokines/chemokines could also play a key role in the brain even in non-pathological conditions. Cytokines/chemokines can influence the brain and the behaviors through several possible pathways: modulating peripheral neurons which project to the brain through the vagus nerve, modulating the levels of hormones such as leptin which can act to the brain through the humoral pathway and acting directly in the brain, through the local production of cytokines and chemokines (Guyon et al., 2008a).

Among cytokines, chemokines are small proteins (7–14 kDa) with chemoattractant properties whose main documented role is leukocyte recruitment at inflammatory sites (Luster, 1998; Ransohoff and Tani, 1998; Glabinski and Ransohoff, 1999; Rossi and Zlotnik, 2000; Luther and Cyster, 2001; Proudfoot, 2002). At least 50 chemokines have been found to date and they have been classified as C, CC, CXC, and CXXXC according to the number and spacing of the conserved cysteine residues at the N-terminal position (Murphy et al., 2000). Phylogenic analyses showed that the large, highly redundant CXC chemokine family is a very recent phenomenon that is exclusive to higher vertebrates. Interestingly, its ancestral role might be within the central nervous system (CNS) and not within the immune system (Huising et al., 2003). Chemokines exert their biological effects through cell surface receptors that belong to the superfamily of seven-transmembrane domain G protein-coupled receptors (GPCRs).

At least 22 chemokine receptors have been characterized, which are designed following the chemokine nomenclature presented before. Most chemokines bind to several chemokine receptors and most chemokine receptors recognize several chemokines (Bacon and Harrison, 2000). Under ligand stimulation, receptor activation usually activates multiple intracellular pathways and undergoes a desensitization and internalization. Besides their role in the immune system, chemokines and their receptors seem to play an important role in the CNS, where they were first detected in immune-like competent cells (microglia and astrocytes), but were next found in neuronal cells (for review, see Cho and Miller, 2002; Banisadr et al., 2005). Local chemokine release is commonly

associated to neurodegenerative and neuroinflammatory disorders such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, and human immunodeficiency virus (HIV)-associated dementia (Streit et al., 2001; Vila et al., 2001; Lee et al., 2002; McGeer and McGeer, 2004; Cartier et al., 2005). In addition, accumulating evidence show that chemokines can modulate the electrical activity of neurons through various mechanisms (Guyon and Nahon, 2007; Rostene et al., 2007).

One of the most studied chemokine is the stromal cell-derived factor 1 alpha (SDF-1α) also named CXCL12. This chemokine was originally described as a secreted product of bone marrow stromal cell line (Tashiro et al., 1993). Three protein isoforms, SDF-1α, SDF-1β, and SDF-1γ, which arise from alternative mRNA splicing, have been characterized (Gleichmann et al., 2000; Pillarisetti and Gupta, 2001; Stumm et al., 2002). The SDF-1β isoform is selectively expressed by endothelial cells of cerebral microvessels and could be involved in cerebral infiltration of CXCR4-carrying leukocytes, whereas neurons synthesize SDF-1α mRNA (Stumm et al., 2002), and most studies have focused on SDF-1α. This chemokine of 67 amino acids, more recently called CXCL12, was first believed to act on a single receptor, the CXCR4. Since then, a second receptor has been found to be another target of CXCL12, namely CXCR7. Contrary to CXCR4, coupling of CXCR7 to G proteins could not be demonstrated, and CXCR7 was first believed to be mainly involved in ligand sequestration (Thelen and Thelen, 2008). However, a recent study shows that ligand binding to CXCR7 activates mitogen-activated protein (MAP) kinases through beta-arrestins (Zabel et al., 2009; Rajagopal et al., 2010; **Figure 1**).

In the immune system, the binding of CXCL12 to CXCR4/CD184 induces intracellular signaling through several divergent pathways initiating signals related to chemotaxis, cell survival and/or proliferation, increase in intracellular calcium, and gene transcription. CXCR4 is expressed on multiple cell types including lymphocytes, hematopoietic stem cells, endothelial and epithelial cells, and cancer cells. The CXCL12/CXCR4 axis is involved in tumor progression, angiogenesis, metastasis, and survival. Besides its roles in the immune system, CXCL12 also plays a major role in the CNS (Adler and Rogers, 2005; **Figure 2**).

In a first part, we will describe the roles of this chemokine system in the brain in pathological as well as physiological conditions, and show that it is acting as a neuro-modulator. In a second part, we will show in more detail the cross-talk of the CXCL12/CXCR4–7 system with other neurotransmitter systems in the brain, particularly γ-aminobutyric acid (GABA) and glutamate systems. Finally, we will show the role of the CXCL12/CXCR4–7 system in the immune–nervous system interaction.

## CXCL12 ACTIONS IN THE BRAIN

In the CNS, CXCL12 is an important chemokine playing a key role in *neurogenesis* (Ma et al., 1998; Zou et al., 1998; Lu et al., 2002) controlling axonal guidance and neurite outgrowth (Xiang et al., 2002; Pujol et al., 2005). For example, it is established that the future interneurons are maintained by a CXCL12/CXCR4

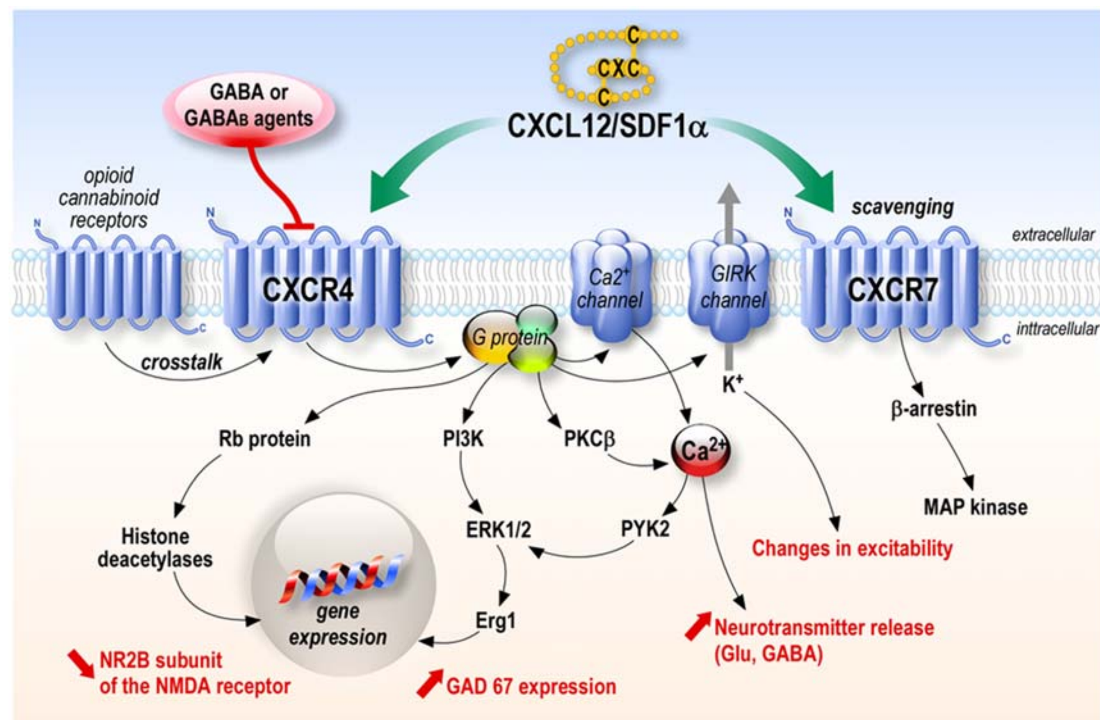



**FIGURE 1 | CXCL12 chemokine signaling.** CXCL12 acts through its receptors CXCR4 and CXCR7. CXCR4 stimulation leads to the activation of numerous signaling pathways depending on the cell types while CXCR7 has mainly been shown to be involved in scavenging CXCL12, although it can activate a MAP kinase pathway through β-arrestin in several systems.

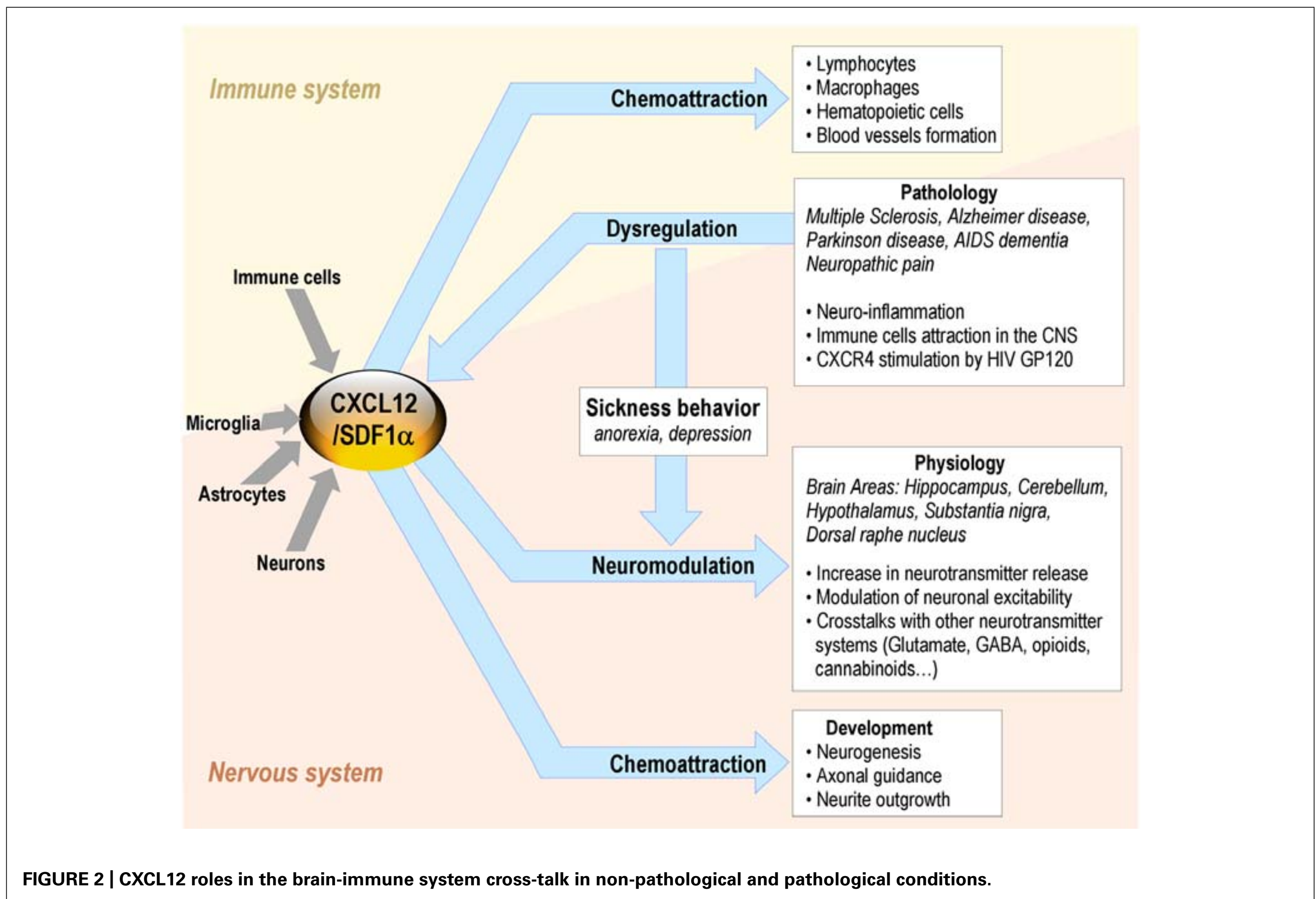



**FIGURE 2 | CXCL12 roles in the brain-immune system cross-talk in non-pathological and pathological conditions.**

mediated attractive interaction in their tangential cortical routes (Stumm et al., 2003; Lopez-Bendito et al., 2008; Lysko et al., 2011). CXCL12/CXCR4 has also been shown to regulate the migration and orientation of processes in A9–A10 dopaminergic neurons (Yang et al., 2013). During development, CXCL12 also regulates the migration rate of gonadotropin-releasing hormone (GnRH) neurons (neuroendocrine cells, located in the hypothalamus, that play an essential role in mammalian reproduction), through CXCR4 and activation of a GIRK channel (Casoni et al., 2012). The migration of GnRH neurons is also indirectly influenced by CXCR7, which regulates CXCL12 availability by acting as a scavenger along the migratory path of these neurons (Memi et al., 2013).

CXCL12 also plays a major role in *neuro-inflammation* as it mediates local immune responses as well as attracting leukocytes which are believed to migrate along a concentration gradient of chemokine across the blood–brain barrier (BBB) to their target (Engelhardt and Ransohoff, 2012). This occurs for example in Alzheimer's disease in the vicinity of the amyloid plaques that attract and/or activate local glial cells (Xia and Hyman, 1999). As the glycoprotein gp120 from the envelope of HIV-1 binds directly to CXCR4 and has direct neurotoxic effects, CXCR4 is likely to be crucial for different aspects of CNS HIV infection and the development of AIDS dementia, and CXCL12 could have *neuroprotective* effects in this context as well as in other forms of damage.

Aside from a role in CNS development and pathology, constitutive *expression* of CXCL12 and its receptor CXCR4 has been demonstrated in different cell types of the adult brain including endothelial, glial, and notably neuronal cells (Ohtani et al., 1998; Bajetto et al., 1999a; Lazarini et al., 2000; Stumm et al., 2002; Banisadr et al., 2003, 2004; Bonavia et al., 2003; Heinisch and Kirby, 2010). *In situ* hybridization and immunocytochemistry showed that CXCR4 neuronal expression was found in many different brain areas, notably cerebral cortex, globus pallidus, caudate putamen and substantia innominata (Banisadr et al., 2002), supraoptic and paraventricular hypothalamic nuclei (Banisadr et al., 2003), lateral hypothalamus (LHA; where CXCR4 is co-localized with neurons expressing the melanin-concentrating hormone (MCH; Guyon et al., 2005a), ventromedial thalamic nucleus and substantia nigra (SN; where CXCR4 is expressed on dopaminergic neurons of the pars compacta; Banisadr et al., 2002), but also on GABAergic neurons of the pars reticulata (Guyon et al., 2006), in the dorsal raphe nucleus (in serotoninergic and non-serotoninergic neurons (Heinisch and Kirby, 2010)) and in the cerebellum (where it is expressed both in Purkinje neurons and granule cells and in glial radial fibers; Ragozzino, 2002). Thus, CXCL12 and CXCR4 proteins were found co-expressed in a number of brain regions and much evidence suggest that they constitute together a functional receptor/ligand system in specific neuronal pathway.

CXCR4 stimulation by CXCL12 activates pertussis toxin (PTX)-sensitive G proteins which activate at least two distinct signaling pathways. The first pathway, involving phosphatidylinositol-3 (PI-3) kinase and extracellular signal-regulated kinase (ERK)1/2, has been described in rodent astrocytes, neuronal progenitors, and cortical neurons (Bacon and Harrison, 2000; Lazarini et al., 2000; Bajetto et al., 2001; Bonavia et al., 2003). The other pathway involves the phospholipase Cβ whose activation leads to an increase in the intracellular calcium in astrocytes, cortical neurons, and cerebellar granule cell, as well as in primate fetal neuron and microglia (Bajetto et al., 1999b; Klein et al., 1999; Zheng et al., 1999). The increase in calcium leads to the activation of proline-rich tyrosine kinase (PYK2), which may itself lead to ERK1/2 activation (Bajetto et al., 2001). CXCR4 stimulation can directly modulate ionic channels of the plasma membrane in neurons, particularly high-threshold calcium channels (Guyon et al., 2008b), and this could also result in intracellular calcium increase and PYK2 activation (Lazarini et al., 2003). Finally, in primary cultures of neurons, CXCR4 can also inhibit cAMP pathways through the Gi component of GPCRs (Liu et al., 2003).

The *neuromodulatory actions* of CXCL12 have been found in various neuronal populations (including CA1 neurons of the hippocampus, granular and Purkinje cells of the cerebellum, MCH neurons of the LHA, vasopressinergic neurons of the supraoptic and the paraventricular nucleus of the hypothalamus, dopaminergic neurons of the SN, serotoninergic and non-serotoninergic neurons of the dorsal raphe nucleus; Limatola et al., 2000; Ragozzino, 2002; Guyon et al., 2005a,b, 2008b; Callewaere et al., 2006; Heinisch and Kirby, 2010).

CXCR4 activation by its ligand can modulate neuronal activity through multiple regulatory pathways including and often combining: (i) modulation of voltage-dependent channels (sodium, potassium, and calcium; Limatola et al., 2000; Guyon et al., 2005b), (ii) activation of the GIRK channels, (iii) increase in neurotransmitter release (GABA, glutamate, DA), often via calcium-dependent mechanisms (Guyon and Nahon, 2007). From one structure to another, CXCL12 has often similar consequences on neuronal transmembrane currents, but through different mechanisms.

CXCL12 has *pre-synaptic actions*, which are similar in the different brain structures where it has been tested, increasing glutamate and/or GABA synaptic activities in LHA (Guyon et al., 2005a), hippocampus (Zheng et al., 1999), cerebellum (Limatola et al., 2000), SN (Guyon et al., 2006), and dorsal raphe nucleus (Heinisch and Kirby, 2010). However, the mechanisms of action of CXCL12 vary from one structure to the other: for example, the increase in frequency of GABA type A ($GABA_A$) post-synaptic events in response to CXCL12 occurs through an indirect mechanism involving glutamate release in the cerebellum (Limatola et al., 2000) and the serotoninergic neurons of the raphe nucleus (Heinisch and Kirby, 2010), while the effect is direct through CXCR4 of dopaminergic neurons in the SN (Guyon et al., 2006). Similarly, the glutamate release is tetrodotoxin (TTX) dependent in the LHA (Guyon et al., 2005a) and in the raphe (Heinisch and Kirby, 2010), while it is TTX independent in the SN (Guyon et al., 2006).

The target effects on the *post-synaptic* neurons also vary depending on the structure and the neuronal population. For example, the CXCL12-induced increase in GABA release in the LHA evokes a tonic $GABA_A$ current in MCH-expressing neurons (Guyon et al., 2005a), opposite to what is observed in DA neurons in which GABA type B ($GABA_B$) receptor stimulation following GABA spillover activates a GIRK current (Guyon et al., 2006). This could be due to various subunit compositions of the $GABA_A$ receptor expressed in the two neuronal populations, with different kinetics, and/or different subcellular localization of the $GABA_A/_B$/CXCR4 receptors and GIRK channels. Interestingly, in MCH neurons, CXCL12 also induced the activation of a GIRK current, but this happened directly through CXCR4 stimulation.

In the dorsal raphe nucleus, CXCR4 stimulation by CXCL12 stimulates spontaneous inhibitory post-synaptic potential (sIPSC) frequency, by a pre-synaptic mechanism on 5HT-neurons, but it acts on sIPSC amplitude by a post-synaptic mechanism in non-5HT neurons (Heinisch and Kirby, 2010). Finally, CXCR4 stimulation is able to modulate various voltage-dependent channels: $Na^+$ and $K^+$ channels of the action potential in MCH neurons (Guyon et al., 2005b) and high voltage activated (HVA) Ca channels, in particular of the N-type, in DA neurons of the SN (Guyon et al., 2008b) and in pre-synaptic glutamatergic terminals of the hippocampus (Zheng et al., 1999).

In conclusion, from one structure to another, CXCL12 has often similar consequences on neuronal transmembrane currents, but through different mechanisms.

Therefore, the CXCL12/CXCR4–7 system exerts neuromodulatory functions in the normal brain.

## CROSS-TALK WITH OTHER NEUROTRANSMITTER SYSTEMS

### CROSS-TALK WITH GABAergic SYSTEM

γ-Aminobutyric acid is the major inhibitory neurotransmitter in the adult nervous system but it also plays important roles in CNS development by regulating neurogenesis and synaptogenesis (LoTurco et al., 1995; Somogyi et al., 1995). In contrast to its inhibitory actions on adult neurons, GABA is capable of depolarizing neuronal progenitor cells and immature neurons (Ben-Ari, 2002; Rheims et al., 2008) and participates in formation of a primitive network-driven pattern of electrical activity called the giant depolarizing potentials (GDPs), an electrical circuit pattern critical to generate large oscillations of intracellular calcium for activity-dependent modulation of neuronal growth and synapse formation (Ben-Ari, 2002). HIV-1 gp120, which binds and stimulates CXCR4, enhances GDPs in neonatal rat hippocampus (Kasyanov et al., 2006), underlying the role played by CXCR4 in the developmental process. Moreover, the developmental function of GABA is in part regulated by GABA production, a process mediated by glutamic acid decarboxylases (GADs), the key rate-limiting enzymes for synthesis of GABA. Two GAD isoforms, GAD65 and GAD67, are expressed in the adult nervous system (Erlander et al., 1991). It has been shown that CXCL12/CXCR4 signaling, via ERKs and the transcription factor Egr1, induces expression of GAD67 in embryonic hippocampal cultured neurons, a mechanism which may promote the maturation of GABAergic neurons during development (Luo et al., 2008).

In adult brain, as previously mentioned, CXCL12, through CXCR4, is also able to modulate pre-synaptic GABA release

(Limatola et al., 2000; Guyon et al., 2006; Heinisch and Kirby, 2010). GABA acts post-synaptically through its receptors. $GABA_A$ receptors are ionotropic receptors permeant to chloride. As CXCR4, $GABA_B$ receptors are GPCRs that mediate metabotropic action of GABA and are located on neurons and immune cells as well. Using diverse approaches, a novel interaction between CXCR4 and GABA/$GABA_B$ receptor agonists/antagonists has been recently reported, which was revealed to be an allosteric action of these agents on CXCR4 (Guyon et al., 2013). This result came first from the observation that $GABA_B$ antagonists and agonists and even GABA itself blocked CXCL12-elicited chemotaxis in human breast cancer cells, and that a $GABA_B$ antagonist blocked the potentiation by CXCL12 of high threshold $Ca^{+2}$ channels in Rat dopaminergic neurons (Guyon et al., 2013). CXCR4 and $GABA_B$ often co-express in the same cell type (Banisadr et al., 2002), have complementary functionality and may be involved in cross-talk (Duthey et al., 2010). CXCR4 and $GABA_B$ receptor could have interacted directly through heterodimerization. Indeed, heterodimerization is known to play a role in signal transduction of other metabotropic receptors, for example, $GABA_B$ receptors interact with metabotropic glutamate receptors (Hirono et al., 2001). Following CXCL12 interaction, CXCR4 undergoes a homo-dimerization which is necessary for its functionality and signaling (Mellado et al., 2001; Toth et al., 2004). Dimerization, which is accompanied by receptor phosphorylation as well as changes in signal transduction processes (Rodriguez-Frade et al., 2001), enables the activation of the JAK/STAT (Janus kinase/signal transducers and activators of transcription ) pathway which allows the subsequent triggering of G protein-dependent signaling events (Vila-Coro et al., 1999). CXCR4 could also form heterodimers with other GPCRs, which could lead to complex responses according to the chemokines/peptides/neuromediator environment present in the extracellular medium. For example, CXCR4 has been shown to form heterodimers with CCR2 and CCR5, delta opioid receptors and CXCR7 (Percherancier et al., 2005; Pello et al., 2008; Levoye et al., 2009; Sohy et al., 2009). However, by co-expressing in the membrane of *Xenopus* oocytes $GABA_B$ receptors tagged with Td tomato (red fluorophore) and CXCR4 receptors tagged with green fluorescent protein (GFP), data obtained in total internal reflection fluorescence (TIRF) microscopy showed that CXCR4 and $GABA_B$ receptors did not co-localize in the membrane (A. Guyon, unpublished data).

γ-Aminobutyric acid and the agonists/antagonists of $GABA_B$ receptors were recently found to act directly on CXCR4 in an allosteric manner (**Figure 3A**). Using electrophysiology in *Xenopus* oocytes and human embryonic kidney (HEK293) cells in which Rat CXCR4 and the GIRK channel were co-expressed, it could be demonstrated that $GABA_B$ antagonist and agonist modify the CXCL12-evoked activation of GIRK channels (**Figures 3B,C**; Guyon et al., 2013). By expressing CXCR4 receptors in heterologous systems lacking $GABA_B$ receptors and performing competition binding experiments it could be investigated whether $GABA_B$ ligands bind to CXCR4. FRET experiments suggested that $GABA_B$ ligands do not bind CXCR4 at the CXCL12 binding pocket suggesting allosteric modulation, in accordance with electrophysiology data (Guyon et al., 2013). Finally, back-scattering interferometry (BSI) on lipoparticles containing only the CXCR4 receptor allowed to quantify the binding affinity for the $GABA_B$ ligands (including GABA), which were in a similar range to the affinity of the ligands for $GABA_B$ receptors themselves, thus confirming that GABA and $GABA_B$ receptor ligands directly interact allosterically with the CXCR4 receptor (**Figure 3D**; Guyon et al., 2013).

As mentioned previously, CXCR4 activation by CXCL12 increases pre-synaptic neurotransmitter release and particularly GABA release in several neuronal populations (Guyon and Nahon, 2007; Bhattacharyya et al., 2008; Qu et al., 2008). If GABA can in turn block the effects of CXCL12, this could represent a negative feedback loop for pre-synaptic chemokine release. Indeed, when applying CXCL12 for several minutes, a transient increase in the frequency of sIPSCs is frequently observed, followed by a reduced activity (see Figure 3 in Guyon et al., 2006). This reduction could be due to an antagonistic effect of GABA, although desensitization of CXCR4 itself cannot be excluded. Similarly, it has been shown that elevated concentrations of CXCL12 exert opposite effect than lower concentrations on the electrical activity of some neuronal populations that receive GABA inputs (Guyon and Nahon, 2007). The antagonistic effect of GABA released pre-synaptically in response to CXCL12 could contribute to these biphasic effects. In the future, it will be of interest to search for putative effects of $GABA_B$ receptor ligands on CXCR7, the other receptor for CXCL12. In addition, the effect of GABAergic agents on CXCR4 suggests new therapeutic potentials for neurological and immune diseases.

### CROSS-TALK WITH GLUTAMATERGIC SYSTEMS

Glutamate is the main excitatory neurotransmitter in the adult brain. CXCL12/CXCR4 also plays a major role in the regulation of crucial components of glutamatergic transmission.

The chemokine CXCR4 receptor is a GPCR widely expressed on glial cells (especially astrocytes and microglia). Activation of the astrocytic CXCR4 by CXCL12 results in a long chain of intracellular and extracellular events [including the release of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) and prostanglandins] leading to glutamate release (Cali et al., 2008; Cali and Bezzi, 2010). Similarly, and as previously mentioned, CXCL12/CXCR4 increases glutamate release from neurons in different structures including LHA (Guyon et al., 2005a), hippocampus (Zheng et al., 1999), cerebellum (Limatola et al., 2000), SN (Guyon et al., 2006), and dorsal raphe nucleus (Heinisch and Kirby, 2010).

In addition, recent studies showed that CXCL12 protects cortical neurons from excitotoxicity by promoting the function of the gene-repressor protein Rb, which is involved in the recruitment of chromatin modifiers [such as histone deacetylases (HDACs)] to gene promoters. In neurons, Rb controls activity-dependent genes essential to neuronal plasticity and survival, such as the *N*-methyl-D-aspartic acid (NMDA) receptor's subunit NR2B (Khan et al., 2008), the expression of which in the tetrameric ion channel largely affects calcium signaling by glutamate. CXCL12 selectively inhibits NR2B expression *in vitro* and *in vivo* altering NMDA-induced calcium responses associated with neuronal death, while promoting prosurvival pathways

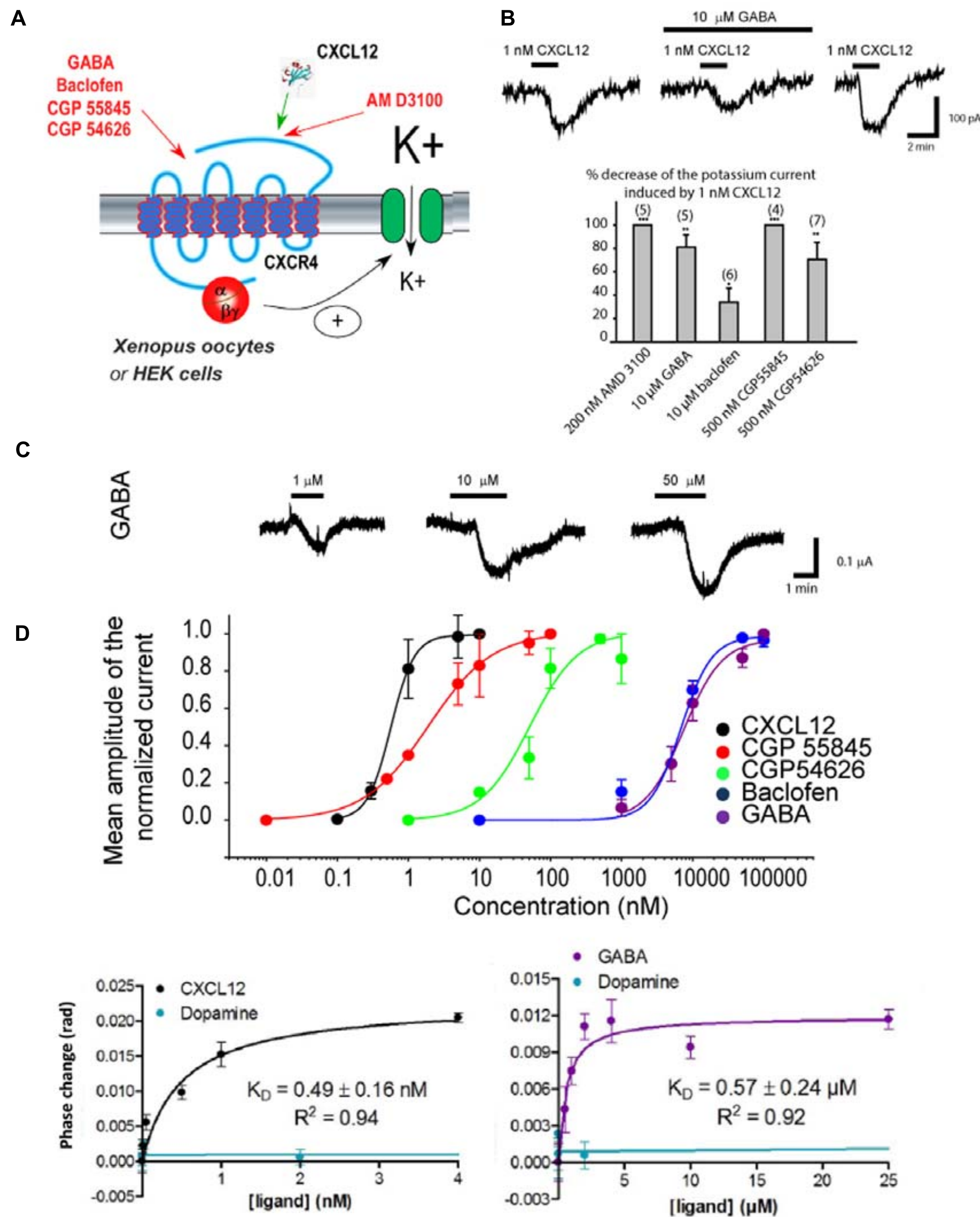



**FIGURE 3 | GABA and agonists/antagonists of the GABA$_B$ receptor are allosteric modulators of CXCR4. (A)** Scheme showing the diverse agents acting on the CXCR4 receptor that have been tested. CXCR4 has been co-expressed together with GIRK channels in *Xenopus* oocytes and human embryonic kidney (HEK293) cells. The activation of CXCR4 by the diverse agent tested was evaluated by measuring the amplitude of the GIRK current activated. **(B)** Results obtained in HEK293 cells expressing CXCR4 and reporting GIRK channel. *Top*: Traces recorded in response to the application of 1 nM CXCL12 with or without GABA. GABA inhibits partially the CXCL12 effect and this effect is reversible upon washout of GABA. *Bottom*: Histogram showing the allosteric actions of the CXCR4 antagonist AMD3100 compared to GABA and other agonists/antagonists of GABA$_B$ receptors. **(C)** Results obtained in *Xenopus* oocytes expressing CXCR4 and reporting GIRK channel. *Top*: Traces recorded in response to the application of three increasing GABA concentrations. *Bottom*: We took advantage of the agonist effect of GABA and of the GABA$_B$ agonists/antagonists in this expression system to build their concentration–response curve on CXCR4. **(D)** Back-scattering interferometry (BSI) results obtained on lipoparticles containing only CXCR4. *Left*: BSI reveals a $K_D$ of CXCL12 on its receptor of 0.49 ± 0.16 nM, which is coherent with the EC50 measured by our electrophysiological experiments. Dopamine, used as a negative control, does not bind to CXCR4. *Right*: BSI reveals that GABA can also directly bind to CXCR4 with a $K_D$ of 0.57 ± 0.24 μM, coherent with the EC50 measured in electrophysiology. Therefore, GABA can directly bind to CXCR4. Adapted from Guyon et al. (2013).

that depend on stimulation of synaptic receptors (Nicolai et al., 2010).

### CROSS-TALK WITH OTHER SYSTEMS

In the periaqueducal gray (PAG), a pretreatment with CXCL12 desensitized the analgesic effects of *opioids* (Szabo et al., 2002; Chen et al., 2007) and a heterologous desensitization mechanism at the GPCR level involving CXCR4 has been suggested (Chen et al., 2007; Heinisch et al., 2011). Moreover, intermediate opioid peptides (pro-enkephalin A-derived peptides secreted by adrenal subcapsular cell hyperplasia) have been shown to be potent activators of CXCR7 (Ikeda et al., 2013).

In the same line, the analgesic activity of the *cannabinoid* agonist WIN 55,212-2 in the brain can be overcome in situations in which there are elevated levels of CXCL12 in the brain (Benamar et al., 2008). There could be a functional interaction between chemokine and cannabinoid systems in the brain as the thermoregulatory action of the cannabinoid agonist WIN 55,212-2 in the preoptic anterior hypothalamus can be antagonized by elevated levels of CXCL12 (Benamar et al., 2009). This could explain why conditions associated with elevated level of chemokines may result in reduction of cannabinoid functions, as is the case with most neuroinflammatory diseases (such as multiple sclerosis and HIV encephalitis).

## ROLE IN THE IMMUNE–NERVOUS SYSTEM INTERACTION

As in the context of the immune system, where low levels of CXCL12 (100 ng/ml) are attractive, whereas higher levels (1 mg/ml) are repulsive for T cells (Zlatopolskiy and Laurence, 2001), CXCL12 often appears to have opposite effects on neuronal function depending on the concentration. For example, in DA neurons, at low concentrations, it acts as a neuromodulator by potentiating $K^+$-induced DA secretion and HVA calcium currents, whereas at higher concentration, it decreases DA release and HVA calcium currents. This can be paralleled to what happens in MCH neurons of the LHA, where CXCL12 also exerts opposite effects on the action potential discharge depending on the concentration (Guyon et al., 2005a). Several putative mechanisms for these opposite effects, which are not mutually exclusive, are reviewed in Guyon and Nahon (2007).

CXCL12 can act in the CNS as a classical neuromediator under normal conditions and modulate the activity of several neuroendocrine networks. Low concentrations of CXCL12 exert a tonic inhibition on MCH neurons, which are known to have a hyperpolarized membrane potential under basal conditions, as compared to orexin neurons of the LHA which are in intrinsic state of membrane depolarization and lack CXCR4 expression (Eggermann et al., 2003). In addition, the CXCR4 antagonist AMD 3100 has its own effects when applied alone which suggests that a tonic activation of CXCR4 occurs, at least in slices preparations, and that low levels of CXCL12 are secreted under basal conditions (although the slice preparation could also be considered as an inflammatory state).

However, pathological state (altered immune response or inflammation) leads to abnormal concentrations of chemokines and/or their presence at unusual sites can be found, due to local production by glial and/or endothelial cells and/or diffusion and transportation through the vascular circulation. This enhanced production of chemokines could interfere with their normal functions, affect neuronal and neuro-endocrine activity and modify the functioning of the brain, leading to pathological behaviors and/or neurotoxicity.

Following inflammation, cytokines are released in the blood and can reach the brain, as the BBB permeability is increased. Cytokine stimulation leads to higher levels of CXCL12 and other chemokines by activation of glial or endothelial cells which release chemokines (Meucci et al., 1998; Ohtani et al., 1998; Lee et al., 2002). The chemokines released can reach neurons expressing CXCR4, bind their neuronal receptors and induce a change in their excitability that could induce an adaptive answer to the inflammation, leading to the "sickness behavior," characterized by depression, anorexia, and fatigue (Reichenberg et al., 2001; Dantzer and Kelley, 2007). Given the abundance of chemokines and their receptors in the CNS, it is not surprising that perturbations of cytokines/chemokines levels during inflammation are causing multiple perturbations in the brain functions and behaviors. The effects of CXCL12 on dorsal raphe neurons could underlie depressive symptoms frequently observed with inflammation (Maes, 1999), as dysfunction of the serotoninergic systems is implicated in depression. Similarly, the effects of CXCL12 on MCH neurons which are part of the circuits controlling feeding behavior and metabolism (Nahon, 2006) could explain the anorexia. These symptoms of sickness behavior are usually reversible when inflammation stops. However, a prolonged inflammation, producing higher levels of CXCL12 could even lead to neurotoxicity and to neuro-degenerescence (Gerashchenko and Shiromani, 2004).

## GENERAL CONCLUSION

CXCL12 have recently attracted much attention because this chemokine seems to play an important role as intermediate in the brain between cytokines and neurons in the cascade linking inflammation to adaptive behavioral changes. Convergent data suggest that CXCL12 could also act in the CNS as a classical neuromediator under normal conditions and could modulate the activity of several neuroendocrine networks. However, during a pathological state (altered immune response or inflammation), elevated concentrations of CXCL12 and/or its presence at unusual sites, due to its local production by glial and/or endothelial cells and/or its diffusion and transportation through the vascular circulation could affect neuronal and neuroendocrine activities and modify the functioning of the brain, leading to pathological behaviors and/or neurotoxicity. In addition, recent evidence show that there are numerous cross-talks between CXCL12/CXCR4–7 systems and other neurotransmitter systems of the CNS, illustrating new pathways by which the CNS and immune system can interact.

## ACKNOWLEDGMENTS

Acknowledgments to Amanda Kussrow, Ian Roys Olmsted, and Darryl J. Bornhop from Department of Chemistry, Vanderbilt University, Nashville, TN, USA for BSI experiments, Dr Joseph Rucker from Integral Molecular for generously providing the

lipoparticles, Prof. Richard Miller, Chicago, USA for providing the rat CXCR4 clone, Prof. Ehud Isacoff and Drs Shashank Bharill, Ryan Arant, Hitomi Okada, and Josh Levitz, University of California at Berkeley, USA for help and advices on the electrophysiological experiments, Audrey Recouly, Fabienne Chevallier, and Florence Servent from CISBIO Bioassays, Codolet, France for the receptor–ligand binding assay using the Tag-lite technology, Ann Fischer and Michelle Richner from the University of California at Berkeley MCB Tissue Culture Facility for cell culture support, Agnès Petit-Paitel and Joelle Chabry for fruitful discussions, Veronique Thieffin for animal care, and Franck Aguila for artwork.

## REFERENCES


Adler, M. W., and Rogers, T. J. (2005). Are chemokines the third major system in the brain? *J. Leukoc. Biol.* 78, 1204–1209. doi: 10.1189/jlb.0405222

Bacon, K. B., and Harrison, J. K. (2000). Chemokines and their receptors in neurobiology: perspectives in physiology and homeostasis. *J. Neuroimmunol.* 104, 92–97. doi: 10.1016/S0165-5728(99)00266-0

Bajetto, A., Barbero, S., Bonavia, R., Piccioli, P., Pirani, P., Florio, T., et al. (2001). Stromal cell-derived factor-1alpha induces astrocyte proliferation through the activation of extracellular signal-regulated kinases 1/2 pathway. *J. Neurochem.* 77, 1226–1236. doi: 10.1046/j.1471-4159.2001.00350.x

Bajetto, A., Bonavia, R., Barbero, S., Florio, T., Costa, A., and Schettini, G. (1999a). Expression of chemokine receptors in the rat brain. *Ann. N. Y. Acad. Sci.* 876, 201–209. doi: 10.1111/j.1749-6632.1999.tb07640.x

Bajetto, A., Bonavia, R., Barbero, S., Piccioli, P., Costa, A., Florio, T., et al. (1999b). Glial and neuronal cells express functional chemokine receptor CXCR4 and its natural ligand stromal cell-derived factor 1. *J. Neurochem.* 73, 2348–2357. doi: 10.1046/j.1471-4159.1999.0732348.x

Banisadr, G., Fontanges, P., Haour, F., Kitabgi, P., Rostene, W., and Melik Parsadaniantz, S. (2002). Neuroanatomical distribution of CXCR4 in adult rat brain and its localization in cholinergic and dopaminergic neurons. *Eur. J. Neurosci.* 16, 1661–1671. doi: 10.1046/j.1460-9568.2002.02237.x

Banisadr, G., Rostene, W., Kitabgi, P., and Melik Parsadaniantz, S. (2005). Chemokines and brain functions. *Curr. Drug Targets Inflamm. Allergy* 4, 387–399. doi: 10.2174/1568010054022097

Banisadr, G., Skrzydelski, D., Callewaere, C., Desarménien, M., Kitabgi, P., Rostène, W., et al. (2004). "Chemokines and chemokine receptors in the brain: possible implications in neuromodulation and neuroendocrine regulation," in *34th Annual Meeting of Society for Neurosciences*, San Diego, USA, 660.6.

Banisadr, G., Skrzydelski, D., Kitabgi, P., Rostene, W., and Parsadaniantz, S. M. (2003). Highly regionalized distribution of stromal cell-derived factor-1/CXCL12 in adult rat brain: constitutive expression in cholinergic, dopaminergic and vasopressinergic neurons. *Eur. J. Neurosci.* 18, 1593–1606. doi: 10.1046/j.1460-9568.2003.02893.x

Benamar, K., Geller, E. B., and Adler, M. W. (2008). First in vivo evidence for a functional interaction between chemokine and cannabinoid systems in the brain. *J. Pharmacol. Exp. Ther.* 325, 641–645. doi: 10.1124/jpet.107.135053

Benamar, K., Yondorf, M., Geller, E. B., Eisenstein, T. K., and Adler, M. W. (2009). Physiological evidence for interaction between the HIV-1 co-receptor CXCR4 and the cannabinoid system in the brain. *Br. J. Pharmacol.* 157, 1225–1231. doi: 10.1111/j.1476-5381.2009.00285.x

Ben-Ari, Y. (2002). Excitatory actions of GABA during development: the nature of the nurture. *Nat. Rev. Neurosci.* 3, 728–739. doi: 10.1038/nrn920

Bhattacharyya, B. J., Banisadr, G., Jung, H., Ren, D., Cronshaw, D. G., Zou, Y., et al. (2008). The chemokine stromal cell-derived factor-1 regulates GABAergic inputs to neural progenitors in the postnatal dentate gyrus. *J. Neurosci.* 28, 6720–6730. doi: 10.1523/JNEUROSCI.1677-08.2008

Bonavia, R., Bajetto, A., Barbero, S., Pirani, P., Florio, T., and Schettini, G. (2003). Chemokines and their receptors in the CNS: expression of CXCL12/SDF-1 and CXCR4 and their role in astrocyte proliferation. *Toxicol. Lett.* 139, 181–189. doi: 10.1016/S0378-4274(02)00432-0

Cali, C., and Bezzi, P. (2010). CXCR4-mediated glutamate exocytosis from astrocytes. *J. Neuroimmunol.* 224, 13–21. doi: 10.1016/j.jneuroim.2010.05.004

Cali, C., Marchaland, J., Regazzi, R., and Bezzi, P. (2008). SDF 1-alpha (CXCL12) triggers glutamate exocytosis from astrocytes on a millisecond time scale: imaging analysis at the single-vesicle level with TIRF microscopy. *J. Neuroimmunol.* 198, 82–91. doi: 10.1016/j.jneuroim.2008.04.015

Callewaere, C., Banisadr, G., Desarmenien, M. G., Mechighel, P., Kitabgi, P., Rostene, W. H., et al. (2006). The chemokine SDF-1/CXCL12 modulates the firing pattern of vasopressin neurons and counteracts induced vasopressin release through CXCR4. *Proc. Natl. Acad. Sci. U.S.A.* 103, 8221–8226. doi: 10.1073/pnas.0602620103

Cartier, L., Hartley, O., Dubois-Dauphin, M., and Krause, K. H. (2005). Chemokine receptors in the central nervous system: role in brain inflammation and neurodegenerative diseases. *Brain Res. Brain Res. Rev.* 48, 16–42. doi: 10.1016/j.brainresrev.2004.07.021

Casoni, F., Hutchins, B. I., Donohue, D., Fornaro, M., Condie, B. G., and Wray, S. (2012). SDF and GABA interact to regulate axophilic migration of GnRH neurons. *J. Cell Sci.* 125, 5015–5025. doi: 10.1242/jcs.101675

Chen, X., Geller, E. B., Rogers, T. J., and Adler, M. W. (2007). Rapid heterologous desensitization of antinociceptive activity between mu or delta opioid receptors and chemokine receptors in rats. *Drug Alcohol Depend.* 88, 36–41. doi: 10.1016/j.drugalcdep.2006.09.010

Cho, C., and Miller, R. J. (2002). Chemokine receptors and neural function. *J. Neurovirol.* 8, 573–584. doi: 10.1080/13550280290101003

Dantzer, R., and Kelley, K. W. (2007). Twenty years of research on cytokine-induced sickness behavior. *Brain Behav. Immun.* 21, 153–160. doi: 10.1016/j.bbi.2006.09.006

Duthey, B., Hubner, A., Diehl, S., Boehncke, S., Pfeffer, J., and Boehncke, W. H. (2010). Anti-inflammatory effects of the GABA(B) receptor agonist baclofen in allergic contact dermatitis. *Exp. Dermatol.* 19, 661–666. doi: 10.1111/j.1600-0625.2010.01076.x

Eggermann, E., Bayer, L., Serafin, M., Saint-Mleux, B., Bernheim, L., Jones, B. E., et al. (2003). The wake-promoting hypocretin-orexin neurons are in an intrinsic state of membrane depolarization. *J. Neurosci.* 23, 1557–1562.

Engelhardt, B., and Ransohoff, R. M. (2012). Capture, crawl, cross: the T cell code to breach the blood–brain barriers. *Trends Immunol.* 33, 579–589. doi: 10.1016/j.it.2012.07.004

Erlander, M. G., Tillakaratne, N. J., Feldblum, S., Patel, N., and Tobin, A. J. (1991). Two genes encode distinct glutamate decarboxylases. *Neuron* 7, 91–100. doi: 10.1016/0896-6273(91)90077-D

Franco, R., Pacheco, R., Lluis, C., Ahern, G. P., and O'Connell, P. J. (2007). The emergence of neurotransmitters as immune modulators. *Trends Immunol.* 28, 400–407. doi: 10.1016/j.it.2007.07.005

Gerashchenko, D., and Shiromani, P. J. (2004). Effects of inflammation produced by chronic lipopolysaccharide administration on the survival of hypocretin neurons and sleep. *Brain Res.* 1019, 162–169. doi: 10.1016/j.brainres.2004.06.016

Glabinski, A. R., and Ransohoff, R. M. (1999). Sentries at the gate: chemokines and the blood–brain barrier. *J. Neurovirol.* 5, 623–634. doi: 10.3109/13550289909021291

Gleichmann, M., Gillen, C., Czardybon, M., Bosse, F., Greiner-Petter, R., Auer, J., et al. (2000). Cloning and characterization of SDF-1gamma, a novel SDF-1 chemokine transcript with developmentally regulated expression in the nervous system. *Eur. J. Neurosci.* 12, 1857–1866. doi: 10.1046/j.1460-9568.2000.00048.x

Guyon, A., Banisadr, G., Rovere, C., Cervantes, A., Kitabgi, P., Melik-Parsadaniantz, S., et al. (2005a). Complex effects of stromal cell-derived factor-1alpha on melanin-concentrating hormone neuron excitability. *Eur. J. Neurosci.* 21, 701–710. doi: 10.1111/j.1460-9568.2005.03890.x

Guyon, A., Rovere, C., Cervantes, C., Allaeys, I., and Nahon, J. L. (2005b). Stromal cell-derived factor-1alpha directly modulates voltage-dependent currents of the action potential in mammalian neuronal cells. *J. Neurochem.* 93, 963–973. doi: 10.1111/j.1471-4159.2005.03083.x

Guyon, A., Kussrow, A., Olmsted, I. R., Sandoz, G., Bornhop, D. J., and Nahon, J. L. (2013). Baclofen and other GABAB receptor agents are allosteric modulators of the CXCL12 chemokine receptor CXCR4. *J. Neurosci.* 33, 11643–11654. doi: 10.1523/JNEUROSCI.6070-11.2013

Guyon, A., Massa, F., Rovere, C., and Nahon, J. L. (2008a). How cytokines can influence the brain: a role for chemokines? *J. Neuroimmunol.* 198, 46–55. doi: 10.1016/j.jneuroim.2008.04.009

Guyon, A., Skrzydelski, D., Rovere, C., Apartis, E., Rostene, W., Kitabgi, P., et al. (2008b). Stromal-cell-derived factor 1alpha /CXCL12 modulates high-threshold

calcium currents in rat substantia nigra. *Eur. J. Neurosci.* 28, 862–870. doi: 10.1111/j.1460-9568.2008.06367.x

Guyon, A., and Nahon, J. L. (2007). Multiple actions of the chemokine stromal cell-derived factor-1alpha on neuronal activity. *J. Mol. Endocrinol.* 38, 365–376. doi: 10.1677/JME-06-0013

Guyon, A., Skrzydelsi, D., Rovere, C., Rostene, W., Parsadaniantz, S. M., and Nahon, J. L. (2006). Stromal cell-derived factor-1alpha modulation of the excitability of rat substantia nigra dopaminergic neurones: presynaptic mechanisms. *J. Neurochem.* 96, 1540–1550. doi: 10.1111/j.1471-4159.2006.03659.x

Heinisch, S., and Kirby, L. G. (2010). SDF-1alpha/CXCL12 enhances GABA and glutamate synaptic activity at serotonin neurons in the rat dorsal raphe nucleus. *Neuropharmacology* 58, 501–514. doi: 10.1016/j.neuropharm.2009.08.022

Heinisch, S., Palma, J., and Kirby, L. G. (2011). Interactions between chemokine and mu-opioid receptors: anatomical findings and electrophysiological studies in the rat periaqueductal grey. *Brain Behav. Immun.* 25, 360–372. doi: 10.1016/j.bbi.2010.10.020

Hirono, M., Yoshioka, T., and Konishi, S. (2001). GABA(B) receptor activation enhances mGluR-mediated responses at cerebellar excitatory synapses. *Nat. Neurosci.* 4, 1207–1216. doi: 10.1038/nn764

Huising, M. O., Stet, R. J., Kruiswijk, C. P., Savelkoul, H. F., and Lidy Verburg-van Kemenade, B. M. (2003). Molecular evolution of CXC chemokines: extant CXC chemokines originate from the CNS. *Trends Immunol.* 24, 307–313. doi: 10.1016/S1471-4906(03)00120-0

Ikeda, Y., Kumagai, H., Skach, A., Sato, M., and Yanagisawa, M. (2013). Modulation of circadian glucocorticoid oscillation via adrenal opioid-CXCR7 signaling alters emotional behavior. *Cell* 155, 1323–1336. doi: 10.1016/j.cell.2013.10.052

Kasyanov, A., Tamamura, H., Fujii, N., and Xiong, H. (2006). HIV-1 gp120 enhances giant depolarizing potentials via chemokine receptor CXCR4 in neonatal rat hippocampus. *Eur. J. Neurosci.* 23, 1120–1128. doi: 10.1111/j.1460-9568.2006.04646.x

Khan, M. Z., Brandimarti, R., Shimizu, S., Nicolai, J., Crowe, E., and Meucci, O. (2008). The chemokine CXCL12 promotes survival of postmitotic neurons by regulating Rb protein. *Cell Death Differ.* 15, 1663–1672. doi: 10.1038/cdd.2008.95

Klein, R. S., Williams, K. C., Alvarez-Hernandez, X., Westmoreland, S., Force, T., Lackner, A. A., et al. (1999). Chemokine receptor expression and signaling in macaque and human fetal neurons and astrocytes: implications for the neuropathogenesis of AIDS. *J. Immunol.* 163, 1636–1646.

Lazarini, F., Casanova, P., Tham, T. N., De Clercq, E., Arenzana-Seisdedos, F., Baleux, F., et al. (2000). Differential signalling of the chemokine receptor CXCR4 by stromal cell-derived factor 1 and the HIV glycoprotein in rat neurons and astrocytes. *Eur. J. Neurosci.* 12, 117–125. doi: 10.1046/j.1460-9568.2000.00894.x

Lazarini, F., Tham, T. N., Casanova, P., Arenzana-Seisdedos, F., and Dubois-Dalcq, M. (2003). Role of the alpha-chemokine stromal cell-derived factor (SDF-1) in the developing and mature central nervous system. *Glia* 42, 139–148. doi: 10.1002/glia.10139

Lee, Y. B., Nagai, A., and Kim, S. U. (2002). Cytokines, chemokines, and cytokine receptors in human microglia. *J. Neurosci. Res.* 69, 94–103. doi: 10.1002/jnr.10253

Levoye, A., Balabanian, K., Baleux, F., Bachelerie, F., and Lagane, B. (2009). CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood* 113, 6085–6093. doi: 10.1182/blood-2008-12-196618

Limatola, C., Giovannelli, A., Maggi, L., Ragozzino, D., Castellani, L., Ciotti, M. T., et al. (2000). SDF-1alpha-mediated modulation of synaptic transmission in rat cerebellum. *Eur. J. Neurosci.* 12, 2497–2504. doi: 10.1046/j.1460-9568.2000.00139.x

Liu, Z., Geng, L., Li, R., He, X., Zheng, J. Q., and Xie, Z. (2003). Frequency modulation of synchronized $Ca^{2+}$ spikes in cultured hippocampal networks through G-protein-coupled receptors. *J. Neurosci.* 23, 4156–4163.

Lopez-Bendito, G., Sanchez-Alcaniz, J. A., Pla, R., Borrell, V., Pico, E., Valdeolmillos, M., et al. (2008). Chemokine signaling controls intracortical migration and final distribution of GABAergic interneurons. *J. Neurosci.* 28, 1613–1624. doi: 10.1523/JNEUROSCI.4651-07.2008

LoTurco, J. J., Owens, D. F., Heath, M. J., Davis, M. B., and Kriegstein, A. R. (1995). GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. *Neuron* 15, 1287–1298. doi: 10.1016/0896-6273(95)90008-X

Lu, M., Grove, E. A., and Miller, R. J. (2002). Abnormal development of the hippocampal dentate gyrus in mice lacking the CXCR4 chemokine receptor. *Proc. Natl. Acad. Sci. U.S.A.* 99, 7090–7095. doi: 10.1073/pnas.092013799

Luo, Y., Lathia, J., Mughal, M., and Mattson, M. P. (2008). SDF1alpha/CXCR4 signaling, via ERKs and the transcription factor Egr1, induces expression of a 67-kDa form of glutamic acid decarboxylase in embryonic hippocampal neurons. *J. Biol. Chem.* 283, 24789–24800. doi: 10.1074/jbc.M800649200

Luster, A. D. (1998). Chemokines – chemotactic cytokines that mediate inflammation. *N. Engl. J. Med.* 338, 436–445. doi: 10.1056/NEJM199802123380706

Luther, S. A., and Cyster, J. G. (2001). Chemokines as regulators of T cell differentiation. *Nat. Immunol.* 2, 102–107. doi: 10.1038/84205

Lysko, D. E., Putt, M., and Golden, J. A. (2011). SDF1 regulates leading process branching and speed of migrating interneurons. *J. Neurosci.* 31, 1739–1745. doi: 10.1523/JNEUROSCI.3118-10.2011

Ma, Q., Jones, D., Borghesani, P. R., Segal, R. A., Nagasawa, T., Kishimoto, T., et al. (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9448–9453. doi: 10.1073/pnas.95.16.9448

Maes, M. (1999). Major depression and activation of the inflammatory response system. *Adv. Exp. Med. Biol.* 461, 25–46. doi: 10.1007/978-0-585-37970-8_2

McGeer, P. L., and McGeer, E. G. (2004). Inflammation and the degenerative diseases of aging. *Ann. N. Y. Acad. Sci.* 1035, 104–116. doi: 10.1196/annals.1332.007

Mellado, M., Rodriguez-Frade, J. M., Vila-Coro, A. J., Fernandez, S., Martin de Ana, A., Jones, D. R., et al. (2001). Chemokine receptor homo- or heterodimerization activates distinct signaling pathways. *EMBO J.* 20, 2497–2507. doi: 10.1093/emboj/20.10.2497

Memi, F., Abe, P., Cariboni, A., MacKay, F., Parnavelas, J. G., and Stumm, R. (2013). CXC chemokine receptor 7 (CXCR7) affects the migration of GnRH neurons by regulating CXCL12 availability. *J. Neurosci.* 33, 17527–17537. doi: 10.1523/JNEUROSCI.0857-13.2013

Meucci, O., Fatatis, A., Simen, A. A., Bushell, T. J., Gray, P. W., and Miller, R. J. (1998). Chemokines regulate hippocampal neuronal signaling and gp120 neurotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* 95, 14500–14505. doi: 10.1073/pnas.95.24.14500

Murphy, P. M., Baggiolini, M., Charo, I. F., Hebert, C. A., Horuk, K., Matsushima, R., et al. (2000). International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol. Rev.* 52, 145–176.

Nahon, J. L. (2006). The melanocortins and melanin-concentrating hormone in the central regulation of feeding behavior and energy homeostasis. *C. R. Biol.* 329, 623–638; discussion 653–655. doi: 10.1016/j.crvi.2006.03.021

Nicolai, J., Burbassi, S., Rubin, J., and Meucci, O. (2010). CXCL12 inhibits expression of the NMDA receptor's NR2B subunit through a histone deacetylase-dependent pathway contributing to neuronal survival. *Cell Death Dis.* 1, e33. doi: 10.1038/cddis.2010.10

Ohtani, Y., Minami, M., Kawaguchi, N., Nishiyori, A., Yamamoto, J., Takami, S., et al. (1998). Expression of stromal cell-derived factor-1 and CXCR4 chemokine receptor mRNAs in cultured rat glial and neuronal cells. *Neurosci. Lett.* 249, 163–166. doi: 10.1016/S0304-3940(98)00425-X

Pello, O. M., Martinez-Munoz, L., Parrillas, V., Serrano, A., Rodriguez-Frade, J. M., Toro, M. J., et al. (2008). Ligand stabilization of CXCR4/delta-opioid receptor heterodimers reveals a mechanism for immune response regulation. *Eur. J. Immunol.* 38, 537–549. doi: 10.1002/eji.200737630

Percherancier, Y., Berchiche, Y. A., Slight, I., Volkmer-Engert, R., Tamamura, H., Fujii, N., et al. (2005). Bioluminescence resonance energy transfer reveals ligand-induced conformational changes in CXCR4 homo- and heterodimers. *J. Biol. Chem.* 280, 9895–9903. doi: 10.1074/jbc.M411151200

Pillarisetti, K., and Gupta, S. K. (2001). Cloning and relative expression analysis of rat stromal cell derived factor-1 (SDF-1)1: SDF-1 alpha mRNA is selectively induced in rat model of myocardial infarction. *Inflammation* 25, 293–300. doi: 10.1023/A:1012808525370

Proudfoot, A. E. (2002). Chemokine receptors: multifaceted therapeutic targets. *Nat. Rev. Immunol.* 2, 106–115. doi: 10.1038/nri722

Pujol, F., Kitabgi, P., and Boudin, H. (2005). The chemokine SDF-1 differentially regulates axonal elongation and branching in hippocampal neurons. *J. Cell Sci.* 118, 1071–1080. doi: 10.1242/jcs.01694

Qu, Y., Mao, M., Li, X., Zhang, L., Huang, X., Yang, C., et al. (2008). Enhanced migration and CXCR4 over-expression in fibroblasts with telomerase reconstitution. *Mol. Cell. Biochem.* 313, 45–52. doi: 10.1007/s11010-008-9740-6

Ragozzino, D. (2002). CXC chemokine receptors in the central nervous system: role in cerebellar neuromodulation and development. *J. Neurovirol.* 8, 559–572. doi: 10.1080/13550280290100932

Rajagopal, S., Kim, J., Ahn, S., Craig, S., Lam, C. M., Gerard, N. P., et al. (2010). Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proc. Natl. Acad. Sci. U.S.A.* 107, 628–632. doi: 10.1073/pnas.0912852107

Ransohoff, R. M., and Tani, M. (1998). Do chemokines mediate leukocyte recruitment in post-traumatic CNS inflammation? *Trends Neurosci.* 21, 154–159. doi: 10.1016/S0166-2236(97)01198-3

Reichenberg, A., Yirmiya, R., Schuld, A., Kraus, T., Haack, M., Morag, A., et al. (2001). Cytokine-associated emotional and cognitive disturbances in humans. *Arch. Gen. Psychiatry* 58, 445–452. doi: 10.1001/archpsyc.58.5.445

Rheims, S., Minlebaev, M., Ivanov, A., Represa, A., Khazipov, R., Holmes, G. L., et al. (2008). Excitatory GABA in rodent developing neocortex in vitro. *J. Neurophysiol.* 100, 609–619. doi: 10.1152/jn.90402.2008

Rodriguez-Frade, J. M., Mellado, M., and Martinez, A. C. (2001). Chemokine receptor dimerization: two are better than one. *Trends Immunol.* 22, 612–617. doi: 10.1016/S1471-4906(01)02036-1

Rossi, D., and Zlotnik, A. (2000). The biology of chemokines and their receptors. *Annu. Rev. Immunol.* 18, 217–242. doi: 10.1146/annurev.immunol.18.1.217

Rostene, W., Kitabgi, P., and Parsadaniantz, S. M. (2007). Chemokines: a new class of neuromodulator? *Nat. Rev. Neurosci.* 8, 895–903. doi: 10.1038/nrn2255

Sohy, D., Yano, H., de Nadai, P., Urizar, E., Guillabert, A., Javitch, J. A., et al. (2009). Hetero-oligomerization of CCR2, CCR5, and CXCR4 and the protean effects of "selective" antagonists. *J. Biol. Chem.* 284, 31270–31279. doi: 10.1074/jbc.M109.054809

Somogyi, R., Wen, X., Ma, W., and Barker, J. L. (1995). Developmental kinetics of GAD family mRNAs parallel neurogenesis in the rat spinal cord. *J. Neurosci.* 15, 2575–2591.

Streit, W. J., Conde, J. R., and Harrison, J. K. (2001). Chemokines and Alzheimer's disease. *Neurobiol. Aging* 22, 909–913. doi: 10.1016/S0197-4580(01)00290-1

Stumm, R. K., Rummel, J., Junker, V., Culmsee, C., Pfeiffer, M., Krieglstein, J., et al. (2002). A dual role for the SDF-1/CXCR4 chemokine receptor system in adult brain: isoform-selective regulation of SDF-1 expression modulates CXCR4-dependent neuronal plasticity and cerebral leukocyte recruitment after focal ischemia. *J. Neurosci.* 22, 5865–5878.

Stumm, R. K., Zhou, C., Ara, T., Lazarini, F., Dubois-Dalcq, M., Nagasawa, T., et al. (2003). CXCR4 regulates interneuron migration in the developing neocortex. *J. Neurosci.* 23, 5123–5130.

Szabo, I., Chen, X. H., Xin, L., Adler, M. W., Howard, O. M., Oppenheim, J. J., et al. (2002). Heterologous desensitization of opioid receptors by chemokines inhibits chemotaxis and enhances the perception of pain. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10276–10281. doi: 10.1073/pnas.102327699

Tashiro, K., Tada, H., Heilker, R., Shirozu, M., Nakano, T., and Honjo, T. (1993). Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. *Science* 261, 600–603. doi: 10.1126/science.8342023

Thelen, M., and Thelen, S. (2008). CXCR7, CXCR4 and CXCL12: an eccentric trio? *J. Neuroimmunol.* 198, 9–13. doi: 10.1016/j.jneuroim.2008.04.020

Toth, P. T., Ren, D., and Miller, R. J. (2004). Regulation of CXCR4 receptor dimerization by the chemokine SDF-1alpha and the HIV-1 coat protein gp120: a fluorescence resonance energy transfer (FRET) study. *J. Pharmacol. Exp. Ther.* 310, 8–17. doi: 10.1124/jpet.103.064956

Vila, M., Jackson-Lewis, V., Guegan, C., Wu, D. C., Teismann, P., Choi, D. K., et al. (2001). The role of glial cells in Parkinson's disease. *Curr. Opin. Neurol.* 14, 483–489. doi: 10.1097/00019052-200108000-00009

Vila-Coro, A. J., Rodriguez-Frade, J. M., Martin De Ana, A., Moreno-Ortiz, M. C., Martinez, A. C., and Mellado, M. (1999). The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. *FASEB J.* 13, 1699–1710.

Xia, M. Q., and Hyman, B. T. (1999). Chemokines/chemokine receptors in the central nervous system and Alzheimer's disease. *J. Neurovirol.* 5, 32–41. doi: 10.3109/13550289909029743

Xiang, Y., Li, Y., Zhang, Z., Cui, K., Wang, S., Yuan, X. B., et al. (2002). Nerve growth cone guidance mediated by G protein-coupled receptors. *Nat. Neurosci.* 5, 843–848. doi: 10.1038/nn899

Yang, S., Edman, L. C., Sanchez-Alcaniz, J. A., Fritz, N., Bonilla, S., Hecht, J., et al. (2013). Cxcl12/Cxcr4 signaling controls the migration and process orientation of A9–A10 dopaminergic neurons. *Development* 140, 4554–4564. doi: 10.1242/dev.098145

Zabel, B. A., Wang, Y., Lewen, S., Berahovich, R. D., Penfold, M. E., Zhang, P., et al. (2009). Elucidation of CXCR7-mediated signaling events and inhibition of CXCR4-mediated tumor cell transendothelial migration by CXCR7 ligands. *J. Immunol.* 183, 3204–3211. doi: 10.4049/jimmunol.0900269

Zheng, J., Thylin, M. R., Ghorpade, A., Xiong, H., Persidsky, Y., Cotter, R., et al. (1999). Intracellular CXCR4 signaling, neuronal apoptosis and neuropathogenic mechanisms of HIV-1-associated dementia. *J. Neuroimmunol.* 98, 185–200. doi: 10.1016/S0165-5728(99)00049-1

Zlatopolskiy, A., and Laurence, J. (2001). "Reverse gear" cellular movement mediated by chemokines. *Immunol. Cell Biol.* 79, 340–344. doi: 10.1046/j.1440-1711.2001.01015.x

Zou, Y. R., Kottmann, A. H., Kuroda, M., Taniuchi, I., and Littman, D. R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 393, 595–599. doi: 10.1038/31269

**Conflict of Interest Statement:** The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 18 December 2013; accepted: 13 February 2014; published online: 06 March 2014.*

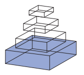
*Citation: Guyon A (2014) CXCL12 chemokine and its receptors as major players in the interactions between immune and nervous systems. Front. Cell. Neurosci.* ***8****:65. doi: 10.3389/fncel.2014.00065*

*This article was submitted to the journal Frontiers in Cellular Neuroscience.*

*Copyright © 2014 Guyon. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*

frontiers in
CELLULAR NEUROSCIENCE

MINI REVIEW ARTICLE
published: 22 December 2014
doi: 10.3389/fncel.2014.00430



# Brain-derived neurotrophic factor: a bridge between inflammation and neuroplasticity

**Francesca Calabrese[1], Andrea C. Rossetti[1], Giorgio Racagni[1], Peter Gass[2], Marco A. Riva[1] and Raffaella Molteni[1]***

[1] Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milan, Italy
[2] Department of Psychiatry and Psychotherapy, Research Group Animal Models in Psychiatry, Central Institute of Mental Health, Medical Faculty Mannheim/Heidelberg University, Mannheim, Germany

***Edited by:***
*Silvia Alboni, University of Modena and Reggio Emilia, Italy*

***Reviewed by:***
*Sasidhar Reddy Murikinati, Yale University school of medicine, USA*
*Shawn Hayley, Carleton University, Canada*
*Zheyu Chen, Shandong University, China*

****Correspondence:***
*Raffaella Molteni, Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Via Balzaretti 9, 20133 Milan, Italy*
*e-mail: raffaella.molteni@unimi.it*

Cytokines are key regulatory mediators involved in the host response to immunological challenges, but also play a critical role in the communication between the immune and the central nervous system. For this, their expression in both systems is under a tight regulatory control. However, pathological conditions may lead to an overproduction of pro-inflammatory cytokines that may have a detrimental impact on central nervous system. In particular, they may damage neuronal structure and function leading to deficits of neuroplasticity, the ability of nervous system to perceive, respond and adapt to external or internal stimuli. In search of the mechanisms by which pro-inflammatory cytokines may affect this crucial brain capability, we will discuss one of the most interesting hypotheses: the involvement of the neurotrophin brain-derived neurotrophic factor (BDNF), which represents one of the major mediators of neuroplasticity.

**Keywords: BDNF, neurogenesis, lipopolysaccaride, pro-inflammatory cytokines, depression**

## NEUROPLASTICITY AND BRAIN-DERIVED NEUROTROPHIC FACTOR

For many years the medical field held the belief that the brain did not make major changes after a certain point in time. It was fixed or set on a specific path. Today, in contrast, we know that the brain is actually capable of changing and developing throughout a lifetime. It is plastic or malleable, and the term *neuroplasticity* is used to describe this tendency for the brain to keep developing, changing, and potentially healing itself.

Specifically, neuroplasticity or neuronal plasticity refers to the ability of the nervous system to respond and adapt to environmental challenges and encompasses a series of functional and structural mechanisms that may lead to neuronal remodeling, formation of novel synapses and birth of new neurons. Neuronal plasticity is intimately linked to cellular responsiveness and may therefore be considered an index of the neuronal capability to adapt its function to a different demand. Failure of such mechanisms might enhance the susceptibility to environmental challenges, such as stress, and ultimately lead to psychopathology.

Among the genes responsive to neuronal activity, neurotrophic factors (NTFs), and in particular the neurotrophin family, play an important role. In fact, besides their classical role in supporting neuronal survival, NTFs finely modulate all the crucial steps of network construction, from neuronal migration to experience-dependent refinement of local connections (Poo, 2001). These functions were first reported based on the observation that, during the development of the nervous system, neuron survival depends on the limited amount of specific NTFs secreted by target cells (Huang and Reichardt, 2001). However, it is now well established that NTFs are important mediators of neuronal plasticity also in adulthood where they modulate axonal and dendritic growth and remodeling, membrane receptor trafficking, neurotransmitter release, synapse formation and function (Lu et al., 2005). The neurotrophin brain-derived neurotrophic factor (BDNF) has emerged as crucial mediator of neuronal plasticity, since it is abundant in brain regions particularly relevant for plasticity, but also because it shows a remarkable activity-dependent regulation of expression and secretion (Bramham and Messaoudi, 2005), suggesting that it might indeed bridge experience with enduring change in neuronal function. BDNF has a complex genomic structure, which results into a sophisticated organization in terms of transcriptional, translational and post-translational regulatory mechanisms (Aid et al., 2007). In particular, the rat BDNF gene—that is similar to the human gene—can generate nine distinct transcripts through the alternative splicing of 5' un-translated exons to a common 3' exon (IX), which encodes the BDNF protein (Aid et al., 2007). These transcripts have different distribution and/or translation efficacy and, more importantly, may sub-serve different functions. For example, transcripts that are primarily localized or targeted to dendrites may sustain local neurotrophin production, thus providing an effective mechanism to regulate synaptic structure and function (An et al., 2008; Wu et al., 2011). Since the transcription of the different isoforms is regulated by specific signaling pathways (Pruunsild et al., 2011), their investigation may provide useful information on the up-stream mechanisms contributing to the changes of BDNF gene expression.

The mechanisms that lie downstream from NTFs and contribute to the maintenance of neuroplasticity are different i.e., adult neurogenesis and neuronal remodeling, but on the purpose of this mini-review we will focus only on adult neurogenesis, the process by which neurons are generated. Neurogenesis occurs under precise spatial and temporal control, but it can be modulated by both internal and external stimuli. Among these, several sources of data indicate the positive impact of BDNF on adult neurogenesis (Lee et al., 2002; Sairanen et al., 2005; Scharfman et al., 2005; Gass and Riva, 2007; Bergami et al., 2008; Chan et al., 2008; Li et al., 2008; Waterhouse et al., 2012), however in this review we will focus our attention on the effects of pro-inflammatory cytokines.

## NEUROGENESIS AND INFLAMMATORY STATE

Neurogenesis has been defined as the process in which newborn neurons are generated from progenitors to functionally integrate in the neuronal network (Ming and Song, 2005; Balu and Lucki, 2009; Aimone et al., 2014). Actually, active neurogenesis take place, in the healthy central nervous system, only in two specific regions: neurons are continuously generated in the sub-ventricular zone (SVZ) and migrate into the olfactory bulb to become interneurons and, in parallel, neurogenesis occurs also in the sub-granular zone (SGZ) of the dentate gyrus of the hippocampus, where new granule neurons are continually generated. Depending on different stimuli, neural stem cells, located in so-called stem cell niches, could divide symmetrically, leading to the generation of two identical cells to maintain the pool of undifferentiated progenitors or, on the other hand, they can divide asymmetrically in order to generate an identical daughter cell and a second cell that starts to differentiate. The *de novo* formation and integration of new neurons into the existing circuitry is one of the various plastic changes that allow the adult brain to adapt to exogenous stimuli (Amrein et al., 2011). In particular, adult neurogenesis within the hippocampus could contribute to enhanced neural plasticity, a process that is fundamental for specific brain functions such as spatial learning, pattern discrimination, contextual memory and mood regulation (Clelland et al., 2009; Sahay et al., 2011; Denny et al., 2014). The important role of hippocampal neurogenesis is underlined by the fact that this system is altered after various types of negative stimuli such as stress, one of the major risk factors for psychiatric diseases. Specifically, repeated restraint and inescapable foot shock, two examples of physical stressors, inhibit one or more steps of adult neurogenesis in the dentate gyrus (Malberg and Duman, 2003; Pham et al., 2003); the social defeat paradigm leads to an inhibitory effect on cell proliferation and survival of newborn granule neurons in rodents (Czéh et al., 2002; Jun et al., 2012); and social isolation, which is associated with decreased neurogenesis and behavioral alterations in rodents, has been recently proven to be deleterious also for hippocampal neurogenesis and behavior in non human primates (Cinini et al., 2014).

As previously mentioned, neurogenesis is conditioned by a very complex microenvironment constituted by the vascular net, different growth and NTFs, changes in electrical and chemical environment and support by glial cells (Kohman and Rhodes, 2013). In this scenario, neuroinflammation is emerging as one of the main actors. In fact the immune system, through cells within the brain (e.g., microglia) and the detrimental or the beneficial action of signaling molecules (pro-inflammatory or anti-inflammatory cytokines) could participate in the response to different exogenous and endogenous stimuli. The negative effects of neuroinflammation on neurogenesis could lead to impaired survival and proliferation of new neurons. For example, intracortical or intraperitoneal administration of lipopolysaccaride (LPS) from *E. coli*, an agent able to induce a strong immune response, decreases new neurons survival and the differentiation of new cells into neurons (Ekdahl et al., 2003; Monje et al., 2003). The consequences of inflammation on neurogenesis could have also functional implications for cognition. In fact, the impact of neuroinflammation could affect also the correct integration of newborn neurons into pre-existing circuits, through changes in cellular morphology and in electrophysiological properties (Jakubs et al., 2008) and reduction in recruitment into hippocampal networks encoding spatial information (Belarbi et al., 2012).

## THE IMPACT OF PRO-INFLAMMATORY CYTOKINES ON NEUROGENESIS

Neuroinflammation has an important role in the pathophysiology of different acute or chronic CNS disorders such as cerebral ischemia, multiple sclerosis, Alzheimer's disease, Parkinson's disease and major depression (Wang and Jin, 2014). These diseases are characterized by the modulation of different mediators of inflammation and among them pro-inflammatory cytokines seem to play a key role. It is important to note that the same cytokines that in a physiological state are involved in the maintenance of neuronal integrity, may instead have detrimental effects under pathological conditions. Accordingly, the impact of the pro-inflammatory cytokines on neurogenesis depends on their concentration, on the specific cells activated (astrocytes and microglia) and on the presence of other factors secreted in the neurogenic niche (Eyre and Baune, 2012). The increase of pro-inflammatory cytokines is not only due to a direct inflammatory stimulus (infection or trauma), but it could be caused by environmental stimuli such as stress (García-Bueno et al., 2008). The main consequence of a dysregulation of cytokine levels within the brain is the production of inflammatory, oxidative and nitrosative molecules that could affect neurogenesis and the neural homeostasis (Kubera et al., 2011; Stepanichev et al., 2014).

The most common pro-inflammatory cytokines are IL-1β, IL-6, TNF-α and IFN-γ and here we will present some examples of the involvement of these molecules in the modulation of neurogenesis.

The main actions of IL-1β are the stimulation of immune cells to produce pro-inflammatory cytokines, the activation of microglia, and the regulation of growth factors activity (Audet and Anisman, 2013). Recently, IL-1β has been proven to influence hippocampal cytogenesis and neurogenesis in different ways: by direct interaction to its receptor (IL-1R1) and the consequent activation of the nuclear factor-kappa B (NFkB; Koo and Duman, 2008) or through the promotion of glucocorticoids secretion

after the exposure to environmental stressors (Goshen et al., 2008). Moreover this cytokine has been proposed as the central mediator of antineurogenic effect of stress (Ben Menachem-Zidon et al., 2008). In fact the blockade of IL-1β signaling, using knockout mice for its receptor or administrating a IL-1R1 antagonist (IL-1Ra), prevents the decrease in neurogenesis observed after acute stressors such as footshock and immobilization in rats (Koo and Duman, 2008). Another relevant cytokine is IL-6 that is involved in a multitude of neuroprotective functions. In physiological conditions IL-6 is able to activate pathways related to neural plasticity, neurogenesis, Long Term Potentiation, and memory (Eyre and Baune, 2012). On the other hand, this cytokine is also responsible of mediating synthesis of acute phase proteins, growth and differentiation of immune cells and regulation of pro-inflammatory factors (Audet and Anisman, 2013). Monje et al. demonstrated that the incubation of hippocampal progenitor cells with recombinant IL-6 decreases neurogenesis by half and reduces neuronal differentiation in favor of astrocytogenesis (Monje et al., 2003; Taga and Fukuda, 2005), an effect mediated by the activation of the JAK/STAT3 pathway via gp130 (Namihira and Nakashima, 2013). Tumor necrosis factor—alpha (TNF-α) is a potent inductor of inflammation and has been linked to decreased neural stem cell proliferation, decreased neurogenesis, neurodegenerative processes, apoptosis and excitotoxicity (Dantzer et al., 2008; Belarbi et al., 2012), but also to the modulation of synaptic strength and synaptic preservation through the increase of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Khairova et al., 2009). The negative action of TNF-α on neurogenesis is mediated by the activation of its receptor TNF-R1, conversely the interaction with TNF-R2 increases proliferation and survival of newborn neurons, as demonstrated by using transgenic animals with deletion of TNF-R1 or TNF-R2 (Iosif et al., 2006). A similar result on the beneficial role of TNF-R2 activation after irradiation injury has been recently reported (Chen and Palmer, 2013). Moreover, the up-regulation of TNF-α observed in the hippocampus of adult rats pre-exposed to maternal deprivation has been associated with impaired memory consolidation (Pinheiro et al., 2014). IFN-γ is a pro-inflammatory cytokine with *in vitro* anti-neurogenic effect able to reduce the number of neural stem cells. The negative action of IFN-γ on neurogenesis may be exerted by the activation of the caspase 3/7, the upregulation of sonic hedgehog (SHH) pathway and promotion of an abnormal marker profile of neural stem cells, expressing both GFAP and βIII tubulin (Walter et al., 2011). Nevertheless, IFN-γ may also exerts positive action on neurogenesis. For example, it enhances neurogenesis in dentate gyrus of adult mice and ameliorates spatial learning and memory performance (Baron et al., 2008). These observations suggest that IFN-γ has different effects depending on tissues involved and on the neurogenic process involved.

Taken together, all these studies indicate that a dysregulation of pro-inflammatory cytokines may have a detrimental effect on neurogenesis and point out the importance of neuroinflammation in the microenvironment around neural stem cell development. On this context, the identification and characterization of the mechanisms by which pro-inflammatory cytokines affect neurogenesis are crucial to develop new strategies to maintain the proper function of stem cell niches within the brain.

## THE IMPACT OF PRO-INFLAMMATORY CYTOKINES ON BDNF

Given the role of BDNF as an important mediator of neuroplasticity and on the basis of its positive contribution on neurogenesis in contrast to the detrimental effect of pro-inflammatory cytokines, we may hypothesize that one of the mechanisms by which inflammation may affect brain function could involve BDNF modulation.

Several *in vivo* studies demonstrated that inflammation clearly affects the expression of BDNF within the brain. In particular, it has been reported that the administration of pro-inflammatory cytokines or of the cytokine-inducer lipopolysaccharide, (LPS; Raetz and Whitfield, 2002) causes a significant reduction of BDNF gene expression. For example, the mRNA levels of BDNF were significantly decreased in the rat hippocampus 4 h after intraperitoneal injection of IL-1β or LPS (Lapchak et al., 1993) and a similar reduction was also observed in several cortical regions and at protein level (Guan and Fang, 2006; Schnydrig et al., 2007). Interestingly, the effect of the systemic inflammatory challenge was not restricted to BDNF: other neurotrophins such as nerve growth factor (NGF) and neurotrophin-3 (NT-3) were similarly reduced although with different magnitude (Guan and Fang, 2006).

Recently, it has also been evaluated the effect of peripheral immune challenge on the different BDNF transcripts, finding that the expression of exons I, II, and IV in the dentate gyrus was reduced in the CA1 and in the dentate gyrus of rats acutely treated with *E. coli* (Chapman et al., 2012), indicating that inflammation may affect specific isoforms of the neurotrophin. Nevertheless, there is a critical lack of information about the effects of inflammation on the expression of specific BDNF transcripts and further studies are demanded in order to clarify the mechanisms involved in the modulation of the neurotrophin by the immune/inflammatory system.

The negative impact of inflammation on BDNF has important implications for a number of pathological conditions. For example, it is known that pro-inflammatory cytokines compromise hippocampus-dependent memory (Pugh et al., 1998), spatial memory (Arai et al., 2001) and increase apoptosis in the brain (Nolan et al., 2003), features that are involved in many aging-associated pathologies and neurodegenerative diseases. In addition, it is well-know that the activation of the immune/inflammatory system may contribute to the development of different psychiatric diseases such as schizophrenia and major depression (Dantzer et al., 2008; Miller et al., 2009; Leonard and Maes, 2012; Zunszain et al., 2013). Regarding depression, there are three main supportive evidences: first, depressed subjects exhibit increased levels of inflammatory markers both in the periphery and in brain (Howren et al., 2009; Dowlati et al., 2010); second: several pathologies associated with moderate inflammatory grade present high depression comorbidity (Benton et al., 2007); third: a high percentage of patients with cancer or hepatitis C treated with interferon-alpha develop major depression (Valentine and Meyers, 2005; Udina et al., 2012). In addition, it has to be noted that animals exposed to immune challenges

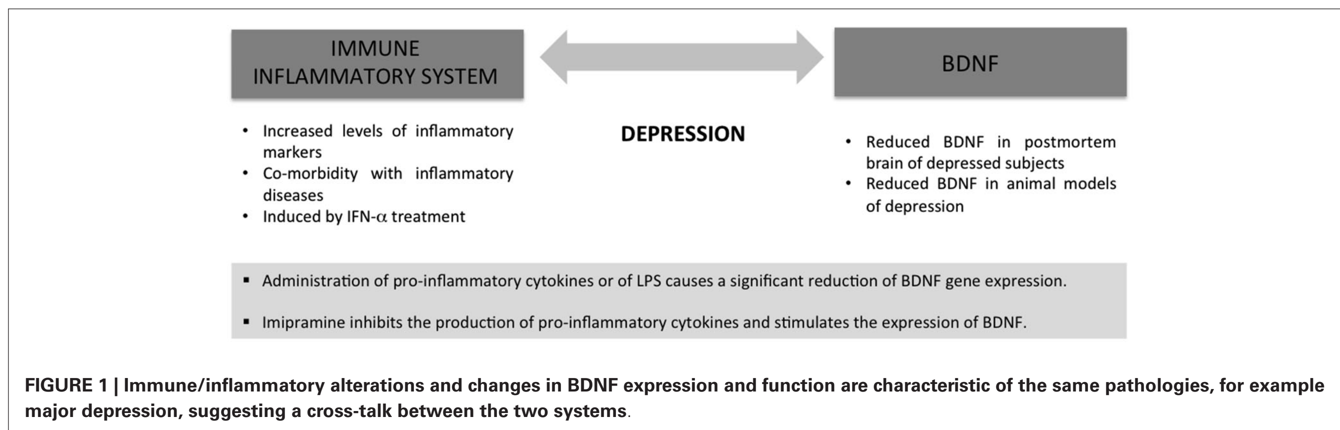



**FIGURE 1 | Immune/inflammatory alterations and changes in BDNF expression and function are characteristic of the same pathologies, for example major depression, suggesting a cross-talk between the two systems.**

display depressive-like behaviors (Yirmiya, 1996; Frenois et al., 2007) that can be normalized, or at least limited, by antidepressant treatment (Yirmiya et al., 2001). In contrast, mice that lack IL-6 are stress resistant and have a reduced disposition for depressive-like behaviors (Chourbaji et al., 2006). The mechanism underlying the anti-inflammatory properties of antidepressant is still unknown and is beyond the aim of our mini-review. However, the results of several *in vitro* and *in vivo* studies indicate that these drugs are able to modulate cytokine functioning through their effects on intracellular cyclic adenosyl monophosphate, serotonin metabolism, the hypothalamo-pituitary-adrenocortical axis (Janssen et al., 2010; Walker, 2013; Leonard, 2014).

It is important to consider that the immune/inflammatory alterations previously described are actually in parallel with changes on BDNF expression and function (**Figure 1**). Indeed, BDNF has a well recognized role in the etiology as well as in the treatment response of patients affected by different psychiatric disorders including major depression (Pezet and Malcangio, 2004; Duman and Monteggia, 2006). For example, decreased expression of the neurotrophin has been found in the hippocampus and prefrontal cortex of postmortem brains from depressed and suicide victims (Dwivedi et al., 2003). Moreover, BDNF mRNA levels are reduced in the brain of genetic animal models of depression (Ridder et al., 2005; Calabrese et al., 2010; Molteni et al., 2010a,c) as well as in animal models based on the environmental component of the disease (Duman and Monteggia, 2006; Tsankova et al., 2006; Chourbaji et al., 2012).

All these findings support the possibility that inflammation contributes to the development of depression by compromising neuroplasticity via reduction of BDNF. In agreement with this line of thinking, it has been recently reported that intranigral LPS infusion induced an anxious and depressive phenotype in the rat that was associated with decreased hippocampal expression of BDNF (Hritcu and Gorgan, 2014).

In order to have a unequivocal proof for causality, inflammation-dependent decrease of BDNF should be normalized or at least attenuated by antidepressant treatment, as occurs in experimental models where BDNF expression is up-regulated in response to prolonged treatment with different antidepressant drugs (Schmidt and Duman, 2007; Calabrese et al., 2010; Molteni et al., 2010b; Park et al., 2011).

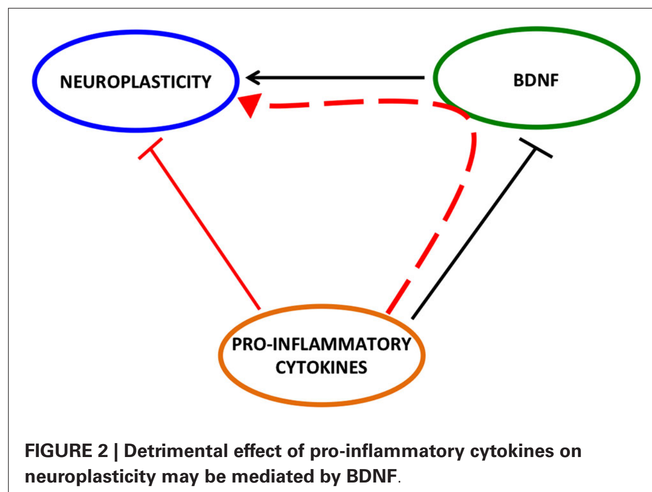



**FIGURE 2 | Detrimental effect of pro-inflammatory cytokines on neuroplasticity may be mediated by BDNF.**

Although there are only few data on this issue, it has been demonstrated that the incubation of rat neural stem cells with the antidepressant imipramine inhibits the production of pro-inflammatory cytokines, whereas stimulates the expression of BDNF (Peng et al., 2008), nevertheless, further studies are demanded to clarify this issue in order to provide unequivocal proof for causality.

## CONCLUDING REMARKS

In conclusion, we attempted to provide evidence on the possibility that one of the mechanisms underlying the negative impact of pro-inflammatory cytokines on neuroplasticity is the reduction of BDNF expression and function (**Figure 2**).

Although several data support this hypothesis, further studies are demanded to better clarify how it occurs. A number of result points out a key role for the pro-inflammatory cytokine IL-1β as it has been shown that the inhibitory effect of stress paradigms on cerebral BDNF expression may be attenuated by intracerebroventricular injection of IL-1 receptor antagonist (Barrientos et al., 2003). However, how this -or others- pro-inflammatory cytokine affects the neurotrophin is still not well understood. Since *in vitro* and *in vivo* studies indicate that glucocorticoids decrease the neurotrophin (Hansson et al., 2003; Gubba et al., 2004;

Hansson and Fuxe, 2008), one possibility is the involvement of the Hypotalamus-Pituitary-Axis (HPA), which is strongly stimulated by pro-inflammatory cytokines (Rivest, 2010). However, we have to be aware that pro-inflammatory cytokines act on a plethora of different targets, for example the neurotransmitters glutamate (Viviani et al., 2007; Di Filippo et al., 2013) and GABA (Galic et al., 2012), both able to modulate BDNF. In this context, it is feasible that the effect of the immune/inflammatory system on BDNF results from the integration of multiple mechanisms. A better knowledge of these events may be useful to develop new therapeutic strategies aimed to normalize, or at least ameliorate, the pathological consequences of the negative impact of inflammation on brain structure and function.

## REFERENCES


Aid, T., Kazantseva, A., Piirsoo, M., Palm, K., and Timmusk, T. (2007). Mouse and rat BDNF gene structure and expression revisited. *J. Neurosci. Res.* 85, 525–535. doi: 10.1002/jnr.21139

Aimone, J. B., Li, Y., Lee, S. W., Clemenson, G. D., Deng, W., and Gage, F. H. (2014). Regulation and function of adult neurogenesis: from genes to cognition. *Physiol. Rev.* 94, 991–1026. doi: 10.1152/physrev.00004.2014

Amrein, I., Isler, K., and Lipp, H. P. (2011). Comparing adult hippocampal neurogenesis in mammalian species and orders: influence of chronological age and life history stage. *Eur. J. Neurosci.* 34, 978–987. doi: 10.1111/j.1460-9568.2011.07804.x

An, J. J., Gharami, K., Liao, G. Y., Woo, N. H., Lau, A. G., Vanevski, F., et al. (2008). Distinct role of long 3′ UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. *Cell* 134, 175–187. doi: 10.1016/j.cell.2008.05.045

Arai, K., Matsuki, N., Ikegaya, Y., and Nishiyama, N. (2001). Deterioration of spatial learning performances in lipopolysaccharide-treated mice. *Jpn. J. Pharmacol.* 87, 195–201. doi: 10.1254/jjp.87.195

Audet, M. C., and Anisman, H. (2013). Interplay between pro-inflammatory cytokines and growth factors in depressive illnesses. *Front. Cell. Neurosci.* 7:68. doi: 10.3389/fncel.2013.00068

Balu, D. T., and Lucki, I. (2009). Adult hippocampal neurogenesis: regulation, functional implications and contribution to disease pathology. *Neurosci. Biobehav. Rev.* 33, 232–252. doi: 10.1016/j.neubiorev.2008.08.007

Baron, R., Nemirovsky, A., Harpaz, I., Cohen, H., Owens, T., and Monsonego, A. (2008). IFN-gamma enhances neurogenesis in wild-type mice and in a mouse model of Alzheimer's disease. *FASEB J.* 22, 2843–2852. doi: 10.1096/fj.08-105866

Barrientos, R. M., Sprunger, D. B., Campeau, S., Higgins, E. A., Watkins, L. R., Rudy, J. W., et al. (2003). Brain-derived neurotrophic factor mRNA downregulation produced by social isolation is blocked by intrahippocampal interleukin-1 receptor antagonist. *Neuroscience* 121, 847–853. doi: 10.1016/s0306-4522(03)00564-5

Belarbi, K., Arellano, C., Ferguson, R., Jopson, T., and Rosi, S. (2012). Chronic neuroinflammation impacts the recruitment of adult-born neurons into behaviorally relevant hippocampal networks. *Brain Behav. Immun.* 26, 18–23. doi: 10.1016/j.bbi.2011.07.225

Ben Menachem-Zidon, O., Goshen, I., Kreisel, T., Ben Menahem, Y., Reinhartz, E., Ben Hur, T., et al. (2008). Intrahippocampal transplantation of transgenic neural precursor cells overexpressing interleukin-1 receptor antagonist blocks chronic isolation-induced impairment in memory and neurogenesis. *Neuropsychopharmacology* 33, 2251–2262. doi: 10.1038/sj.npp.1301606

Benton, T., Staab, J., and Evans, D. L. (2007). Medical co-morbidity in depressive disorders. *Ann. Clin. Psychiatry* 19, 289–303. doi: 10.1080/10401230701653542

Bergami, M., Rimondini, R., Santi, S., Blum, R., Götz, M., and Canossa, M. (2008). Deletion of TrkB in adult progenitors alters newborn neuron integration into hippocampal circuits and increases anxiety-like behavior. *Proc. Natl. Acad. Sci. U S A* 105, 15570–15575. doi: 10.1073/pnas.0803702105

Bramham, C. R., and Messaoudi, E. (2005). BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. *Prog. Neurobiol.* 76, 99–125. doi: 10.1016/j.pneurobio.2005.06.003

Calabrese, F., Molteni, R., Cattaneo, A., Macchi, F., Racagni, G., Gennarelli, M., et al. (2010). Long-Term duloxetine treatment normalizes altered brain-derived neurotrophic factor expression in serotonin transporter knockout rats through the modulation of specific neurotrophin isoforms. *Mol. Pharmacol.* 77, 846–853. doi: 10.1124/mol.109.063081

Chan, J. P., Cordeira, J., Calderon, G. A., Iyer, L. K., and Rios, M. (2008). Depletion of central BDNF in mice impedes terminal differentiation of new granule neurons in the adult hippocampus. *Mol. Cell. Neurosci* 39, 372–383. doi: 10.1016/j.mcn.2008.07.017

Chapman, T. R., Barrientos, R. M., Ahrendsen, J. T., Hoover, J. M., Maier, S. F., and Patterson, S. L. (2012). Aging and infection reduce expression of specific brain-derived neurotrophic factor mRNAs in hippocampus. *Neurobiol. Aging* 33, 832.e1–832.e14. doi: 10.1016/j.neurobiolaging.2011.07.015

Chen, Z., and Palmer, T. D. (2013). Differential roles of TNFR1 and TNFR2 signaling in adult hippocampal neurogenesis. *Brain Behav. Immun.* 30, 45–53. doi: 10.1016/j.bbi.2013.01.083

Chourbaji, S., Hörtnagl, H., Molteni, R., Riva, M. A., Gass, P., and Hellweg, R. (2012). The impact of environmental enrichment on sex-specific neurochemical circuitries—effects on brain-derived neurotrophic factor and the serotonergic system. *Neuroscience* 220, 267–276. doi: 10.1016/j.neuroscience.2012.06.016

Chourbaji, S., Urani, A., Inta, I., Sanchis-Segura, C., Brandwein, C., Zink, M., et al. (2006). IL-6 knockout mice exhibit resistance to stress-induced development of depression-like behaviors. *Neurobiol. Dis.* 23, 587–594. doi: 10.1016/j.nbd.2006.05.001

Cinini, S. M., Barnabe, G. F., Galvão-Coelho, N., de Medeiros, M. A., Perez-Mendes, P., Sousa, M. B., et al. (2014). Social isolation disrupts hippocampal neurogenesis in young non-human primates. *Front. Neurosci.* 8:45. doi: 10.3389/fnins.2014.00045

Clelland, C. D., Choi, M., Romberg, C., Clemenson, G. D. Jr., Fragniere, A., Tyers, P., et al. (2009). A functional role for adult hippocampal neurogenesis in spatial pattern separation. *Science* 325, 210–213. doi: 10.1126/science.1173215

Czéh, B., Welt, T., Fischer, A. K., Erhardt, A., Schmitt, W., Müller, M. B., et al. (2002). Chronic psychosocial stress and concomitant repetitive transcranial magnetic stimulation: effects on stress hormone levels and adult hippocampal neurogenesis. *Biol. Psychiatry* 52, 1057–1065. doi: 10.1016/s0006-3223(02)01457-9

Dantzer, R., O'connor, J. C., Freund, G. G., Johnson, R. W., and Kelley, K. W. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat. Rev. Neurosci.* 9, 46–56. doi: 10.1038/nrn2297

Denny, C. A., Kheirbek, M. A., Alba, E. L., Tanaka, K. F., Brachman, R. A., Laughman, K. B., et al. (2014). Hippocampal memory traces are differentially modulated by experience, time, and adult neurogenesis. *Neuron* 83, 189–201. doi: 10.1016/j.neuron.2014.05.018

Di Filippo, M., Chiasserini, D., Gardoni, F., Viviani, B., Tozzi, A., Giampà, C., et al. (2013). Effects of central and peripheral inflammation on hippocampal synaptic plasticity. *Neurobiol. Dis.* 52, 229–236. doi: 10.1016/j.nbd.2012.12.009

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E. K., et al. (2010). A meta-analysis of cytokines in major depression. *Biol. Psychiatry* 67, 446–457. doi: 10.1016/j.biopsych.2009.09.033

Duman, R. S., and Monteggia, L. M. (2006). A neurotrophic model for stress-related mood disorders. *Biol. Psychiatry* 59, 1116–1127. doi: 10.1016/j.biopsych.2006.02.013

Dwivedi, Y., Rizavi, H. S., Conley, R. R., Roberts, R. C., Tamminga, C. A., and Pandey, G. N. (2003). Altered gene expression of brain-derived neurotrophic factor and receptor tyrosine kinase B in postmortem brain of suicide subjects. *Arch. Gen. Psychiatry* 60, 804–815. doi: 10.1001/archpsyc.60.8.804

Ekdahl, C. T., Claasen, J. H., Bonde, S., Kokaia, Z., and Lindvall, O. (2003). Inflammation is detrimental for neurogenesis in adult brain. *Proc. Natl. Acad. Sci. U S A* 100, 13632–13637. doi: 10.1073/pnas.2234031100

Eyre, H., and Baune, B. T. (2012). Neuroplastic changes in depression: a role for the immune system. *Psychoneuroendocrinology* 37, 1397–1416. doi: 10.1016/j.psyneuen.2012.03.019

Frenois, F., Moreau, M., O'connor, J., Lawson, M., Micon, C., Lestage, J., et al. (2007). Lipopolysaccharide induces delayed FosB/DeltaFosB immunostaining within the mouse extended amygdala, hippocampus and hypothalamus, that parallel the expression of depressive-like behavior. *Psychoneuroendocrinology* 32, 516–531. doi: 10.1016/j.psyneuen.2007.03.005

Galic, M. A., Riazi, K., and Pittman, Q. J. (2012). Cytokines and brain excitability. *Front. Neuroendocrinol.* 33, 116–125. doi: 10.1016/j.yfrne.2011.12.002

García-Bueno, B., Caso, J. R., and Leza, J. C. (2008). Stress as a neuroinflammatory condition in brain: damaging and protective mechanisms. *Neurosci. Biobehav. Rev.* 32, 1136–1151. doi: 10.1016/j.neubiorev.2008.04.001

Gass, P., and Riva, M. A. (2007). CREB, neurogenesis and depression. *Bioessays* 29, 957–961. doi: 10.1002/bies.20658

Goshen, I., Kreisel, T., Ben-Menachem-Zidon, O., Licht, T., Weidenfeld, J., Ben-Hur, T., et al. (2008). Brain interleukin-1 mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis suppression. *Mol. Psychiatry* 13, 717–728. doi: 10.1038/sj.mp.4002055

Guan, Z., and Fang, J. (2006). Peripheral immune activation by lipopolysaccharide decreases neurotrophins in the cortex and hippocampus in rats. *Brain Behav. Immun.* 20, 64–71. doi: 10.1016/j.bbi.2005.04.005

Gubba, E. M., Fawcett, J. W., and Herbert, J. (2004). The effects of corticosterone and dehydroepiandrosterone on neurotrophic factor mRNA expression in primary hippocampal and astrocyte cultures. *Brain Res. Mol. Brain Res.* 127, 48–59. doi: 10.1016/j.molbrainres.2004.05.004

Hansson, A. C., and Fuxe, K. (2008). Time-course of immediate early gene expression in hippocampal subregions of adrenalectomized rats after acute corticosterone challenge. *Brain Res.* 1215, 1–10. doi: 10.1016/j.brainres.2008.03.080

Hansson, A. C., Sommer, W., Rimondini, R., Andbjer, B., Strömberg, I., and Fuxe, K. (2003). c-fos reduces corticosterone-mediated effects on neurotrophic factor expression in the rat hippocampal CA1 region. *J. Neurosci.* 23, 6013–6022.

Howren, M. B., Lamkin, D. M., and Suls, J. (2009). Associations of depression with C-reactive protein, IL-1 and IL-6: a meta-analysis. *Psychosom. Med.* 71, 171–186. doi: 10.1097/psy.0b013e3181907c1b

Hritcu, L., and Gorgan, L. D. (2014). Intranigral lipopolysaccharide induced anxiety and depression by altered BDNF mRNA expression in rat hippocampus. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 51, 126–132. doi: 10.1016/j.pnpbp.2014.01.016

Huang, E. J., and Reichardt, L. F. (2001). Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci.* 24, 677–736. doi: 10.1146/annurev.neuro.24.1.677

Iosif, R. E., Ekdahl, C. T., Ahlenius, H., Pronk, C. J., Bonde, S., Kokaia, Z., et al. (2006). Tumor necrosis factor receptor 1 is a negative regulator of progenitor proliferation in adult hippocampal neurogenesis. *J. Neurosci.* 26, 9703–9712. doi: 10.1523/jneurosci.2723-06.2006

Jakubs, K., Bonde, S., Iosif, R. E., Ekdahl, C. T., Kokaia, Z., Kokaia, M., et al. (2008). Inflammation regulates functional integration of neurons born in adult brain. *J. Neurosci.* 28, 12477–12488. doi: 10.1523/jneurosci.3240-08.2008

Janssen, D. G., Caniato, R. N., Verster, J. C., and Baune, B. T. (2010). A psychoneuroimmunological review on cytokines involved in antidepressant treatment response. *Hum. Psychopharmacol.* 25, 201–215. doi: 10.1002/hup.1103

Jun, H., Mohammed Qbsim Hussaini, S., Rigby, M. J., and Jang, M. H. (2012). Functional role of adult hippocampal neurogenesis as a therapeutic strategy for mental disorders. *Neural Plast.* 2012:854285. doi: 10.1155/2012/854285

Khairova, R. A., Machado-Vieira, R., Du, J., and Manji, H. K. (2009). A potential role for pro-inflammatory cytokines in regulating synaptic plasticity in major depressive disorder. *Int. J. Neuropsychopharmacol.* 12, 561–578. doi: 10.1017/s1461145709009924

Kohman, R. A., and Rhodes, J. S. (2013). Neurogenesis, inflammation and behavior. *Brain Behav. Immun.* 27, 22–32. doi: 10.1016/j.bbi.2012.09.003

Koo, J. W., and Duman, R. S. (2008). IL-1beta is an essential mediator of the antineurogenic and anhedonic effects of stress. *Proc. Natl. Acad. Sci. U S A* 105, 751–756. doi: 10.1073/pnas.0708092105

Kubera, M., Obuchowicz, E., Goehler, L., Brzeszcz, J., and Maes, M. (2011). In animal models, psychosocial stress-induced (neuro)inflammation, apoptosis and reduced neurogenesis are associated to the onset of depression. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 35, 744–759. doi: 10.1016/j.pnpbp.2010.08.026

Lapchak, P. A., Araujo, D. M., and Hefti, F. (1993). Systemic interleukin-1 beta decreases brain-derived neurotrophic factor messenger RNA expression in the rat hippocampal formation. *Neuroscience* 53, 297–301. doi: 10.1016/0306-4522(93)90196-m

Lee, J., Duan, W., and Mattson, M. P. (2002). Evidence that brain-derived neurotrophic factor is required for basal neurogenesis and mediates, in part, the enhancement of neurogenesis by dietary restriction in the hippocampus of adult mice. *J. Neurochem.* 82, 1367–1375. doi: 10.1046/j.1471-4159.2002.01085.x

Leonard, B. E. (2014). Impact of inflammation on neurotransmitter changes in major depression: an insight into the action of antidepressants. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 48, 261–267. doi: 10.1016/j.pnpbp.2013.10.018

Leonard, B., and Maes, M. (2012). Mechanistic explanations how cell-mediated immune activation, inflammation and oxidative and nitrosative stress pathways and their sequels and concomitants play a role in the pathophysiology of unipolar depression. *Neurosci. Biobehav. Rev.* 36, 764–785. doi: 10.1016/j.neubiorev.2011.12.005

Li, Y., Luikart, B. W., Birnbaum, S., Chen, J., Kwon, C. H., Kernie, S. G., et al. (2008). TrkB regulates hippocampal neurogenesis and governs sensitivity to antidepressive treatment. *Neuron* 59, 399–412. doi: 10.1016/j.neuron.2008.06.023

Lu, B., Pang, P. T., and Woo, N. H. (2005). The yin and yang of neurotrophin action. *Nat. Rev. Neurosci.* 6, 603–614. doi: 10.1038/nrn1726

Malberg, J. E., and Duman, R. S. (2003). Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. *Neuropsychopharmacology* 28, 1562–1571. doi: 10.1038/sj.npp.1300234

Miller, A. H., Maletic, V., and Raison, C. L. (2009). Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. *Biol. Psychiatry* 65, 732–741. doi: 10.1016/j.biopsych.2008.11.029

Ming, G. L., and Song, H. (2005). Adult neurogenesis in the mammalian central nervous system. *Annu. Rev. Neurosci.* 28, 223–250. doi: 10.1146/annurev.neuro.28.051804.101459

Molteni, R., Calabrese, F., Chourbaji, S., Brandwein, C., Racagni, G., Gass, P., et al. (2010a). Depression-prone mice with reduced glucocorticoid receptor expression display an altered stress-dependent regulation of brain-derived neurotrophic factor and activity-regulated cytoskeleton-associated protein. *J. Psychopharmacol.* 24, 595–603. doi: 10.1177/0269881108099815

Molteni, R., Calabrese, F., Pisoni, S., Gabriel, C., Mocaer, E., Racagni, G., et al. (2010b). Synergistic mechanisms in the modulation of the neurotrophin BDNF in the rat prefrontal cortex following acute agomelatine administration. *World J. Biol. Psychiatry* 11, 148–153. doi: 10.3109/15622970903447659

Molteni, R., Cattaneo, A., Calabrese, F., Macchi, F., Olivier, J. D., Racagni, G., et al. (2010c). Reduced function of the serotonin transporter is associated with decreased expression of BDNF in rodents as well as in humans. *Neurobiol. Dis.* 37, 747–755. doi: 10.1016/j.nbd.2009.12.014

Monje, M. L., Toda, H., and Palmer, T. D. (2003). Inflammatory blockade restores adult hippocampal neurogenesis. *Science* 302, 1760–1765. doi: 10.1126/science.1088417

Namihira, M., and Nakashima, K. (2013). Mechanisms of astrocytogenesis in the mammalian brain. *Curr. Opin. Neurobiol.* 23, 921–927. doi: 10.1016/j.conb.2013.06.002

Nolan, Y., Vereker, E., Lynch, A. M., and Lynch, M. A. (2003). Evidence that lipopolysaccharide-induced cell death is mediated by accumulation of reactive oxygen species and activation of p38 in rat cortex and hippocampus. *Exp. Neurol.* 184, 794–804. doi: 10.1016/s0014-4886(03)00301-7

Park, S. W., Phuong, V. T., Lee, C. H., Lee, J. G., Seo, M. K., Cho, H. Y., et al. (2011). Effects of antipsychotic drugs on BDNF, GSK-3beta and beta-catenin expression in rats subjected to immobilization stress. *Neurosci. Res.* 71, 335–340. doi: 10.1016/j.neures.2011.08.010

Peng, C. H., Chiou, S. H., Chen, S. J., Chou, Y. C., Ku, H. H., Cheng, C. K., et al. (2008). Neuroprotection by Imipramine against lipopolysaccharide-induced apoptosis in hippocampus-derived neural stem cells mediated by activation of BDNF and the MAPK pathway. *Eur. Neuropsychopharmacol.* 18, 128–140. doi: 10.1016/j.euroneuro.2007.05.002

Pezet, S., and Malcangio, M. (2004). Brain-derived neurotrophic factor as a drug target for CNS disorders. *Expert Opin. Ther. Targets* 8, 391–399. doi: 10.1517/14728222.8.5.391

Pham, K., Nacher, J., Hof, P. R., and Mcewen, B. S. (2003). Repeated restraint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus. *Eur. J. Neurosci.* 17, 879–886. doi: 10.1046/j.1460-9568.2003.02513.x

Pinheiro, R. M., de Lima, M. N., Portal, B. C., Busato, S. B., Falavigna, L., Ferreira, R. D., et al. (2014). Long-lasting recognition memory impairment and alterations in brain levels of cytokines and BDNF induced by maternal deprivation: effects of valproic acid and topiramate. *J. Neural Transm.* doi: 10.1007/s00702-014-1303-2. [Epub ahead of print].

Poo, M. M. (2001). Neurotrophins as synaptic modulators. *Nat. Rev. Neurosci.* 2, 24–32. doi: 10.1038/35049004

Pruunsild, P., Sepp, M., Orav, E., Koppel, I., and Timmusk, T. (2011). Identification of cis-elements and transcription factors regulating neuronal activity-dependent transcription of human BDNF gene. *J. Neurosci.* 31, 3295–3308. doi: 10.1523/jneurosci.4540-10.2011

Pugh, C. R., Kumagawa, K., Fleshner, M., Watkins, L. R., Maier, S. F., and Rudy, J. W. (1998). Selective effects of peripheral lipopolysaccharide administration on contextual and auditory-cue fear conditioning. *Brain Behav. Immun.* 12, 212–229. doi: 10.1006/brbi.1998.0524

Raetz, C. R., and Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* 71, 635–700. doi: 10.1146/annurev.biochem.71.110601.135414

Ridder, S., Chourbaji, S., Hellweg, R., Urani, A., Zacher, C., Schmid, W., et al. (2005). Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions. *J. Neurosci.* 25, 6243–6250. doi: 10.1523/jneurosci.0736-05.2005

Rivest, S. (2010). Interactions between the immune and neuroendocrine systems. *Prog. Brain Res.* 181, 43–53. doi: 10.1016/S0079-6123(08)81004-7

Sahay, A., Scobie, K. N., Hill, A. S., O'carroll, C. M., Kheirbek, M. A., Burghardt, N. S., et al. (2011). Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature* 472, 466–470. doi: 10.1038/nature09817

Sairanen, M., Lucas, G., Ernfors, P., Castrén, M., and Castrén, E. (2005). Brain-derived neurotrophic factor and antidepressant drugs have different but coordinated effects on neuronal turnover, proliferation and survival in the adult dentate gyrus. *J. Neurosci.* 25, 1089–1094. doi: 10.1523/jneurosci.3741-04.2005

Scharfman, H., Goodman, J., Macleod, A., Phani, S., Antonelli, C., and Croll, S. (2005). Increased neurogenesis and the ectopic granule cells after intrahippocampal BDNF infusion in adult rats. *Exp. Neurol.* 192, 348–356. doi: 10.1016/j.expneurol.2004.11.016

Schmidt, H. D., and Duman, R. S. (2007). The role of neurotrophic factors in adult hippocampal neurogenesis, antidepressant treatments and animal models of depressive-like behavior. *Behav. Pharmacol.* 18, 391–418. doi: 10.1097/fbp.0b013e3282ee2aa8

Schnydrig, S., Korner, L., Landweer, S., Ernst, B., Walker, G., Otten, U., et al. (2007). Peripheral lipopolysaccharide administration transiently affects expression of brain-derived neurotrophic factor, corticotropin and proopiomelanocortin in mouse brain. *Neurosci. Lett.* 429, 69–73. doi: 10.1016/j.neulet.2007.09.067

Stepanichev, M., Dygalo, N. N., Grigoryan, G., Shishkina, G. T., and Gulyaeva, N. (2014). Rodent models of depression: neurotrophic and neuroinflammatory biomarkers. *Biomed Res. Int.* 2014:932757. doi: 10.1155/2014/932757

Taga, T., and Fukuda, S. (2005). Role of IL-6 in the neural stem cell differentiation. *Clin. Rev. Allergy Immunol.* 28, 249–256. doi: 10.1385/criai:28:3:249

Tsankova, N. M., Berton, O., Renthal, W., Kumar, A., Neve, R. L., and Nestler, E. J. (2006). Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat. Neurosci.* 9, 519–525. doi: 10.1038/nn1659

Udina, M., Castellví, P., Moreno-España, J., Navinés, R., Valdés, M., Forns, X., et al. (2012). Interferon-induced depression in chronic hepatitis C: a systematic review and meta-analysis. *J. Clin. Psychiatry* 73, 1128–1138. doi: 10.4088/jcp.12r07694

Valentine, A. D., and Meyers, C. A. (2005). Neurobehavioral effects of interferon therapy. *Curr. Psychiatry Rep.* 7, 391–395. doi: 10.1007/s11920-005-0042-3

Viviani, B., Gardoni, F., and Marinovich, M. (2007). Cytokines and neuronal ion channels in health and disease. *Int. Rev. Neurobiol.* 82, 247–263. doi: 10.1016/s0074-7742(07)82013-7

Walker, F. R. (2013). A critical review of the mechanism of action for the selective serotonin reuptake inhibitors: do these drugs possess anti-inflammatory properties and how relevant is this in the treatment of depression? *Neuropharmacology* 67, 304–317. doi: 10.1016/j.neuropharm.2012.10.002

Walter, J., Honsek, S. D., Illes, S., Wellen, J. M., Hartung, H. P., Rose, C. R., et al. (2011). A new role for interferon gamma in neural stem/precursor cell dysregulation. *Mol. Neurodegener.* 6:18. doi: 10.1186/1750-1326-6-18

Wang, B., and Jin, K. (2014). Current perspectives on the link between neuroinflammation and neurogenesis. *Metab. Brain Dis.* doi: 10.1007/s11011-014-9523-6. [Epub ahead of print].

Waterhouse, E. G., An, J. J., Orefice, L. L., Baydyuk, M., Liao, G. Y., Zheng, K., et al. (2012). BDNF promotes differentiation and maturation of adult-born neurons through GABAergic transmission. *J. Neurosci.* 32, 14318–14330. doi: 10.1523/jneurosci.0709-12.2012

Wu, Y. C., Williamson, R., Li, Z., Vicario, A., Xu, J., Kasai, M., et al. (2011). Dendritic trafficking of brain-derived neurotrophic factor mRNA: regulation by translin-dependent and -independent mechanisms. *J. Neurochem.* 116, 1112–1121. doi: 10.1111/j.1471-4159.2010.07166.x

Yirmiya, R. (1996). Endotoxin produces a depressive-like episode in rats. *Brain Res.* 711, 163–174. doi: 10.1016/0006-8993(95)01415-2

Yirmiya, R., Pollak, Y., Barak, O., Avitsur, R., Ovadia, H., Bette, M., et al. (2001). Effects of antidepressant drugs on the behavioral and physiological responses to lipopolysaccharide (LPS) in rodents. *Neuropsychopharmacology* 24, 531–544. doi: 10.1016/s0893-133x(00)00226-8

Zunszain, P. A., Hepgul, N., and Pariante, C. M. (2013). Inflammation and depression. *Curr. Top. Behav. Neurosci.* 14, 135–151. doi: 10.1007/7854_2012_211

**Conflict of Interest Statement**: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 30 September 2014; accepted: 29 November 2014; published online: 22 December 2014.*

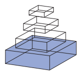
*Citation: Calabrese F, Rossetti AC, Racagni G, Gass P, Riva MA and Molteni R (2014) Brain-derived neurotrophic factor: a bridge between inflammation and neuroplasticity. Front. Cell. Neurosci. **8**:430. doi: 10.3389/fncel.2014.00430*

*This article was submitted to the journal Frontiers in Cellular Neuroscience.*

*Copyright © 2014 Calabrese, Rossetti, Racagni, Gass, Riva and Molteni. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution and reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 31 March 2015
doi: 10.3389/fncel.2015.00040



# Inflammation and neuronal plasticity: a link between childhood trauma and depression pathogenesis

**Annamaria Cattaneo[1,2]*, Flavia Macchi[3], Giona Plazzotta[2], Begni Veronica[3], Luisella Bocchio-Chiavetto[2,4], Marco Andrea Riva[3] and Carmine Maria Pariante[1]**

[1] Stress, Psychiatry and Immunology Laboratory, Department of Psychological Medicine, Institute of Psychiatry, King's College London, London, UK
[2] IRCCS Centro S Giovanni di Dio, Fatebenefratelli, Brescia, Italy
[3] Department of Pharmacological and Biomolecular Sciences, University of Milan, Milan, Italy
[4] Faculty of Psychology, eCampus University, Novedrate (Como), Italy

**Edited by:**
Laura Maggi, Sapienza University, Italy

**Reviewed by:**
Andrew Coogan, Swansea University, UK
Fulvio D'Acquisto, Queen Mary University of London, UK

***Correspondence:**
Annamaria Cattaneo, Stress, Psychiatry and Immunology Laboratory, Department of Psychological Medicine, Institute of Psychiatry, King's College London, Coldharbour Lane, SE5 9NU, London, UK
e-mail: annamaria.cattaneo@kcl.ac.uk

During the past two decades, there has been increasing interest in understanding and characterizing the role of inflammation in major depressive disorder (MDD). Indeed, several are the evidences linking alterations in the inflammatory system to Major Depression, including the presence of elevated levels of pro-inflammatory cytokines, together with other mediators of inflammation. However, it is still not clear whether inflammation represents a cause or whether other factors related to depression result in these immunological effects. Regardless, exposure to early life stressful events, which represent a vulnerability factor for the development of psychiatric disorders, act through the modulation of inflammatory responses, but also of neuroplastic mechanisms over the entire life span. Indeed, early life stressful events can cause, possibly through epigenetic changes that persist over time, up to adulthood. Such alterations may concur to increase the vulnerability to develop psychopathologies. In this review we will discuss the role of inflammation and neuronal plasticity as relevant processes underlying depression development. Moreover, we will discuss the role of epigenetics in inducing alterations in inflammation-immune systems as well as dysfunction in neuronal plasticity, thus contributing to the long-lasting negative effects of stressful life events early in life and the consequent enhanced risk for depression. Finally we will provide an overview on the potential role of inflammatory system to aid diagnosis, predict treatment response, enhance treatment matching, and prevent the onset or relapse of Major Depression.

**Keywords: childhood trauma, inflammation, stress, depression, neuroplasticity**

## BACKGROUND

Major depressive disorder (MDD) is a highly prevalent complex neuropsychiatric condition characterized by a broad range of symptoms, which causes significant distress as well as impairment of normal functioning and that should not be attributable to a recent loss or to a general medical condition (American Psychiatric Association, 2000). Beside the classical monoaminergic hypothesis of depression, at least two major hypotheses have emerged based on dysfunction in immune-inflammatory systems (cytokine hypothesis) or in neuronal plasticity (neurotrophic hypothesis) (Schiepers et al., 2005; Calabrese et al., 2009; Maes et al., 2009; Miller et al., 2009; Castrén, 2014).

The cytokine hypothesis suggests that different environmental stressors as well as organic inflammatory conditions may trigger depression via inflammatory processes (Maes et al., 2009). Indeed, systemic infections, cancer or autoimmune diseases, as well as stressful life events, are characterized by an activation of the peripheral immune system, which is part of the required response of the body to cope with the adverse condition. However, when the activation of the immune system is prolonged, for example because of a persistence of the adverse event, cytokines and other immune modulators can access the brain and affect different brain systems that play a role in enhancing vulnerability to depressive disorders (Dantzer et al., 2008).

The neurotrophic hypothesis has been put forward based on a number of clinical and preclinical evidence suggesting that, beyond neurotransmitter changes, depression may be associated with structural abnormalities in different brain regions as well as defects in cell-cell communication (Frodl and O'Keane, 2013; Zhao et al., 2014). These alterations may be particularly relevant for core disease symptoms implying that therapeutic interventions should correct such defects in order to restore brain function in depressed subjects.

The goal of this review is to recapitulate the alterations in inflammation and neuronal plasticity that may be relevant for depression. Moreover, considering that the etiology of depression has been associated, at least in some individuals, with the exposure to stressful events early in life, we will discuss the possibility that alterations in inflammation-immune systems as

well as dysfunction in neuronal plasticity may contribute to the long-lasting negative effects of stressful life events early in life and the consequent enhanced risk for depression.

## DEPRESSION AND PERIPHERAL INFLAMMATION

There is strong evidence indicating that depression is associated with an activation of the innate immune system (Dantzer et al., 2008). This theory has been supported over the last 20 years by an increasing body of evidence showing alterations in the functional activity of the immune system in the blood and in the brain of depressed patients, as compared to control subjects (Kronfol et al., 1983; Maes, 1995; Maes et al., 1995a,b,c,d,e; Howren et al., 2009; Dowlati et al., 2010; Liu et al., 2012; Valkanova et al., 2013). To date, several studies have investigated blood and/or cerebrospinal fluid concentrations of one or more pro-inflammatory cytokines (e.g., interleukin IL-1β, IL-6, interferon gamma (IFN-γ)) and/or acute phase proteins (e.g., C reactive protein (CRP), an acute phase protein that promotes resistance to infection and repair of damages tissues) in depressed patients.

The majority of these studies, whose main results have been summarized in several meta-analyses (Howren et al., 2009; Dowlati et al., 2010; Liu et al., 2012; Valkanova et al., 2013) reported increased levels of IL-1β, IL-6, TNF-α and CRP in the serum and/or plasma of depressed patients. For example, Hestad et al. (2003) observed that subjects with depressive disorders had markedly increased TNF-α plasma levels compared with healthy controls and, similarly to TNF- α, also IL-6 plasma levels were increased in similar clinical samples (Sluzewska et al., 1996; Pike and Irwin, 2006). Changes of cytokine mRNA levels were also found when investigating peripheral blood cells. Indeed, Tsao and colleagues found higher mRNA levels of TNF-α, IL-1β, IL-6 and INF-α in the Peripheral Blood Mononuclear Cells (PBMCs) of patients suffering from MDD (Tsao et al., 2006), and our group has also shown an increased expression of cytokine mRNA levels in the leukocytes of drug free depressed patients as compared to controls (Cattaneo et al., 2013). Of note, the same cytokines have been significantly correlated with several clinical depressive "traits". In particular higher cytokines levels have been associated with higher depression severity (Thomas et al., 2005) as well as with poor antidepressant response (Cattaneo et al., 2013; Powell et al., 2013; Stelzhammer et al., 2014). Similarly, CRP blood levels that, as mentioned above, are significantly elevated in depressed patients, may also represent a predictor of a poor outcome to antidepressant therapies (Danner et al., 2003; Ford and Erlinger, 2004; Ford et al., 2004; Howren et al., 2009; Pikhart et al., 2009; Uher et al., 2014).

Emerging evidence has proposed a role for cytokines also in child and adolescent depression (Mills et al., 2013), which is estimated to occur in approximately 2% of children and 4–8% of adolescents (Birmaher et al., 1996) and this may carry its own burden of disadvantages, often persisting or re-emerging at adulthood (Dunn and Goodyer, 2006; Weissman, 2009; Weissman and Talati, 2009). Moreover, similarly to adult depression, a de-regulation of the immune system, characterized by an imbalance between pro- and anti- inflammatory cytokines, has been observed in adolescent depression (Gabbay et al., 2009). To this regard, increased levels of pro-inflammatory cytokines, including IFN-γ, IL-6 and CRP, have been observed in depressed adolescents as compared to controls as well as in adolescents with a history of childhood trauma (Mills et al., 2013). Furthermore, the transition vs. depression development is accompanied by a further increase of these cytokines, which remain higher even after the depressive episode is improved (Miller and Cole, 2012).

Abnormalities in the immune and inflammatory systems occurring in depression are also found in post-mortem brains of depressed and suicide patients. Shelton and colleagues reported, for example, increased inflammatory pattern in the brain of depressed suicide patients (Shelton et al., 2011). Moreover, recent studies in the hypothalamus of depressed subjects have identified abnormalities in protein and mRNA levels of Toll Like Receptors (TLRs), which are involved in neuronal function as well as in the production of cytokines and chemokines in response to inflammation or stressful insults (Wang et al., 2008).

The role for inflammation in the pathogenesis of depression has been supported also by evidence showing that the administration of pro-inflammatory agents, like the endotoxin lipopolysaccharide (LPS), induces the development of depressive symptoms in humans (Grigoleit et al., 2011). In line with this, around the 30–40% of hepatitis C patients treated with the pro-inflammatory cytokine peg-interferon-alpha (pegIFN-α) develop clinically relevant depression (Miyaoka et al., 1999; Raison et al., 2005; Asnis and De La Garza, 2006). Finally, depression shows elevated comorbidity with several immune-related diseases, such as cancer, cardiovascular and neurodegenerative diseases, which are all clinical conditions characterized by the presence of inflammatory alterations (Benton et al., 2007; Anisman et al., 2008).

## PUTATIVE MECHANISMS UNDERLYING THE ASSOCIATION BETWEEN DEPRESSION AND INFLAMMATION

There are several mechanisms by which cytokines can access the brain, influence central neuronal functions and cause behavioral changes known as "sickness behavior", a coordinated set of psychological and physiological modifications that develop during the course of an infection (Dantzer, 2004) and that resemble depressive symptoms. One pathway may involve macrophage-like cells located in the circumventricular organs and the choroid plexus, which detect and respond to circulating pathogen-associated molecular patterns by producing pro-inflammatory cytokines; these cytokines can then cross the Blood Brain Barrier (BBB) and affect neuronal function and microglia activation. Another mechanism by which cytokines can reach the brain is via binding with their specific transporters, which are located on the BBB. Moreover, microglia cells in the brain produce cytokines receptors and thus amplify the inflammatory signals (Besedovsky and del Rey, 1996; Capuron and Miller, 2004). Once in the brain, cytokines can affect brain function in a variety of ways, including the modulation of neurotransmitter metabolism and neurotoxic mechanisms. As an example, cytokines induce the enzyme Indoleamine 2,3 Dioxygenase (IDO), which breaks down the serotonin precursor tryptophan into kynurenine that, once converted into quinolinic acid, may lead to neurotoxicity through the activation of the glutamatergic system (Myint and Kim, 2014). Cytokines have

also been shown to decrease the neurotrophic support and to reduce neurogenesis in several brain areas, particularly in the hippocampus (Hashmi et al., 2013; Williamson and Bilbo, 2013). This may eventually contribute to the reduction of neuronal plasticity that represents a core feature of depression-related dysfunction (see below). Furthermore, as we have also represented in **Figure 1**, cytokines can increase the levels of stress hormones, including corticotrophin releasing hormone (CRH), adreno-corticotrophin hormone (ACTH) and cortisol, which have been reported to be elevated in patients with depression (Besedovsky and del Rey, 1996; Pariante and Miller, 2001) and may therefore participate to HPA dysfunction (Miller et al., 2009).

Deregulation of microglia function has been associated with neurologic and psychiatric diseases and may lead to critical changes in neuronal activity and function (Beumer et al., 2012; Stertz et al., 2013; Paolicelli et al., 2014; Najjar and Pearlman, 2015).

One major mechanism through which microglia can alter brain functions associated with psychiatric diseases is neurogenesis. The impact of inflammation on adult hippocampal neurogenesis was originally discovered by the groups of Lindvall and Palmer, demonstrating that systemic or intra-hippocampal administration of LPS reduces the formation of newborn neurons in the adult hippocampus, an effect that can be prevented by indomethacin, a non steroidal anti-inflammatory drug, which act by inhibiting the synthesis of pro-inflammatory prostaglandins (Ekdahl et al., 2003; Monje et al., 2003).

Microglia can exert a positive or negative influence on proliferation, survival, or differentiation of newborn cells, depending on the inflammatory context. For instance, microglia can compromise the neurogenic cascade during chronic stress,

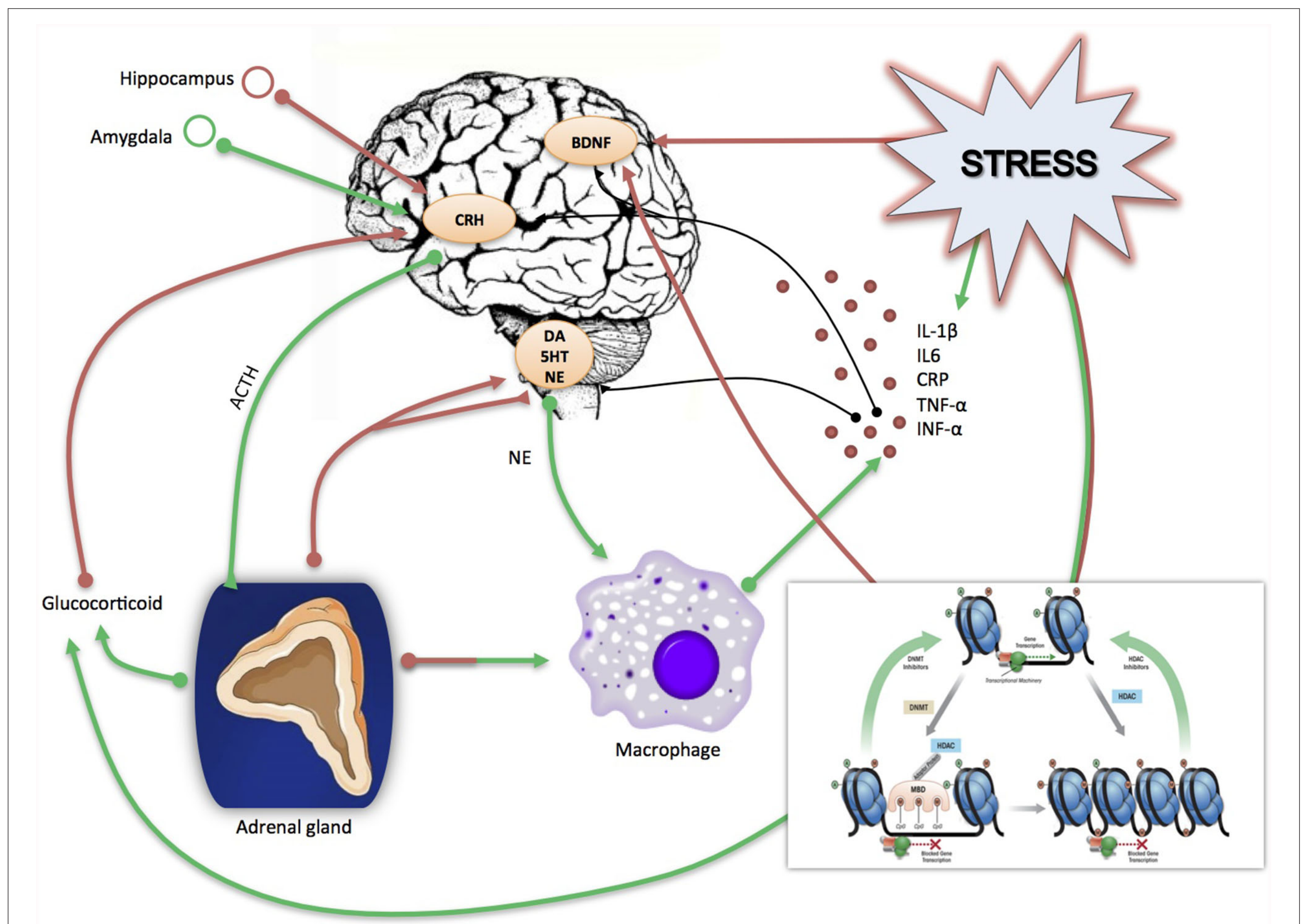



**FIGURE 1 | Schematic rappresentation of the direct and indirect effect of stress on inflammation and neuroplasticity related processes**. Stress induces directly an immediate release of glucocorticoids and pro-inflammatory cytokines (IL-1β, IL-6, CRP, TNF-α, INF-α); in turn incresead levels of glucocorticoids act on the brain by altering the CRH-ACTH signaling and, in turn, negatively affecting neurogenesis as well as the production of neurotrophic factors, including Brain Derived neurotrophic Factor (BDNF). Similarly, proinflammatory cytokines can negatively affect brain functioning and neurotrophins production and release. Stress can also work indirectly by activating epigenetic mechanisms (methylation, deacetylation, miRNAs), which may act on the same target stress related genes i.e., glucocorticid receptors, cytokines and BDNF. Red arrows indicate a suppressive effect, green arrows a stimulating effect.

through the release of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α. Microglia has been also shown to phagocyte the excess of newborn neurons undergoing apoptosis in the hippocampal neurogenic niche during normal physiological conditions, while a similar role in the synaptic integration of newborn cells was also proposed in light of microglial cells to phagocyte synaptic elements (Sierra et al., 2014). Kreisel et al. provided also a link between stress-induced alterations in microglia and the development of stress-induced depression (Kreisel et al., 2014). Indeed they showed a role of dynamic alterations in microglia activation status in the development of chronic unpredictable stress (CUS)-induced depressive-like condition in rodents and the ability of minocycline and of the transgenic interleukin-1 receptor antagonist to rescue the subsequent microglial apoptosis, as well as the CUS-induced depressive-like behavior and suppressed neurogenesis.

It has to be mentioned that depending on its activation state, microglia may have opposite effects on adult neurogenesis and it is likely that pro-neurogenic and anti-neurogenic microglial cells may co-exist, with a different responsiveness to external stimulus, such as voluntary running and housing conditions. Thus it may be inferred that the overall impact on adult neurogenesis may depend on the outcome of the interaction between environmental factors and microglial state (Gebara et al., 2013).

Cytokines alterations in depression have also important implications with respect to the response to pharmacological treatments. On one end, different studies have demonstrated the ability of some antidepressants to reduce cytokines activation in depressed patients (Sluzewska et al., 1995; Frommberger et al., 1997; Tuglu et al., 2003; Basterzi et al., 2005). Our research group has recently demonstrated that cytokine expression in the leukocytes from depressed patients are reduced following escitalopram and nortriptyline treatment with a significant correlation between these changes and treatment response (Cattaneo et al., 2013).

Moreover, depressed patients who are non responders to antidepressant therapies or who are treatment resistant show higher plasma concentrations of several pro-inflammatory cytokines and CRP as compared to responders (Sluzewska et al., 1997; Lanquillon et al., 2000; Fitzgerald et al., 2006; Uher et al., 2014). In line with these results, we found that patients who do not respond to two different classes of antidepressants have higher baseline mRNA levels of IL-1β, macrophage migration inhibitory factor (MIF), and TNF-α (Cattaneo et al., 2013). Similar results on the role of TNF-α in treatment response were also reported by Powell et al. (2013).

It may be argued that peripheral inflammation could alter behavioral response to monoaminergic drugs because high levels of cytokines are known to modulate monoamine synthesis, reuptake and metabolism, for example by altering the function of the serotonin transporter, which is a key target of antidepressant drugs (Tynan et al., 2012). Thus, cytokine-induced changes in monoaminergic signaling may not only induce depressive states, but may conceivably compromise the therapeutic effects of monoamine reuptake inhibitors, leading to first-line treatment resistance. Conversely, monoaminergic drugs may impact directly the inflammatory gene expression or peripheral immune cells, although this possibility has yet to be fully tested and established (Pollak and Yirmiya, 2002).

## DEPRESSION AND NEURONAL PLASTICITY

Neuronal plasticity is a concept that refers to a number of mechanisms crucial for brain function and its ability to perceive, adapt and respond to a variety of internal and external stimuli. It is thought that such mechanisms can be defective in different psychiatric disorders and this may eventually enhance disease susceptibility (Manji et al., 2003; de Kloet et al., 2005; Duman and Monteggia, 2006; Calabrese et al., 2009).

A large body of evidence has demonstrated that stress, a major environmental challenge for depression, can lead to an impairment of neuronal plasticity (McEwen et al., 2012; Bohacek et al., 2014). Among the systems contributing to the maintenance of neuronal plasticity, neurotrophic factors, and in particular the neurotrophin Brain-Derived Neurotrophic Factor (BDNF), have emerged as important mediators for long-term functional deterioration associated with mental illness (Bramham and Messaoudi, 2005; Lu et al., 2005; Duman and Monteggia, 2006; McClung and Nestler, 2008; Cirulli et al., 2009; Castrén and Rantamäki, 2010a; Calabrese et al., 2011b; Chourbaji et al., 2011). BDNF, in fact, is not only important during brain development, but it exerts a pivotal role for neuronal remodeling as well as synaptic function (Lu et al., 2008; Waterhouse and Xu, 2009). Several studies have demonstrated that, in depressed subjects, the expression of BDNF is reduced in brain structures, such as the hippocampus and the prefrontal cortex, which represent key anatomical targets for stress-induced structural changes. Preclinical studies have confirmed the association between stress exposure and BDNF, since chronic exposure to different stress paradigms leads to a consistent reduction of neurotrophin expression (Pittenger and Duman, 2008) (Tsankova et al., 2006). The expression of BDNF is also reduced in the hippocampus and prefrontal cortex of serotonin transporter knockout rats, a genetic model of depression and anxiety (Molteni et al., 2010), suggesting that changes of neuronal plasticity may also contribute to the genetic susceptibility to mood disorders.

Changes of BDNF expression may represent a relevant component for functional disability. For example it has been shown that targeted or inducible deletion of the BDNF gene produces behavioral dysfunction related to anxiety and depression (Chourbaji et al., 2011; Burke et al., 2013), suggesting that such changes may contribute to the pathologic condition. Furthermore BDNF expression plays a critical role in resilience to chronic stress and in the development of neural circuits that control coping mechanisms (Taliaz et al., 2011).

Since the expression of trophic factors is reduced in depression and this may contribute to functional defects associated with the pathologic condition, it may be inferred that effective pharmacological intervention should be able to normalize such alterations. Indeed, a key step in long-term adaptive changes brought about by antidepressants appears to be their ability to modulate the expression of BDNF as well as of other growth factors (Berton and Nestler, 2006; Groves, 2007; Martinowich et al., 2007; Calabrese et al., 2009, 2011a; Castrén and Rantamäki, 2010b; Cattaneo et al., 2013). The majority of the studies focusing

on BDNF have demonstrated that these drugs can modulate neurotrophin transcription (Coppell et al., 2003; Molteni et al., 2006; Calabrese et al., 2007, 2011a; Nair et al., 2007; Kozisek et al., 2008), its translation and trafficking to specific sub-cellular compartments (Calabrese et al., 2007), as well as BDNF receptor activation and signaling (Saarelainen et al., 2003; Fumagalli et al., 2005; Duman et al., 2007). The ability to modulate BDNF has also been demonstrated for the rapid acting antidepressant ketamine (Autry et al., 2011). A number of experimental studies have shown that defective BDNF expression or release may limit the antidepressant activity (Wolkowitz et al., 2011; Dreimüller et al., 2012), suggesting that neurotrophin modulation may represent an important mechanism of antidepressant drugs. This possibility is in accordance with clinical data demonstrating that serum BDNF levels, which are reduced in depressed subjects, can be normalized only in patients that are responsive to pharmacological intervention (Bocchio-Chiavetto et al., 2010; Yoshida et al., 2012; Molendijk et al., 2014).

The modulation of neurotrophic proteins can lead to functional and structural changes affecting brain regions key to depressive symptoms. One of the mechanisms that lie downstream from neurotrophic factors is neurogenesis, the process by which neurons are generated from stem cells. Indeed chronic antidepressant treatment can increase neurogenesis in the adult brain, primarily in the subgranular zone of hippocampal dentate gyrus, a mechanism that depends on the modulation of trophic factors and that appears to be relevant for the behavioral action of antidepressant drugs (Cameron et al., 1998; Duman et al., 2001; Santarelli et al., 2003; Malberg, 2004; Sairanen et al., 2005; Banasr and Duman, 2008).

## CHILDHOOD TRAUMA AS VULNERABILITY FACTOR FOR DEPRESSIVE PHENOTYPES

A recent European Report from WHO indicates that at least 18 million children in Europe suffer from early life trauma, harming mental and physical health, and with enormous societal costs, including for medical and social care (Europe WHO of European report on preventing child maltreatment).

Childhood maltreatment is defined as acts of commission or omission by parents or caregivers resulting in potential harm to the child's health, and includes experiences such as physical, sexual and psychological abuse, as well as physical or emotional neglect. Among substantiated reports, 60% of the childhood maltreatment is classified as neglect, 20% as physical abuse, and 10% as sexual abuse (Holmes and Slap, 1998). The prevalence of emotional abuse and neglect is likely much higher than that of sexual and physical abuse, but more difficult to measure and quantify (Holmes and Slap, 1998).

A number of studies have shown that the onset of mood disorders, such as depression, is undoubtedly influenced by stressful life events that occur in childhood (Kendler et al., 2004a,b; Horesh et al., 2008). In one community-based study of approximately 2,000 women, those with a history of childhood physical or sexual abuse had an increased risk of depression and anxiety and were more likely to have attempted suicide than women without such a history (Kendler et al., 2004a,b). It is also evident that different types of child maltreatment have long-term adverse consequences for mental health (Cicchetti and Toth, 2005; Gonzalez, 2013; Allen et al., 2014; Bailer et al., 2014; Cummings and Berkowitz, 2014; Hagan et al., 2014; Roth et al., 2014). Among the different types of maltreatment, sexual abuse is probably the most relevant with respect to increased risk for psychiatric disorders, such as depression and anxiety (Booth and Gulati, 2014; Kanamüller et al., 2014; Letourneau et al., 2014; Visser et al., 2014). On these bases, there is high interest in understanding, which are the mechanisms that may link the exposure to adversities early in life with the enhanced susceptibility to mood disorders.

## CHILDHOOD TRAUMA AND ALTERATIONS IN THE INFLAMMATORY SYSTEM

Although the association between early life stressful events and depression may occur via several biological processes, a number of studies have suggested a role for increased inflammation or increased sensitivity of inflammatory responses. Taking advantage of the Dunedin cohort subjects, Danese et al. were the first to demonstrate that elevated CRP blood levels were significantly associated with maltreatment during childhood (Danese et al., 2008) and such association was particular strong in individuals that developed depression later in life (Danese et al., 2008, 2009). Similarly Slopen et al. reported that exposure to childhood adversities is associated with higher levels of IL-6 and CRP in teenagers (Slopen et al., 2014).

It has also been shown that depressed subjects with a history of early life stress show an increased inflammatory response when re-exposed to an acute psychological stress at adulthood, as indicated by an exaggerated IL-6 response as well as increased DNA binding of the key pro-inflammatory transcriptio factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in PBMCs (Pace et al., 2006).

Based on this evidence, it is possible to speculate that individuals who experience major stressors early in life are more vulnerable to an immune dysregulation at adulthood, regardless of whether they subsequent develop adverse physical or mental health consequences. Miller and Chen have proposed a model suggesting that stress that occurs during a sensitive period in life, when immune function is highly plastic, gets embedded in the functioning of the cells that regulate inflammation (Miller and Chen, 2007). Therefore, brain inflammatory cells including macrophages, microglia and dendritic cells, will develop a hyper-sensitivity that leads to a chronic pro-inflammatory state, due to an activation of pro-inflammatory transcription factors such as NF-κB and down-regulation of anti-inflammatory transcriptions factors such as the glucocorticoid receptor, thus increasing the levels of circulating cytokines. In addition, an altered response of innate immune cells to stimuli causes abnormalities in other leucocytes, particularly the T- and B- cells that orchestrate adaptive immune responses.

How childhood trauma generates a "pro-inflammatory" phenotype is still an open question but it is probably the result of a deregulation in complex networks within biological pathways affected by such experiences (see **Figure 1**). With this respect, the study of epigenetic processes holds a substantial

promise to explain many of these unsolved questions, since epigenetic operates at the interface between the individual genetic background and the environment.

Studies in rodents have also shown that early life stress induces a premature activation of the immune system that can significantly shift the developmental trajectory of microglia, changing the long-term patterns of activation of these cells (Schwarz et al., 2011; Williamson et al., 2011). As a consequence of these changes, rats exposed to stress early in life are more vulnerable to increase in pro-inflammatory cytokines production following an LPS challenge in the adulthood, suggesting that this pro-inflammatory state persists in time and can be responsible of an enhanced vulnerability and sensitivity to a novel insult in adulthood (Sominsky et al., 2013).

## EARLY LIFE ADVERSITIES AND LONG-TERM CHANGES IN NEURONAL PLASTICITY

Since neuronal plasticity may contribute to structural modifications and to the inability to respond or adapt to environmental challenges (Berton and Nestler, 2006; Krishnan and Nestler, 2008; McClung and Nestler, 2008; Pittenger and Duman, 2008; Calabrese et al., 2009), it is feasible to hypothesize that alterations of these mechanisms may also represent the long-lasting consequence of stressful experience occurring early in life.

In accordance with this possibility, a long-term reduction of BDNF expression and function may represent a common endpoint for adverse experience early in life, although the anatomical specificity of such changes depends on the type, timing and duration of the manipulation. Indeed, BDNF mRNA levels are reduced in the hippocampus of adult rats that were exposed to 24 h of maternal deprivation at postnatal day 9 (Roceri et al., 2002), whereas more protracted manipulations during gestation or the early phase of postnatal life (such as prenatal stress or repeated maternal deprivation) reduce the levels of the neurotrophin, primarily in the prefrontal cortex (Koo et al., 2003; Fumagalli et al., 2004; Roceri et al., 2004; Roth et al., 2009). The time course analysis of BDNF changes in rats exposed to prenatal stress (PNS) suggests that the reduced expression observed in adult animals is not directly linked to stress exposure, but is dependent on the maturational stage of the prefrontal cortex, becoming fully manifest after adolescence (Luoni et al., 2014). Moreover we have recently demonstrated that exposure to PNS leads to a significant down-regulation of the pool of BDNF transcripts with long 3'UTR that are responsible for targeting BDNF mRNA to dendrites, where activity-dependent translation may occur (An et al., 2008; Lau et al., 2010). Hence, the selective decrease of long 3'UTR BDNF mRNA levels after PNS may contribute to defects in local, activity-dependent neurotrophin synthesis (Lau et al., 2010), which may eventually lead to reduced cell-cell communication and synaptic function and ultimately contribute to cognitive and emotional deterioration associated with exposure to early life adversities (Murmu et al., 2006; Michelsen et al., 2007). Interestingly, reduced neurogenesis was also found in response to stress early in life. For example, PNS in rats induced lifespan reduction of neurogenesis in the dentate gyrus and leads to an impairment of hippocampal-related spatial tasks (Lemaire et al., 2000). Similar stressful experiences in monkeys can result in reduced hippocampal volume and an inhibition of neurogenesis in the dentate gyrus, which is associated with increased pituitary-adrenal activity, as well as with behavioral profiles indicative of greater emotionality (Coe et al., 2003). Furthermore, it has been demonstrated that the exposure to prolonged, but not brief, bouts of maternal separation during the first 2 weeks of life determines a long-lasting suppression of adult neurogenesis and diminished plasticity in this parameter after exposure to stress in adulthood (Mirescu et al., 2004). Interestingly, some of the neuroplastic alterations brought about by early life stress can be normalized or even prevented by pharmacological intervention during early life, adolescence as well as adulthood (Matrisciano et al., 2012; Luoni et al., 2014).

## CHILDHOOD TRAUMA, INFLAMMATION AND DEPRESSION: IS EPIGENETIC THE LINKING MECHANISM?

The term "epigenetics" refers to long-lasting changes in gene expression without alterations of the DNA sequence, which are associated with several potentially reversible processes including DNA methylation, histone modifications and aberrant expression of micro-ribonucleic acid (miRNA; Maffioletti et al., 2014; Provençal and Binder, 2014a). Among different epigenetic modifications, DNA methylation is one of the best-characterized mechanisms in relation to childhood adversities (Essex et al., 2013). Indeed changes of DNA methylation at sensitive gene promoters may explain the persistence of early life effects into adulthood, rendering the subject more vulnerable and sensitive to subsequent insults and challenges.

In humans, DNA methylation occurs, almost exclusively, through covalent modification of DNA, where methyl groups are coupled to cytosine residues of CpG dinucleotides. DNA methylation has been shown to be associated with variations in gene expression (Szyf, 2013; Reul, 2014), thus serving as a possible mechanism for regulating the transcriptional response to extracellular events. Several preclinical studies have highlighted how exposure to environmental stressors can produce long-lasting behavioral alterations and may affect coping abilities later in life through epigenetic modifications and in particular through changes in DNA methylation within selected brain regions (Szyf and Bick, 2013; Provençal and Binder, 2014a; Booij et al., 2015; Desplats, 2015). For example, in rats, reduced maternal care produces long lasting effects on the offspring, including an anxious phenotype and higher corticosterone levels in response to stress. These behavioral abnormalities are associated with reduced hippocampal expression of glucocorticoid receptors that appears to be the consequence of increased methylation at gene promoter (Meaney and Szyf, 2005; Szyf et al., 2005; Kofink et al., 2013). Also, maternal separation in mice is able to induce an hypomethylation in the vasopressin gene enhancer region, which leads to increased expression of hypothalamic vasopressin, accompanied by enhanced corticosterone secretion (Murgatroyd et al., 2009). Some of these changes have been shown to occur also in humans. Indeed, McGowan et al. have demonstrated that in human post-mortem brain studies early life abuse was associated with increased methylation of the GR exon 1f promoter in the hippocampus, in support of the "translational"

implications for the epigenetic changes brought about by the exposure to early life stress (McGowan et al., 2009). In addition to the stress-responsive systems, also neuroplastic genes can undergo epigenetic regulation, which may be responsible for the changes observed in mental illness. At experimental level, it was demonstrated that the persistent reduction of BDNF expression in the social defeat stress paradigm is due to epigenetic changes in the promoter region of two of its transcripts (Tsankova et al., 2006). Similarly, we have recently shown that the expression of BDNF is significantly reduced in the prefrontal cortex of serotonin transporter knockout rats through an increased methylation in the promoter region of exons VI and reduced H3 acetylation at exon IV (Molteni et al., 2010). These results are in line with post mortem studies since increased BDNF promoter methylation has been found in the brain of suicide subjects (Keller et al., 2010). Such modification may also represent the consequence of early life adversities. Indeed, maltreatment during infancy in rodents produces a persistent increase of the methylation in BDNF exon-4 and exon-9 that leads to reduced neurotrophin expression in the adult prefrontal cortex (Roth et al., 2009).

A growing number of studies is addressing the consequences of early life stress on DNA methylation at genome wide level in the brain as well as in peripheral tissues (Mehta et al., 2013; Nieratschker et al., 2014; Provençal and Binder, 2014a) in order to identify signatures that may be associated with the long-term pathologic consequences of such experiences. With this respect epigenetic changes in peripheral tissues may correlate to some extent with measures in the brain. As an example differential rearing conditions of rhesus macaques is associated with differential methylation in early adulthood in both the brain and T cells, suggesting that the response to early-life adversity is system-wide and genome-wide and persists to adulthood (Provençal et al., 2012). Furthermore the observation that ELS-associated DNA methylation changes are not limited to the brain but can be found in peripheral systems suggests that such changes may also be relevant for additional health problems, such as the described increased risk for cardiovascular and metabolic diseases (Provencal and Binder, 2014b).

With this respect it will be extremely important to investigate and characterize inflammatory-immune methylation signatures as a consequence of early life stress, which will eventually provide key information not only for their role in mental illness but also as a potential mechanism to explain the comorbidity of depression with different medical conditions.

## CONCLUSIONS

As discussed in this review, there is evidence linking early life stressful events, peripheral inflammation, alterations in neuroplastic mechanisms and depression, although the underlying biological mechanisms still need to be clarified. We have discussed the role of epigenetics, and in particular of DNA methylation, as one such mechanism. Indeed, early life stressful events can activate epigenetic mechanisms at global levels as well as at the promoter regions of key target genes, producing long-lasting and stable changes in gene expression, which persist up to adulthood and may be responsible of an increased vulnerability to develop mental disorders. Through a better understanding of how epigenetic mechanisms underlie psychiatric disorders, we could also better characterize how these modifications can have an impact on specific genes that, in turn, contribute to the pathogenesis of these disorders. Moreover, as increased inflammation is clearly observed in depressed patients and, in particular, in those do not respond to antidepressant therapies, future research will aim to clarify whether increased inflammation actually identifies a single group of depressed patients that has experienced childhood maltreatment and is also resistant to conventional antidepressants. Moreover, inflammatory biomarkers may be used as strategy to screen patients who may benefit from drugs that target inflammatory mechanisms. Finally, future studies should also provide new insights on the reversibility of the damage associated with childhood stress experiences, including studies testing whether pharmacological and non-pharmacological interventions could reverse the abnormalities induced by childhood adversities on the functionality of the immune and stress response systems and thus also minimize the risk for mood disorders, both in the individuals affected and in the next generations.

## AUTHORS STATEMENT

AC designed the work and performed most of the literature work; she achieved the first draft of the paper; she approved the final version of the manuscript and she agreed to have accounted for all the aspects of the work. FM performed the literature work and contributed to the interpretation of the data; she drafted the manuscript, approved the final version of the manuscript and she ensured that all the aspect of the work have been appropriately investigated. GP contributed to the conception of the work and to draft the manuscript; he approved the final version of the manuscript and agreed to have accounted for all the aspects of the work. VB contributed substantially to the revised version of the manuscript and to the data interpretation; she drafted of the manuscript; she approved the final version of the manuscript and she ensured that all the aspects of the work have been appropriately investigated.

LBC contributed to the interpretation of data for the work; she revised critically the manuscript, and she ensured that all the aspects of the work have been appropriately investigated.

MAR contributed substantially to the conception of the work, he revised the manuscript critically for important intellectual content, he approved the final version of the manuscript and agreed to have accounted for all the aspects of the work.

CMP contributed substantially to the conception of the work and to the data discussion; he revised the manuscript critically for important intellectual content, he approved the final version of the manuscript and agreed to have accounted for all the aspects of the work.

All the authors approve and confirm their role in the manuscript and also the order in which they do appear.

## ACKNOWLEDGMENTS

Dr. Cattaneo and Prof. Pariante have been funded through research obtained from the Medical Research Council (UK) (MR/J002739/1) and from the Psychiatry Research Trust, UK (McGregor 97). Prof. Pariante has been also funded by the

Commission of European Communities Seventh Framework Programme (Collaborative Project Grant Agreement no. 22963, Mood Inflame); by the National Institute for Health Research Biomedical Research Centre in Mental Health at South London and Maudsley NHS Foundation Trust and King's College London. Dr. Cattaneo has additionally been supported by the Italian Ministry of Health (Ricerca Corrente) and by ERANET Neuron Grant.

Prof. M.A. Riva has been funded by grants from the Italian Ministry of University and Research (PRIN 20107MSMA4), from Fondazione CARIPLO (grant n. 2012-0503) and from European Union/ERANET. Dr. Bocchio-Chiavetto was supported by grants from Cariplo Foundation (MICROMOOD Project 2009-2701) and from the Italian Ministry of Health (Ricerca Corrente).

## REFERENCES

Allen, B., Tellez, A., Wevodau, A., Woods, C. L., and Percosky, A. (2014). The impact of sexual abuse committed by a child on mental health in adulthood. *J. Interpers. Violence* 29, 2257–2272. doi: 10.1177/0886260513517550

American Psychiatric Association. (2000). *Diagnostic and Statistical Manual of Mental Disorders.* Arlington, VA: American Psychiatric Association.

An, J. J., Gharami, K., Liao, G. Y., Woo, N. H., Lau, A. G., Vanevski, F., et al. (2008). Distinct role of long 3' UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. *Cell* 134, 175–187. doi: 10.1016/j.cell.2008.05.045

Anisman, H., Merali, Z., and Hayley, S. (2008). Neurotransmitter, peptide and cytokine processes in relation to depressive disorder: comorbidity between depression and neurodegenerative disorders. *Prog. Neurobiol.* 85, 1–74. doi: 10.1016/j.pneurobio.2008.01.004

Asnis, G. M., and De La Garza, R. 2nd (2006). Interferon-induced depression in chronic hepatitis C: a review of its prevalence, risk factors, biology and treatment approaches. *J. Clin. Gastroenterol.* 40, 322–335. doi: 10.1097/01.mcg.0000210099.36500.fe

Autry, A. E., Adachi, M., Nosyreva, E., Na, E. S., Los, M. F., Cheng, P. F., et al. (2011). NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature* 475, 91–95. doi: 10.1038/nature10130

Bailer, J., Witthöft, M., Wagner, H., Mier, D., Diener, C., and Rist, F. (2014). Childhood maltreatment is associated with depression but not with hypochondriasis in later life. *J. Psychosom. Res.* 77, 104–108. doi: 10.1016/j.jpsychores.2014.06.004

Banasr, M., and Duman, R. S. (2008). Keeping 'trk' of antidepressant actions. *Neuron* 59, 349–351. doi: 10.1016/j.neuron.2008.07.028

Basterzi, A. D., Aydemir, C., Kisa, C., Aksaray, S., Tuzer, V., Yazici, K., et al. (2005). IL-6 levels decrease with SSRI treatment in patients with major depression. *Hum. Psychopharmacol.* 20, 473–476. doi: 10.1002/hup.717

Benton, T., Staab, J., and Evans, D. L. (2007). Medical co-morbidity in depressive disorders. *Ann. Clin. Psychiatry* 19, 289–303. doi: 10.1080/10401230701653542

Berton, O., and Nestler, E. J. (2006). New approaches to antidepressant drug discovery: beyond monoamines. *Nat. Rev. Neurosci.* 7, 137–151. doi: 10.1038/nrn1846

Besedovsky, H. O., and del Rey, A. (1996). Immune-neuro-endocrine interactions: facts and hypotheses. *Endocr. Rev.* 17, 64–102. doi: 10.1210/er.17.1.64

Beumer, W., Gibney, S. M., Drexhage, R. C., Pont-Lezica, L., Doorduin, J., Klein, H. C., et al. (2012). The immune theory of psychiatric diseases: a key role for activated microglia and circulating monocytes. *J. Leukoc. Biol.* 92, 959–975. doi: 10.1189/jlb.0212100

Birmaher, B., Ryan, N. D., Williamson, D. E., Brent, D. A., Kaufman, J., Dahl, R. E., et al. (1996). Childhood and adolescent depression: a review of the past 10 years. Part I. *J. Am. Acad. Child Adolesc. Psychiatry* 35, 1427–1439. doi: 10.1097/00004583-199611000-00011

Bocchio-Chiavetto, L., Bagnardi, V., Zanardini, R., Molteni, R., Nielsen, M. G., Placentino, A., et al. (2010). Serum and plasma BDNF levels in major depression: a replication study and meta-analyses. *World J. Biol. Psychiatry* 11, 763–773. doi: 10.3109/15622971003611319

Bohacek, J., Farinelli, M., Mirante, O., Steiner, G., Gapp, K., Coiret, G., et al. (2014). Pathological brain plasticity and cognition in the offspring of males subjected to postnatal traumatic stress. *Mol. Psychiatry* doi: 10.1038/mp.2014.80. [Epub ahead of print].

Booij, L., Tremblay, R. E., Szyf, M., and Benkelfat, C. (2015). Genetic and early environmental influences on the serotonin system: consequences for brain development and risk for psychopathology. *J. Psychiatry Neurosci.* 40, 5–18. doi: 10.1503/jpn.140099

Booth, B. D., and Gulati, S. (2014). Mental illness and sexual offending. *Psychiatr. Clin. North Am.* 37, 183–194. doi: 10.1016/j.psc.2014.03.007

Bramham, C. R., and Messaoudi, E. (2005). BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. *Prog. Neurobiol.* 76, 99–125. doi: 10.1016/j.pneurobio.2005.06.003

Burke, T. F., Advani, T., Adachi, M., Monteggia, L. M., and Hensler, J. G. (2013). Sensitivity of hippocampal 5-HT1A receptors to mild stress in BDNF-deficient mice. *Int. J. Neuropsychopharmacol.* 16, 631–645. doi: 10.1017/s1461145712000466

Calabrese, F., Molteni, R., Gabriel, C., Mocaer, E., Racagni, G., and Riva, M. A. (2011a). Modulation of neuroplastic molecules in selected brain regions after chronic administration of the novel antidepressant agomelatine. *Psychopharmacology (Berl)* 215, 267–275. doi: 10.1007/s00213-010-2129-8

Calabrese, F., Molteni, R., Maj, P. F., Cattaneo, A., Gennarelli, M., Racagni, G., et al. (2007). Chronic duloxetine treatment induces specific changes in the expression of BDNF transcripts and in the subcellular localization of the neurotrophin protein. *Neuropsychopharmacology* 32, 2351–2359. doi: 10.1038/sj.npp.1301360

Calabrese, F., Molteni, R., Racagni, G., and Riva, M. A. (2009). Neuronal plasticity: a link between stress and mood disorders. *Psychoneuroendocrinology* 34(Suppl 1), S208–S216. doi: 10.1016/j.psyneuen.2009.05.014

Calabrese, F., Molteni, R., and Riva, M. A. (2011b). Antistress properties of antidepressant drugs and their clinical implications. *Pharmacol. Ther.* 132, 39–56. doi: 10.1016/j.pharmthera.2011.05.007

Cameron, H. A., Hazel, T. G., and McKay, R. D. (1998). Regulation of neurogenesis by growth factors and neurotransmitters. *J. Neurobiol.* 36, 287–306. doi: 10.1002/(sici)1097-4695(199808)36:2<287::aid-neu13>3.3.co;2-e

Capuron, L., and Miller, A. H. (2004). Cytokines and psychopathology: lessons from interferon-alpha. *Biol. Psychiatry* 56, 819–824. doi: 10.1016/j.biopsych.2004.02.009

Castrén, E. (2014). Neurotrophins and psychiatric disorders. *Handb. Exp. Pharmacol.* 220, 461–479. doi: 10.1007/978-3-642-45106-5_17

Castrén, E., and Rantamäki, T. (2010a). The role of BDNF and its receptors in depression and antidepressant drug action: reactivation of developmental plasticity. *Dev. Neurobiol.* 70, 289–297. doi: 10.1002/dneu.20758

Castrén, E., and Rantamäki, T. (2010b). Role of brain-derived neurotrophic factor in the aetiology of depression: implications for pharmacological treatment. *CNS Drugs* 24, 1–7. doi: 10.2165/11530010-000000000-00000

Cattaneo, A., Gennarelli, M., Uher, R., Breen, G., Farmer, A., Aitchison, K. J., et al. (2013). Candidate genes expression profile associated with antidepressants response in the GENDEP study: differentiating between baseline 'predictors' and longitudinal 'targets'. *Neuropsychopharmacology* 38, 377–385. doi: 10.1038/npp.2012.191

Chourbaji, S., Brandwein, C., and Gass, P. (2011). Altering BDNF expression by genetics and/or environment: impact for emotional and depression-like behaviour in laboratory mice. *Neurosci. Biobehav. Rev.* 35, 599–611. doi: 10.1016/j.neubiorev.2010.07.003

Cicchetti, D., and Toth, S. L. (2005). Child maltreatment. *Annu. Rev. Clin. Psychol.* 1, 409–438. doi: 10.1146/annurev.clinpsy.1.102803.144029

Cirulli, F., Francia, N., Berry, A., Aloe, L., Alleva, E., and Suomi, S. J. (2009). Early life stress as a risk factor for mental health: role of neurotrophins from rodents to non-human primates. *Neurosci. Biobehav. Rev.* 33, 573–585. doi: 10.1016/j.neubiorev.2008.09.001

Coe, C. L., Kramer, M., Czéh, B., Gould, E., Reeves, A. J., Kirschbaum, C., et al. (2003). Prenatal stress diminishes neurogenesis in the dentate gyrus of juvenile rhesus monkeys. *Biol. Psychiatry* 54, 1025–1034. doi: 10.1016/s0006-3223(03)00698-x

Coppell, A. L., Pei, Q., and Zetterström, T. S. (2003). Bi-phasic change in BDNF gene expression following antidepressant drug treatment. *Neuropharmacology* 44, 903–910. doi: 10.1016/s0028-3908(03)00077-7

Cummings, M., and Berkowitz, S. J. (2014). Evaluation and treatment of childhood physical abuse and neglect: a review. *Curr. Psychiatry Rep.* 16:429. doi: 10.1007/s11920-013-0429-5

Danese, A., Moffitt, T. E., Harrington, H., Milne, B. J., Polanczyk, G., Pariante, C. M., et al. (2009). Adverse childhood experiences and adult risk factors for age-related disease: depression, inflammation and clustering of metabolic risk markers. *Arch. Pediatr. Adolesc. Med.* 163, 1135–1143. doi: 10.1001/archpediatrics.2009.214

Danese, A., Moffitt, T. E., Pariante, C. M., Ambler, A., Poulton, R., and Caspi, A. (2008). Elevated inflammation levels in depressed adults with a history of childhood maltreatment. *Arch. Gen. Psychiatry* 65, 409–415. doi: 10.1001/archpsyc.65.4.409

Danner, M., Kasl, S. V., Abramson, J. L., and Vaccarino, V. (2003). Association between depression and elevated C-reactive protein. *Psychosom. Med.* 65, 347–356. doi: 10.1097/01.psy.0000041542.29808.01

Dantzer, R. (2004). Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity. *Eur. J. Pharmacol.* 500, 399–411. doi: 10.1016/j.ejphar.2004.07.040

Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W., and Kelley, K. W. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat. Rev. Neurosci.* 9, 46–56. doi: 10.1038/nrn2297

de Kloet, E. R., Joëls, M., and Holsboer, F. (2005). Stress and the brain: from adaptation to disease. *Nat. Rev. Neurosci.* 6, 463–475. doi: 10.1038/nrn1683

Desplats, P. A. (2015). Perinatal programming of neurodevelopment: epigenetic mechanisms and the prenatal shaping of the brain. *Adv. Neurobiol.* 10, 335–361. doi: 10.1007/978-1-4939-1372-5_16

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E. K., et al. (2010). A meta-analysis of cytokines in major depression. *Biol. Psychiatry* 67, 446–457. doi: 10.1016/j.biopsych.2009.09.033

Dreimüller, N., Schlicht, K. F., Wagner, S., Peetz, D., Borysenko, L., Hiemke, C., et al. (2012). Early reactions of brain-derived neurotrophic factor in plasma (pBDNF) and outcome to acute antidepressant treatment in patients with Major Depression. *Neuropharmacology* 62, 264–269. doi: 10.1016/j.neuropharm.2011.07.017

Duman, R. S., and Monteggia, L. M. (2006). A neurotrophic model for stress-related mood disorders. *Biol. Psychiatry* 59, 1116–1127. doi: 10.1016/j.biopsych.2006.02.013

Duman, R. S., Nakagawa, S., and Malberg, J. (2001). Regulation of adult neurogenesis by antidepressant treatment. *Neuropsychopharmacology* 25, 836–844. doi: 10.1016/s0893-133x(01)00358-x

Duman, C. H., Schlesinger, L., Kodama, M., Russell, D. S., and Duman, R. S. (2007). A role for MAP kinase signaling in behavioral models of depression and antidepressant treatment. *Biol. Psychiatry* 61, 661–670. doi: 10.1016/j.biopsych.2006.05.047

Dunn, V., and Goodyer, I. M. (2006). Longitudinal investigation into childhood- and adolescence-onset depression: psychiatric outcome in early adulthood. *Br. J. Psychiatry* 188, 216–222. doi: 10.1192/bjp.188.3.216

Ekdahl, C. T., Claasen, J. H., Bonde, S., Kokaia, Z., and Lindvall, O. (2003). Inflammation is detrimental for neurogenesis in adult brain. *Proc. Natl. Acad. Sci. U S A* 100, 13632–13637. doi: 10.1073/pnas.2234031100

Essex, M. J., Boyce, W. T., Hertzman, C., Lam, L. L., Armstrong, J. M., Neumann, S. M., et al. (2013). Epigenetic vestiges of early developmental adversity: childhood stress exposure and DNA methylation in adolescence. *Child Dev.* 84, 58–75. doi: 10.1111/j.1467-8624.2011.01641.x

Fitzgerald, P., O'Brien, S. M., Scully, P., Rijkers, K., Scott, L. V., and Dinan, T. G. (2006). Cutaneous glucocorticoid receptor sensitivity and pro-inflammatory cytokine levels in antidepressant-resistant depression. *Psychol. Med.* 36, 37–43. doi: 10.1017/s003329170500632x

Ford, D. E., and Erlinger, T. P. (2004). Depression and C-reactive protein in US adults: data from the third national health and nutrition examination survey. *Arch. Intern. Med.* 164, 1010–1014. doi: 10.1001/archinte.164.9.1010

Ford, E. S., Giles, W. H., Mokdad, A. H., and Myers, G. L. (2004). Distribution and correlates of C-reactive protein concentrations among adult US women. *Clin. Chem.* 50, 574–581. doi: 10.1373/clinchem.2003.027359

Frodl, T., and O'Keane, V. (2013). How does the brain deal with cumulative stress? A review with focus on developmental stress, HPA axis function and hippocampal structure in humans. *Neurobiol. Dis.* 52, 24–37. doi: 10.1016/j.nbd.2012.03.012

Frommberger, U. H., Bauer, J., Haselbauer, P., Fräulin, A., Riemann, D., and Berger, M. (1997). Interleukin-6-(IL-6) plasma levels in depression and schizophrenia: comparison between the acute state and after remission. *Eur. Arch. Psychiatry Clin. Neurosci.* 247, 228–233. doi: 10.1007/bf02900219

Fumagalli, F., Bedogni, F., Perez, J., Racagni, G., and Riva, M. A. (2004). Corticostriatal brain-derived neurotrophic factor dysregulation in adult rats following prenatal stress. *Eur. J. Neurosci.* 20, 1348–1354. doi: 10.1111/j.1460-9568.2004.03592.x

Fumagalli, F., Molteni, R., Calabrese, F., Frasca, A., Racagni, G., and Riva, M. A. (2005). Chronic fluoxetine administration inhibits extracellular signal-regulated kinase 1/2 phosphorylation in rat brain. *J. Neurochem.* 93, 1551–1560. doi: 10.1111/j.1471-4159.2005.03149.x

Gabbay, V., Klein, R. G., Alonso, C. M., Babb, J. S., Nishawala, M., De Jesus, G., et al. (2009). Immune system dysregulation in adolescent major depressive disorder. *J. Affect. Disord.* 115, 177–182. doi: 10.1016/j.jad.2008.07.022

Gebara, E., Sultan, S., Kocher-Braissant, J., and Toni, N. (2013). Adult hippocampal neurogenesis inversely correlates with microglia in conditions of voluntary running and aging. *Front. Neurosci.* 7:145. doi: 10.3389/fnins.2013.00145

Gonzalez, A. (2013). The impact of childhood maltreatment on biological systems: implications for clinical interventions. *Paediatr. Child Health* 18, 415–418.

Grigoleit, J. S., Kullmann, J. S., Wolf, O. T., Hammes, F., Wegner, A., Jablonowski, S., et al. (2011). Dose-dependent effects of endotoxin on neurobehavioral functions in humans. *PLoS One* 6:e28330. doi: 10.1371/journal.pone.0028330

Groves, J. O. (2007). Is it time to reassess the BDNF hypothesis of depression? *Mol. Psychiatry* 12, 1079–1088. doi: 10.1038/sj.mp.4002075

Hagan, M. J., Roubinov, D. S., Mistler, A. K., and Luecken, L. J. (2014). Mental health outcomes in emerging adults exposed to childhood maltreatment: the moderating role of stress reactivity. *Child Maltreat.* doi: 10.1177/1077559514539753

Hashmi, A. M., Butt, Z., and Umair, M. (2013). Is depression an inflammatory condition? A review of available evidence. *J. Pak. Med. Assoc.* 63, 899–906.

Hestad, K. A., Tønseth, S., Støen, C. D., Ueland, T., and Aukrust, P. (2003). Raised plasma levels of tumor necrosis factor alpha in patients with depression: normalization during electroconvulsive therapy. *J. ECT* 19, 183–188. doi: 10.1097/00124509-200312000-00002

Holmes, W. C., and Slap, G. B. (1998). Sexual abuse of boys: definition, prevalence, correlates, sequelae and management. *JAMA* 280, 1855–1862. doi: 10.1001/jama.280.21.1855

Horesh, N., Klomek, A. B., and Apter, A. (2008). Stressful life events and major depressive disorders. *Psychiatry Res.* 160, 192–199. doi: 10.1016/j.psychres.2007.06.008

Howren, M. B., Lamkin, D. M., and Suls, J. (2009). Associations of depression with C-reactive protein, IL-1 and IL-6: a meta-analysis. *Psychosom. Med.* 71, 171–186. doi: 10.1097/psy.0b013e3181907c1b

Kanamüller, J., Riala, K., Nivala, M., Hakko, H., and Räsänen, P. (2014). Correlates of sexual abuse in a sample of adolescent girls admitted to psychiatric inpatient care. *J. Child Sex. Abus.* 23, 804–823. doi: 10.1080/10538712.2014.950401

Keller, S., Sarchiapone, M., Zarrilli, F., Videtic, A., Ferraro, A., Carli, V., et al. (2010). Increased BDNF promoter methylation in the Wernicke area of suicide subjects. *Arch. Gen. Psychiatry* 67, 258–267. doi: 10.1001/archgenpsychiatry.2010.9

Kendler, K. S., Kuhn, J., and Prescott, C. A. (2004a). The interrelationship of neuroticism, sex and stressful life events in the prediction of episodes of major depression. *Am. J. Psychiatry* 161, 631–636. doi: 10.1176/appi.ajp.161.4.631

Kendler, K. S., Kuhn, J. W., and Prescott, C. A. (2004b). Childhood sexual abuse, stressful life events and risk for major depression in women. *Psychol. Med.* 34, 1475–1482. doi: 10.1017/s003329170400265x

Kofink, D., Boks, M. P., Timmers, H. T., and Kas, M. J. (2013). Epigenetic dynamics in psychiatric disorders: environmental programming of neurodevelopmental processes. *Neurosci. Biobehav. Rev.* 37, 831–845. doi: 10.1016/j.neubiorev.2013.03.020

Koo, J. W., Park, C. H., Choi, S. H., Kim, N. J., Kim, H. S., Choe, J. C., et al. (2003). The postnatal environment can counteract prenatal effects on cognitive ability, cell proliferation and synaptic protein expression. *FASEB J.* 17, 1556–1558. doi: 10.1096/fj.02-1032fje

Kozisek, M. E., Middlemas, D., and Bylund, D. B. (2008). Brain-derived neurotrophic factor and its receptor tropomyosin-related kinase B in the

mechanism of action of antidepressant therapies. *Pharmacol. Ther.* 117, 30–51. doi: 10.1016/j.pharmthera.2007.07.001

Kreisel, T., Frank, M. G., Licht, T., Reshef, R., Ben-Menachem-Zidon, O., Baratta, M. V., et al. (2014). Dynamic microglial alterations underlie stress-induced depressive-like behavior and suppressed neurogenesis. *Mol. Psychiatry* 19, 699–709. doi: 10.1038/mp.2013.155

Krishnan, V., and Nestler, E. J. (2008). The molecular neurobiology of depression. *Nature* 455, 894–902. doi: 10.1038/nature07455

Kronfol, Z., Silva, J. Jr., Greden, J., Dembinski, S., Gardner, R., and Carroll, B. (1983). Impaired lymphocyte function in depressive illness. *Life Sci.* 33, 241–247. doi: 10.1016/0024-3205(83)90382-x

Lanquillon, S., Krieg, J. C., Bening-Abu-Shach, U., and Vedder, H. (2000). Cytokine production and treatment response in major depressive disorder. *Neuropsychopharmacology* 22, 370–379. doi: 10.1016/s0893-133x(99)00134-7

Lau, A. G., Irier, H. A., Gu, J., Tian, D., Ku, L., Liu, G., et al. (2010). Distinct 3'UTRs differentially regulate activity-dependent translation of brain-derived neurotrophic factor (BDNF). *Proc. Natl. Acad. Sci. U S A* 107, 15945–15950. doi: 10.1073/pnas.1002929107

Lemaire, V., Koehl, M., Le Moal, M., and Abrous, D. N. (2000). Prenatal stress produces learning deficits associated with an inhibition of neurogenesis in the hippocampus. *Proc. Natl. Acad. Sci. U S A* 97, 11032–11037. doi: 10.1073/pnas.97.20.11032

Letourneau, E. J., Eaton, W. W., Bass, J., Berlin, F. S., and Moore, S. G. (2014). The need for a comprehensive public health approach to preventing child sexual abuse. *Public Health Rep.* 129, 222–228.

Liu, Y., Ho, R. C., and Mak, A. (2012). Interleukin (IL)-6, tumour necrosis factor alpha (TNF-alpha) and soluble interleukin-2 receptors (sIL-2R) are elevated in patients with major depressive disorder: a meta-analysis and meta-regression. *J. Affect. Disord.* 139, 230–239. doi: 10.1016/j.jad.2011.08.003

Lu, Y., Christian, K., and Lu, B. (2008). BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory? *Neurobiol. Learn. Mem.* 89, 312–323. doi: 10.1016/j.nlm.2007.08.018

Lu, B., Pang, P. T., and Woo, N. H. (2005). The yin and yang of neurotrophin action. *Nat. Rev. Neurosci.* 6, 603–614. doi: 10.1038/nrn1726

Luoni, A., Berry, A., Calabrese, F., Capoccia, S., Bellisario, V., Gass, P., et al. (2014). Delayed BDNF alterations in the prefrontal cortex of rats exposed to prenatal stress: preventive effect of lurasidone treatment during adolescence. *Eur. Neuropsychopharmacol.* 24, 986–995. doi: 10.1016/j.euroneuro.2013.12.010

Maes, M. (1995). Evidence for an immune response in major depression: a review and hypothesis. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 19, 11–38. doi: 10.1016/0278-5846(94)00101-m

Maes, M., Meltzer, H. Y., Bosmans, E., Bergmans, R., Vandoolaeghe, E., Ranjan, R., et al. (1995a). Increased plasma concentrations of interleukin-6, soluble interleukin-6, soluble interleukin-2 and transferrin receptor in major depression. *J. Affect. Disord.* 34, 301–309. doi: 10.1016/0165-0327(95)00028-l

Maes, M., Meltzer, H. Y., Buckley, P., and Bosmans, E. (1995b). Plasma-soluble interleukin-2 and transferrin receptor in schizophrenia and major depression. *Eur. Arch. Psychiatry Clin. Neurosci.* 244, 325–329. doi: 10.1007/bf02190412

Maes, M., Smith, R., and Scharpe, S. (1995c). The monocyte-T-lymphocyte hypothesis of major depression. *Psychoneuroendocrinology* 20, 111–116. doi: 10.1016/0306-4530(94)00066-j

Maes, M., Vandoolaeghe, E., Ranjan, R., Bosmans, E., Bergmans, R., and Desnyder, R. (1995d). Increased serum interleukin-1-receptor-antagonist concentrations in major depression. *J. Affect. Disord.* 36, 29–36. doi: 10.1016/0165-0327(95)00049-6

Maes, M., Wauters, A., Neels, H., Scharpé, S., Van Gastel, A., D'Hondt, P., et al. (1995e). Total serum protein and serum protein fractions in depression: relationships to depressive symptoms and glucocorticoid activity. *J. Affect. Disord.* 34, 61–69. doi: 10.1016/0165-0327(94)00106-j

Maes, M., Yirmyia, R., Noraberg, J., Brene, S., Hibbeln, J., Perini, G., et al. (2009). The inflammatory & neurodegenerative (I&ND) hypothesis of depression: leads for future research and new drug developments in depression. *Metab. Brain Dis.* 24, 27–53. doi: 10.1007/s11011-008-9118-1

Maffioletti, E., Tardito, D., Gennarelli, M., and Bocchio-Chiavetto, L. (2014). Micro spies from the brain to the periphery: new clues from studies on microRNAs in neuropsychiatric disorders. *Front. Cell. Neurosci.* 8:75. doi: 10.3389/fncel.2014.00075

Malberg, J. E. (2004). Implications of adult hippocampal neurogenesis in antidepressant action. *J. Psychiatry Neurosci.* 29, 196–205.

Manji, H. K., Quiroz, J. A., Sporn, J., Payne, J. L., Denicoff, K., Gray, N. A., et al. (2003). Enhancing neuronal plasticity and cellular resilience to develop novel, improved therapeutics for difficult-to-treat depression. *Biol. Psychiatry* 53, 707–742. doi: 10.1016/s0006-3223(03)00117-3

Martinowich, K., Manji, H., and Lu, B. (2007). New insights into BDNF function in depression and anxiety. *Nat. Neurosci.* 10, 1089–1093. doi: 10.1038/nn1971

Matrisciano, F., Tueting, P., Maccari, S., Nicoletti, F., and Guidotti, A. (2012). Pharmacological activation of group-II metabotropic glutamate receptors corrects a schizophrenia-like phenotype induced by prenatal stress in mice. *Neuropsychopharmacology* 37, 929–938. doi: 10.1038/npp.2011.274

McClung, C. A., and Nestler, E. J. (2008). Neuroplasticity mediated by altered gene expression. *Neuropsychopharmacology* 33, 3–17. doi: 10.1038/sj.npp.1301544

McEwen, B. S., Eiland, L., Hunter, R. G., and Miller, M. M. (2012). Stress and anxiety: structural plasticity and epigenetic regulation as a consequence of stress. *Neuropharmacology* 62, 3–12. doi: 10.1016/j.neuropharm.2011.07.014

McGowan, P. O., Sasaki, A., D'Alessio, A. C., Dymov, S., Labonté, B., Szyf, M., et al. (2009). Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat. Neurosci.* 12, 342–348. doi: 10.1038/nn.2270

Meaney, M. J., and Szyf, M. (2005). Environmental programming of stress responses through DNA methylation: life at the interface between a dynamic environment and a fixed genome. *Dialogues Clin. Neurosci.* 7, 103–123.

Mehta, D., Klengel, T., Conneely, K. N., Smith, A. K., Altmann, A., Pace, T. W., et al. (2013). Childhood maltreatment is associated with distinct genomic and epigenetic profiles in posttraumatic stress disorder. *Proc. Natl. Acad. Sci. U S A* 110, 8302–8307. doi: 10.1073/pnas.1217750110

Michelsen, K. A., van den Hove, D. L., Schmitz, C., Segers, O., Prickaerts, J., and Steinbusch, H. W. (2007). Prenatal stress and subsequent exposure to chronic mild stress influence dendritic spine density and morphology in the rat medial prefrontal cortex. *BMC Neurosci.* 8:107. doi: 10.1186/1471-2202-8-107

Miller, G., and Chen, E. (2007). Unfavorable socioeconomic conditions in early life presage expression of proinflammatory phenotype in adolescence. *Psychosom. Med.* 69, 402–409. doi: 10.1097/psy.0b013e318068fcf9

Miller, G. E., and Cole, S. W. (2012). Clustering of depression and inflammation in adolescents previously exposed to childhood adversity. *Biol. Psychiatry* 72, 34–40. doi: 10.1016/j.biopsych.2012.02.034

Miller, A. H., Maletic, V., and Raison, C. L. (2009). Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. *Biol. Psychiatry* 65, 732–741. doi: 10.1016/j.biopsych.2008.11.029

Mills, N. T., Scott, J. G., Wray, N. R., Cohen-Woods, S., and Baune, B. T. (2013). Research review: the role of cytokines in depression in adolescents: a systematic review. *J. Child Psychol. Psychiatry* 54, 816–835. doi: 10.1111/jcpp.12080

Mirescu, C., Peters, J. D., and Gould, E. (2004). Early life experience alters response of adult neurogenesis to stress. *Nat. Neurosci.* 7, 841–846. doi: 10.1038/nn1290

Miyaoka, H., Otsubo, T., Kamijima, K., Ishii, M., Onuki, M., and Mitamura, K. (1999). Depression from interferon therapy in patients with hepatitis C. *Am. J. Psychiatry* 156, 1120.

Molendijk, M. L., Spinhoven, P., Polak, M., Bus, B. A., Penninx, B. W., and Elzinga, B. M. (2014). Serum BDNF concentrations as peripheral manifestations of depression: evidence from a systematic review and meta-analyses on 179 associations (N=9484). *Mol. Psychiatry* 19, 791–800. doi: 10.1038/mp.2013.105

Molteni, R., Calabrese, F., Bedogni, F., Tongiorgi, E., Fumagalli, F., Racagni, G., et al. (2006). Chronic treatment with fluoxetine up-regulates cellular BDNF mRNA expression in rat dopaminergic regions. *Int. J. Neuropsychopharmacol.* 9, 307–317. doi: 10.1017/s1461145705005766

Molteni, R., Cattaneo, A., Calabrese, F., Macchi, F., Olivier, J. D., Racagni, G., et al. (2010). Reduced function of the serotonin transporter is associated with decreased expression of BDNF in rodents as well as in humans. *Neurobiol. Dis.* 37, 747–755. doi: 10.1016/j.nbd.2009.12.014

Monje, M. L., Toda, H., and Palmer, T. D. (2003). Inflammatory blockade restores adult hippocampal neurogenesis. *Science* 302, 1760–1765. doi: 10.1126/science.1088417

Murgatroyd, C., Patchev, A. V., Wu, Y., Micale, V., Bockmühl, Y., Fischer, D., et al. (2009). Dynamic DNA methylation programs persistent adverse effects of early-life stress. *Nat. Neurosci.* 12, 1559–1566. doi: 10.1038/nn.2436

Murmu, M. S., Salomon, S., Biala, Y., Weinstock, M., Braun, K., and Bock, J. (2006). Changes of spine density and dendritic complexity in the prefrontal cortex in

offspring of mothers exposed to stress during pregnancy. *Eur. J. Neurosci.* 24, 1477–1487. doi: 10.1111/j.1460-9568.2006.05024.x

Myint, A. M., and Kim, Y. K. (2014). Network beyond IDO in psychiatric disorders: revisiting neurodegeneration hypothesis. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 48, 304–313. doi: 10.1016/j.pnpbp.2013.08.008

Nair, A., Vadodaria, K. C., Banerjee, S. B., Benekareddy, M., Dias, B. G., Duman, R. S., et al. (2007). Stressor-specific regulation of distinct brain-derived neurotrophic factor transcripts and cyclic AMP response element-binding protein expression in the postnatal and adult rat hippocampus. *Neuropsychopharmacology* 32, 1504–1519. doi: 10.1038/sj.npp.1301276

Najjar, S., and Pearlman, D. M. (2015). Neuroinflammation and white matter pathology in schizophrenia: systematic review. *Schizophr. Res.* 161, 102–112. doi: 10.1016/j.schres.2014.04.041

Nieratschker, V., Massart, R., Gilles, M., Luoni, A., Suderman, M. J., Krumm, B., et al. (2014). MORC1 exhibits cross-species differential methylation in association with early life stress as well as genome-wide association with MDD. *Transl. Psychiatry* 4:e429. doi: 10.1038/tp.2014.75

Pace, T. W., Mletzko, T. C., Alagbe, O., Musselman, D. L., Nemeroff, C. B., Miller, A. H., et al. (2006). Increased stress-induced inflammatory responses in male patients with major depression and increased early life stress. *Am. J. Psychiatry* 163, 1630–1633. doi: 10.1176/appi.ajp.163.9.1630

Paolicelli, R. C., Bisht, K., and Tremblay, M. E. (2014). Fractalkine regulation of microglial physiology and consequences on the brain and behavior. *Front. Cell. Neurosci.* 8:129. doi: 10.3389/fncel.2014.00129

Pariante, C. M., and Miller, A. H. (2001). Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment. *Biol. Psychiatry* 49, 391–404. doi: 10.1016/s0006-3223(00)01088-x

Pike, J. L., and Irwin, M. R. (2006). Dissociation of inflammatory markers and natural killer cell activity in major depressive disorder. *Brain Behav. Immun.* 20, 169–174. doi: 10.1016/j.bbi.2005.05.004

Pikhart, H., Hubacek, J. A., Kubinova, R., Nicholson, A., Peasey, A., Capkova, N., et al. (2009). Depressive symptoms and levels of C-reactive protein: a population-based study. *Soc. Psychiatry Psychiatr. Epidemiol.* 44, 217–222. doi: 10.1007/s00127-008-0422-1

Pittenger, C., and Duman, R. S. (2008). Stress, depression and neuroplasticity: a convergence of mechanisms. *Neuropsychopharmacology* 33, 88–109. doi: 10.1038/sj.npp.1301574

Pollak, Y., and Yirmiya, R. (2002). Cytokine-induced changes in mood and behaviour: implications for 'depression due to a general medical condition', immunotherapy and antidepressive treatment. *Int. J. Neuropsychopharmacol.* 5, 389–399. doi: 10.1017/s1461145702003152

Powell, T. R., Schalkwyk, L. C., Heffernan, A. L., Breen, G., Lawrence, T., Price, T., et al. (2013). Tumor necrosis factor and its targets in the inflammatory cytokine pathway are identified as putative transcriptomic biomarkers for escitalopram response. *Eur. Neuropsychopharmacol.* 23, 1105–1114. doi: 10.1016/j.euroneuro.2012.09.009

Provençal, N., and Binder, E. B. (2014a). The effects of early life stress on the epigenome: from the womb to adulthood and even before. *Exp. Neurol.* doi: 10.1016/j.expneurol.2014.09.001. [Epub ahead of print].

Provencal, N., and Binder, E. B. (2014b). The neurobiological effects of stress as contributors to psychiatric disorders: focus on epigenetics. *Curr. Opin. Neurobiol.* 30C, 31–37. doi: 10.1016/j.conb.2014.08.007

Provençal, N., Suderman, M. J., Guillemin, C., Massart, R., Ruggiero, A., Wang, D., et al. (2012). The signature of maternal rearing in the methylome in rhesus macaque prefrontal cortex and T cells. *J. Neurosci.* 32, 15626–15642. doi: 10.1523/JNEUROSCI.1470-12.2012

Raison, C. L., Borisov, A. S., Broadwell, S. D., Capuron, L., Woolwine, B. J., Jacobson, I. M., et al. (2005). Depression during pegylated interferon-alpha plus ribavirin therapy: prevalence and prediction. *J. Clin. Psychiatry* 66, 41–48. doi: 10.4088/jcp.v66n0106

Reul, J. M. (2014). Making memories of stressful events: a journey along epigenetic, gene transcription and signaling pathways. *Front. Psychiatry* 5:5. doi: 10.3389/fpsyt.2014.00005

Roceri, M., Cirulli, F., Pessina, C., Peretto, P., Racagni, G., and Riva, M. A. (2004). Postnatal repeated maternal deprivation produces age-dependent changes of brain-derived neurotrophic factor expression in selected rat brain regions. *Biol. Psychiatry* 55, 708–714. doi: 10.1016/j.biopsych.2003.12.011

Roceri, M., Hendriks, W., Racagni, G., Ellenbroek, B. A., and Riva, M. A. (2002). Early maternal deprivation reduces the expression of BDNF and NMDA receptor subunits in rat hippocampus. *Mol. Psychiatry* 7, 609–616. doi: 10.1038/sj.mp.4001036

Roth, T. L., Lubin, F. D., Funk, A. J., and Sweatt, J. D. (2009). Lasting epigenetic influence of early-life adversity on the BDNF gene. *Biol. Psychiatry* 65, 760–769. doi: 10.1016/j.biopsych.2008.11.028

Roth, M., Neuner, F., and Elbert, T. (2014). Transgenerational consequences of PTSD: risk factors for the mental health of children whose mothers have been exposed to the Rwandan genocide. *Int. J. Ment. Health Syst.* 8:12. doi: 10.1186/1752-4458-8-12

Saarelainen, T., Hendolin, P., Lucas, G., Koponen, E., Sairanen, M., MacDonald, E., et al. (2003). Activation of the TrkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral effects. *J. Neurosci.* 23, 349–357.

Sairanen, M., Lucas, G., Ernfors, P., Castrén, M., and Castrén, E. (2005). Brain-derived neurotrophic factor and antidepressant drugs have different but coordinated effects on neuronal turnover, proliferation and survival in the adult dentate gyrus. *J. Neurosci.* 25, 1089–1094. doi: 10.1523/jneurosci.3741-04.2005

Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., et al. (2003). Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* 301, 805–809. doi: 10.1126/science.1083328

Schiepers, O. J., Wichers, M. C., and Maes, M. (2005). Cytokines and major depression. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 29, 201–217. doi: 10.1016/j.pnpbp.2004.11.003

Schwarz, J. M., Hutchinson, M. R., and Bilbo, S. D. (2011). Early-life experience decreases drug-induced reinstatement of morphine CPP in adulthood via microglial-specific epigenetic programming of anti-inflammatory IL-10 expression. *J. Neurosci.* 31, 17835–17847. doi: 10.1523/JNEUROSCI.3297-11.2011

Shelton, R. C., Claiborne, J., Sidoryk-Wegrzynowicz, M., Reddy, R., Aschner, M., Lewis, D. A., et al. (2011). Altered expression of genes involved in inflammation and apoptosis in frontal cortex in major depression. *Mol. Psychiatry* 16, 751–762. doi: 10.1038/mp.2010.52

Sierra, A., Beccari, S., Diaz-Aparicio, I., Encinas, J. M., Comeau, S., and Tremblay, M. È. (2014). Surveillance, phagocytosis and inflammation: how never-resting microglia influence adult hippocampal neurogenesis. *Neural Plast.* 2014:610343. doi: 10.1155/2014/610343

Slopen, N., Koenen, K. C., and Kubzansky, L. D. (2014). Cumulative adversity in childhood and emergent risk factors for long-term health. *J. Pediatr.* 164, 631–638.e2. doi: 10.1016/j.jpeds.2013.11.003

Sluzewska, A., Rybakowski, J. K., Laciak, M., Mackiewicz, A., Sobieska, M., and Wiktorowicz, K. (1995). Interleukin-6 serum levels in depressed patients before and after treatment with fluoxetine. *Ann. N Y Acad. Sci.* 762, 474–476. doi: 10.1111/j.1749-6632.1995.tb32372.x

Sluzewska, A., Rybakowski, J. K., Sobieska, M., and Wiktorowicz, K. (1996). Concentration and microheterogeneity glycophorms of alpha-1-acid glycoprotein in major depressive disorder. *J. Affect. Disord.* 39, 149–155. doi: 10.1016/0165-0327(96)00026-2

Sluzewska, A., Sobieska, M., and Rybakowski, J. K. (1997). Changes in acute-phase proteins during lithium potentiation of antidepressants in refractory depression. *Neuropsychobiology* 35, 123–127. doi: 10.1159/000119332

Sominsky, L., Fuller, E. A., Bondarenko, E., Ong, L. K., Averell, L., Nalivaiko, E., et al. (2013). Functional programming of the autonomic nervous system by early life immune exposure: implications for anxiety. *PLoS One* 8:e57700. doi: 10.1371/journal.pone.0057700

Stelzhammer, V., Haenisch, F., Chan, M. K., Cooper, J. D., Steiner, J., Steeb, H., et al. (2014). Proteomic changes in serum of first onset, antidepressant drug-naive major depression patients. *Int. J. Neuropsychopharmacol.* 17, 1599–1608. doi: 10.1017/S1461145714000819

Stertz, L., Magalhães, P. V., and Kapczinski, F. (2013). Is bipolar disorder an inflammatory condition? The relevance of microglial activation. *Curr. Opin. Psychiatry* 26, 19–26. doi: 10.1097/YCO.0b013e32835aa4b4

Szyf, M. (2013). The genome- and system-wide response of DNA methylation to early life adversity and its implication on mental health. *Can. J. Psychiatry* 58, 697–704.

Szyf, M., and Bick, J. (2013). DNA methylation: a mechanism for embedding early life experiences in the genome. *Child Dev.* 84, 49–57. doi: 10.1111/j.1467-8624.2012.01793.x

Szyf, M., Weaver, I. C., Champagne, F. A., Diorio, J., and Meaney, M. J. (2005). Maternal programming of steroid receptor expression and phenotype through DNA methylation in the rat. *Front. Neuroendocrinol.* 26, 139–162. doi: 10.1016/j.yfrne.2005.10.002

Taliaz, D., Loya, A., Gersner, R., Haramati, S., Chen, A., and Zangen, A. (2011). Resilience to chronic stress is mediated by hippocampal brain-derived neurotrophic factor. *J. Neurosci.* 31, 4475–4483. doi: 10.1523/JNEUROSCI.5725-10.2011

Thomas, A. J., Davis, S., Morris, C., Jackson, E., Harrison, R., and O'Brien, J. T. (2005). Increase in interleukin-1beta in late-life depression. *Am. J. Psychiatry* 162, 175–177. doi: 10.1176/appi.ajp.162.1.175

Tsankova, N. M., Berton, O., Renthal, W., Kumar, A., Neve, R. L., and Nestler, E. J. (2006). Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat. Neurosci.* 9, 519–525. doi: 10.1038/nn1659

Tsao, C. W., Lin, Y. S., Chen, C. C., Bai, C. H., and Wu, S. R. (2006). Cytokines and serotonin transporter in patients with major depression. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 30, 899–905. doi: 10.1016/j.pnpbp.2006.01.029

Tuglu, C., Kara, S. H., Caliyurt, O., Vardar, E., and Abay, E. (2003). Increased serum tumor necrosis factor-alpha levels and treatment response in major depressive disorder. *Psychopharmacology (Berl)* 170, 429–433. doi: 10.1007/s00213-003-1566-z

Tynan, R. J., Weidenhofer, J., Hinwood, M., Cairns, M. J., Day, T. A., and Walker, F. R. (2012). A comparative examination of the anti-inflammatory effects of SSRI and SNRI antidepressants on LPS stimulated microglia. *Brain Behav. Immun.* 26, 469–479. doi: 10.1016/j.bbi.2011.12.011

Uher, R., Tansey, K. E., Dew, T., Maier, W., Mors, O., Hauser, J., et al. (2014). An inflammatory biomarker as a differential predictor of outcome of depression treatment with Escitalopram and Nortriptyline. *Am. J. Psychiatry* doi: 10.1176/appi.ajp.2014.14010094. [Epub ahead of print].

Valkanova, V., Ebmeier, K. P., and Allan, C. L. (2013). CRP, IL-6 and depression: a systematic review and meta-analysis of longitudinal studies. *J. Affect. Disord.* 150, 736–744. doi: 10.1016/j.jad.2013.06.004

Visser, H. A., van Minnen, A., van Megen, H., Eikelenboom, M., Hoogendoorn, A. W., Kaarsemaker, M., et al. (2014). The relationship between adverse childhood experiences and symptom severity, chronicity and comorbidity in patients with obsessive-compulsive disorder. *J. Clin. Psychiatry* 75, 1034–1039. doi: 10.4088/JCP.13m08825

Wang, H., Garcia, C. A., Rehani, K., Cekic, C., Alard, P., Kinane, D. F., et al. (2008). IFN-beta production by TLR4-stimulated innate immune cells is negatively regulated by GSK3-beta. *J. Immunol.* 181, 6797–6802. doi: 10.4049/jimmunol.181.10.6797

Waterhouse, E. G., and Xu, B. (2009). New insights into the role of brain-derived neurotrophic factor in synaptic plasticity. *Mol. Cell. Neurosci.* 42, 81–89. doi: 10.1016/j.mcn.2009.06.009

Weissman, M. (2009). Depression. *Ann. Epidemiol.* 19, 264–267. doi: 10.1016/j.annepidem.2009.01.021

Weissman, M. M., and Talati, A. (2009). Depression in at-risk adolescents and their parents. *JAMA* 302, 1167; author reply 1167–1168. doi: 10.1001/jama.2009.1329

Williamson, L. L., and Bilbo, S. D. (2013). Chemokines and the hippocampus: a new perspective on hippocampal plasticity and vulnerability. *Brain Behav. Immun.* 30, 186–194. doi: 10.1016/j.bbi.2013.01.077

Williamson, L. L., Sholar, P. W., Mistry, R. S., Smith, S. H., and Bilbo, S. D. (2011). Microglia and memory: modulation by early-life infection. *J. Neurosci.* 31, 15511–15521. doi: 10.1523/JNEUROSCI.3688-11.2011

Wolkowitz, O. M., Wolf, J., Shelly, W., Rosser, R., Burke, H. M., Lerner, G. K., et al. (2011). Serum BDNF levels before treatment predict SSRI response in depression. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 35, 1623–1630. doi: 10.1016/j.pnpbp.2011.06.013

Yoshida, T., Ishikawa, M., Niitsu, T., Nakazato, M., Watanabe, H., Shiraishi, T., et al. (2012). Decreased serum levels of mature brain-derived neurotrophic factor (BDNF), but not its precursor proBDNF, in patients with major depressive disorder. *PLoS One* 7:e42676. doi: 10.1371/journal.pone.0042676

Zhao, Y. J., Du, M. Y., Huang, X. Q., Lui, S., Chen, Z. Q., Liu, J., et al. (2014). Brain grey matter abnormalities in medication-free patients with major depressive disorder: a meta-analysis. *Psychol. Med.* 44, 2927–2937. doi: 10.1017/s0033291714000518

**Conflict of Interest Statement**: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 14 August 2014; paper pending published: 31 December 2014; accepted: 27 January 2015; published online: 31 March 2015.*

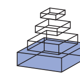
*Citation: Cattaneo A, Macchi F, Plazzotta G, Veronica B, Bocchio-Chiavetto L, Riva MA and Pariante CM (2015) Inflammation and neuronal plasticity: a link between childhood trauma and depression pathogenesis. Front. Cell. Neurosci.* ***9****:40. doi: 10.3389/fncel.2015.00040*

*This article was submitted to the journal Frontiers in Cellular Neuroscience.*

*Copyright © 2015 Cattaneo, Macchi, Plazzotta, Veronica, Bocchio-Chiavetto, Riva and Pariante. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution and reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 03 April 2014
doi: 10.3389/fncel.2014.00097



# Cellular and molecular mechanisms of immunomodulation in the brain through environmental enrichment

**Gaurav Singhal[1], Emily J. Jaehne[1], Frances Corrigan[2] and Bernhard T. Baune[1]***

[1] Psychiatric Neuroscience Lab, Discipline of Psychiatry, School of Medicine, University of Adelaide, Adelaide, SA, Australia
[2] Discipline of Anatomy and Physiology, School of Medical Sciences, University of Adelaide, Adelaide, SA, Australia

**Edited by:**
Laura Maggi, Sapienza, Italy

**Reviewed by:**
Ping Liu, University of Connecticut Health Center, USA
Michele Zoli, Università di Modena e Reggio Emilia, Italy

***Correspondence:**
Bernhard T. Baune, Psychiatric Neuroscience Lab, Discipline of Psychiatry, School of Medicine, University of Adelaide, Level 4, Eleanor Harrald Building, Royal Adelaide Hospital, Adelaide, SA 5005, Australia
e-mail: bernhard.baune@adelaide.edu.au

Recent studies on environmental enrichment (EE) have shown cytokines, cellular immune components [e.g., T lymphocytes, natural killer (NK) cells], and glial cells in causal relationship to EE in bringing out changes to neurobiology and behavior. The purpose of this review is to evaluate these neuroimmune mechanisms associated with neurobiological and behavioral changes in response to different EE methods. We systematically reviewed common research databases. After applying all inclusion and exclusion criteria, 328 articles remained for this review. Physical exercise (PE), a form of EE, elicits anti-inflammatory and neuromodulatory effects through interaction with several immune pathways including interleukin (IL)-6 secretion from muscle fibers, reduced expression of Toll-like receptors on monocytes and macrophages, reduced secretion of adipokines, modulation of hippocampal T cells, priming of microglia, and upregulation of mitogen-activated protein kinase phosphatase-1 in central nervous system. In contrast, immunomodulatory roles of other enrichment methods are not studied extensively. Nonetheless, studies showing reduction in the expression of IL-1β and tumor necrosis factor-α in response to enrichment with novel objects and accessories suggest anti-inflammatory effects of novel environment. Likewise, social enrichment, though considered a necessity for healthy behavior, results in immunosuppression in socially defeated animals. This has been attributed to reduction in T lymphocytes, NK cells and IL-10 in subordinate animals. EE through sensory stimuli has been investigated to a lesser extent and the effect on immune factors has not been evaluated yet. Discovery of this multidimensional relationship between immune system, brain functioning, and EE has paved a way toward formulating environ-immuno therapies for treating psychiatric illnesses with minimal use of pharmacotherapy. While the immunomodulatory role of PE has been evaluated extensively, more research is required to investigate neuroimmune changes associated with other enrichment methods.

**Keywords: environmental enrichment, immune, cytokines, glial cells, T cells, neurobiology, cognition, behavior**

## INTRODUCTION

Cognitive deficit, memory loss, and behavioral impairment underpin most psychiatric disorders. Several etiologies such as age, gender, and race (Piccinelli and Wilkinson, 2000; Gottlieb et al., 2004; Hedden and Gabrieli, 2004), stress (Lupien et al., 2009), socioeconomic status (Gilman et al., 2002; Lorant et al., 2003), metabolic disorders (Simon et al., 2006; Rinaldi et al., 2014), gene–environment interactions (Caspi and Moffitt, 2006), and neuroinflammation (Campbell, 2004; Ownby, 2010; Tansey and Goldberg, 2010) have been implicated for the impairment of brain function. Contrary to this, environmental enrichment (EE), a concept of "modifying the environment of captive animals to enhance their physical and psychological well-being by providing stimuli meeting their species-specific need" (Baumans, 2005), has been shown to slow down neuronal aging (Gould et al., 2000; Kempermann et al., 2002) and improve cognition, memory, behavior, and motor coordination in pre-clinical models of dementia, depression, Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (Faherty et al., 2005; Jankowsky et al., 2005; Hannan, 2014).

Although most EE paradigms used rodents for research, other animals like rabbits (Hansen and Berthelsen, 2000), pigs (van de Weerd and Day, 2009), fish (Batzina et al., 2014), and primates (e.g., marmosets; Kozorovitskiy et al., 2005) have also been used in EE studies. Researchers have used several methods of EE for rodents in cages, either alone or in combination. Physical exercise (PE), social housing, and enrichment with novel objects and accessories are the most commonly used methods. On the other hand, the sensory method of enrichment, where a sensory stimulus such as visual, auditory, and olfactory stimuli is given to stimulate brain functions, has been used to a lesser extent.

Equivalent treatments of EE in rodents can be seen in human literature. Although not operationally similar, these treatments promote mental stimulation and can provide enrichment to the standard human environment, similar to EE in rodent studies. These could include aerobic exercise (Colcombe et al., 2006; Hillman et al., 2008), an active and socially integrated lifestyle (Fratiglioni et al., 2004), cognitive training with brain storming exercises (Willis et al., 2006; Miller et al., 2013), learning of complex tasks (e.g., learning to juggle balls; Draganski et al.,

2004; Boyke et al., 2008), extensive learning during examinations (Draganski et al., 2006), food supplementation (Fuglestad et al., 2008), and sensory enhancement (e.g., listening to favorite music; Särkämö et al., 2008; Koelsch, 2010). Like EE in rodents, these treatments have shown similar effects in humans, improving cognition and memory, which means there may be similar mechanisms of action of both on the central nervous system (CNS).

Recent studies have shown that EE is able to affect cytokines, various immune components and glial cells suggesting this may be a potential mechanism of action for how it may modulate brain function. The discovery of the modulation of neuroimmune mechanisms by EE has provided a potential mechanism of action of this intervention. Circulating immune cells and proteins (e.g., T cells and cytokines) maintain the brain homeostasis (Ron-Harel et al., 2011) forming a bi-directional neuroimmune pathway which can affect behavior, mood, and cognition (Maier and Watkins, 1998). It has been suggested that there are modifications in several immune markers, for example, cytokines (Pedersen and Hoffman-Goetz, 2000), chemokines (Trøseid et al., 2004), T cells (Marashi et al., 2003, 2004), natural killer (NK) cells (Benaroya-Milshtein et al., 2004), Toll-like receptors (TLRs; Gleeson et al., 2006), C-reactive protein (CRP; Koletzko, 2003), and glial cells (Ehninger and Kempermann, 2003; Williamson et al., 2012) alongside neurobiological and behavioral alterations in rodents treated with different enrichment techniques.

Regardless of this significant relationship, the immunomodulatory role of EE has received less attention than its neurobiological and behavioral effects. While independent rodent studies predominantly put forward a role of PE in inducing changes to neuroimmune markers such as cytokines [e.g., tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6], T lymphocytes, NK cells, glial cells (e.g., microglia), CRP, and the complement system, less information is available about the immunomodulatory role of other enrichment methods, like social housing and enrichment with novel objects and accessories in rodents. In this review, we aim to fill this gap in the existing literature by conducting a critical analysis of randomized controlled trials (RCTs) on rodents and analogous human studies, exploring the neuroimmune modulatory effects of EE and how this may improve cognition and memory. A particular emphasis is placed on how neuroimmune mechanisms that modulate brain function differ in response to various EE methods in this review.

## MATERIALS AND METHODS

### PRISMA CRITERIA

We followed the guidelines prescribed by PRISMA (preferred reporting items for systematic reviews and meta-analyses; Liberati et al., 2009; Moher et al., 2009) while constructing this review. The checklist items from PRISMA as relevant to this review, for example, those related to search and writing approaches, were included and the items not relevant, for example, those related to meta-analyses, were excluded.

### SEARCH AND SELECTION PROCESS

An electronic database search of PubMed, Google Scholar, and ScienceDirect with the following key terms in various permutations was performed: environmental enrichment, immune, neuroimmune, cytokine, glial cells, T cells, B cells, immunoglobulins, NK cells, Toll-like receptor, C-reactive protein, complement system, neuroplasticity, neuropathology, inflammation, neuroinflammation, cognition, cognitive stimulation, cognitive remediation, cognitive training, cognitive rehabilitation, memory, behavior, physical exercise, novelty, rodents, social enrichment, social interaction, social factors, cage enrichment, nesting, nutrition, sensory enrichment, visual cortex, auditory cortex, olfactory bulb, music, and motor activity. At each stage of the search, titles and abstracts were scrutinized and the most appropriate organized into separate folders using End Note X6.0.1 software. In addition, articles relevant to our discussion were retrieved from the reference list of other online articles on each subtopic. This in total yielded approximately 1700 papers. After placing all inclusion and exclusion criteria into our search (depicted in **Figure 1**), 328 articles closely related to the aims set forth for this review were selected and hence utilized.

### INCLUSION AND EXCLUSION CRITERIA

Articles on EE paradigms in rodents were mainly selected for detailed analysis. Articles which investigated the effects of an environmental stimulus on nervous and/or immune systems, but not specifically discussing EE were consulted in less depth and are cited wherever required for the convenience of readers. Similarly, analogous evidence to each EE method in human studies have also been included and cited. This raised the total number of articles cited to 328. EE paradigms on animals other than rodents were excluded due to their small numbers and to maintain uniformity. All articles included in this review have been published between 1988 and 2014. Articles without the full text available were excluded from the review.

## ROLE OF IMMUNE FACTORS IN NEUROBIOLOGY AND BEHAVIOR

Various cytokines and circulating T cells have been shown to play important role in hippocampal neurogenesis and in the molecular and cellular mechanisms responsible for learning, memory, and cognition under physiological conditions (McAfoose and Baune, 2009; Yirmiya and Goshen, 2011). Further, these immune factors also maintain homeostasis and influence molecular mechanisms involved in monoamine metabolism, the sensitivity of the hypothalamic–pituitary–adrenal (HPA) axis to cortisol and certain other cellular neuroimmune functions in constitutive levels (Eyre and Baune, 2012). Conversely, levels of both pro-inflammatory and anti-inflammatory cytokines in the peripheral circulation and CNS rise during several brain disorders such as depression, schizophrenia, and AD (Schwarz et al., 2001).

In the course of pathological conditions, such as stroke and related diseases (Kim, 1996) and environment adversities, such as social stress (Avitsur et al., 2005), a cytokine cascade is initiated and brain cells express various pro-inflammatory cytokines, chemokines, and adhesion molecules. The first two cytokines in the cytokine cascade, TNF-α and IL-1β (Th1 type, stimulatory), are pro-inflammatory and produced locally. They further activate granulocytes, monocytes/macrophages, NK cells, and T and B cells, and recruit them to the sites of inflammation (Petersen

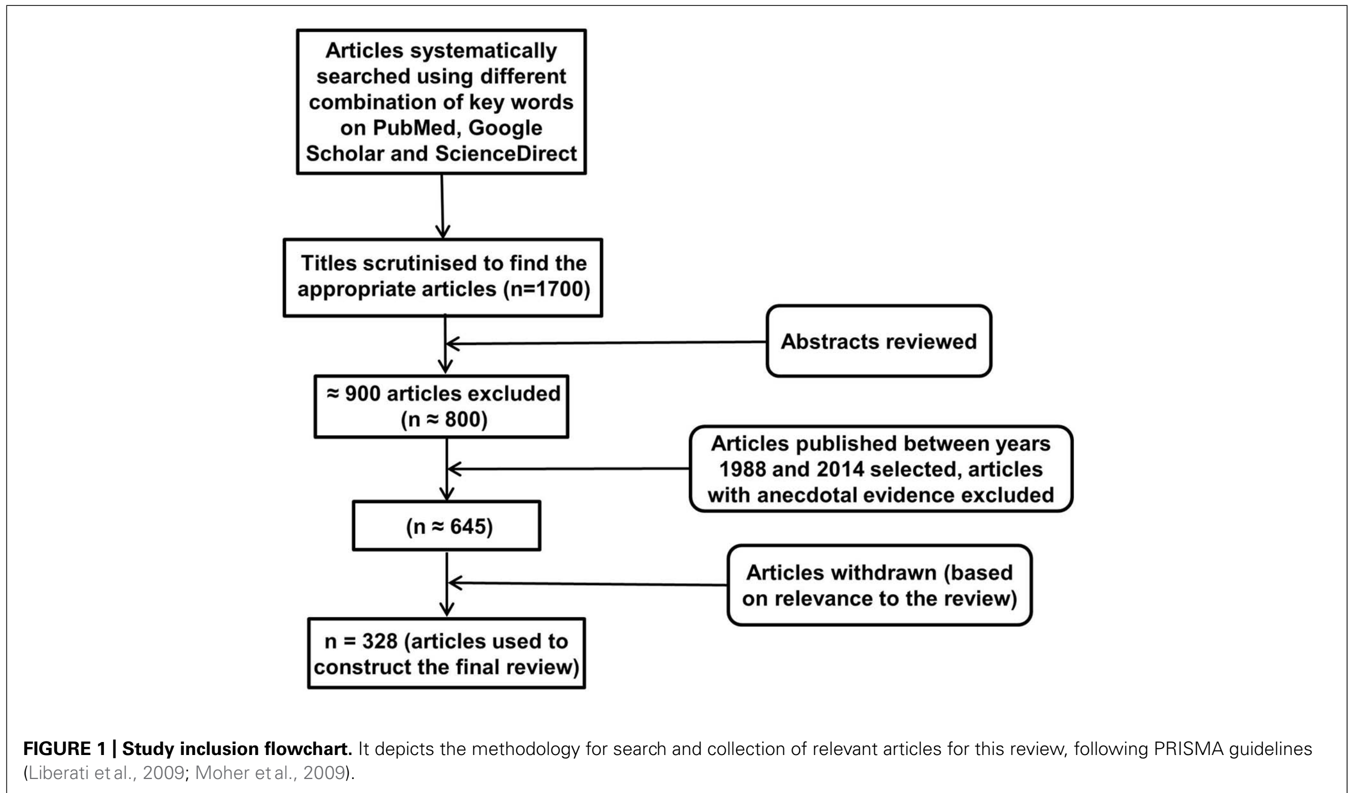



**FIGURE 1 | Study inclusion flowchart.** It depicts the methodology for search and collection of relevant articles for this review, following PRISMA guidelines (Liberati et al., 2009; Moher et al., 2009).

and Pedersen, 2005, 2006). Importantly, studies investigating the correlation between cytokine levels and occurrence of AD have reported the presence of cytokines TNF-α and IL-1β in cerebrospinal fluid (Tarkowski et al., 2003), and elevated plasma levels of cytokines IL-1β and IL-6 (Licastro et al., 2000) in patients, which suggests their active role in the pathophysiology of AD. Indeed, elevated levels of TNF-α in particular have been shown to cause a reduction in hippocampal volumes through the neurodegenerative TNF receptor 1 (TNFR1) pathway (Baune et al., 2012) and can lead to the development of depressive-like behavior (Eyre et al., 2013). A regression analysis on a cohort of non-demented community-dwelling adults aged between 70 and 90 years showed that increased levels of cytokines during systemic inflammation are related to cognitive deficit in a non-clinical community-dwelling population, independent of depression, cardiovascular and metabolic risk factors (Trollor et al., 2012), highlighting the significance of levels of cytokines in systemic circulation for brain function.

Experiments in rodents have revealed that the level of pro-inflammatory cytokines in the brain rise with aging and are directly related to age-related impairments in learning, memory, and cognition (Tha et al., 2000). This indicates that pro-inflammatory cytokines are involved in promoting neuroinflammation during old age and play a role in associated psychiatric disorders which are generally accompanied by memory and cognitive deficits. It should, however, be noted that pro-inflammatory cytokines can also stimulate anti-inflammatory pathways, through, for example, enhancement of the production of anti-inflammatory cytokines such as IL-1ra and IL-10. These anti-inflammatory cytokines can then inhibit the production of TNF-α and IL-1β (Opal and DePalo, 2000; Sredni-Kenigsbuch, 2002; Petersen and Pedersen, 2005), thereby reducing inflammation and marking the end of the cytokine cascade.

Several other humoral immune factors have also been reported to modify brain anatomy and functions. These include TLRs, mitogen-activated protein kinases (MAPKs), CRP, the complement system, chemokines, and immunoglobulins (Igs). The enhanced expression of TLR-3 and -4, the proteins expressed by glial cells and oligodendrocytes in the brain, has been observed in inflamed CNS tissues during immunohistochemical post-mortem brain analysis (Bsibsi et al., 2002). They are indeed reported to be actively involved in the modulation of innate (Medzhitov, 2001) and adaptive immune responses, and regulation of dendritic cell functions (Iwasaki and Medzhitov, 2004). Likewise, MAPKs which are specific protein kinases (serine–threonine specific), elicit pro-inflammatory and immunomodulatory functions (Lee et al., 1994; Dong et al., 2002) in the brain and their signaling is controlled by MKP-1 (MAPK phosphatase-1), a dual-specificity phosphatase. CRP, which is an acute phase reactant protein, has been shown to enhance inflammation and tissue damage by promoting phagocytosis by opsonization (Du Clos, 2000) and activating the complement system (Padilla et al., 2003). The latter consists of distinct plasma proteins that also act as opsonins and initiate a series of inflammatory responses (Janeway et al., 2001). Researchers have observed upregulation of the complement system in human brain during AD and other neurodegenerative diseases (McGeer and McGeer, 1995; Yasojima et al., 1999). Similarly, high levels of CRP in the brain

have been linked to cognitive impairment and dementia (Kuo et al., 2005), and AD (McGeer et al., 2000). Chemokines are small cytokines that promote inflammation by attracting leucocytes to the point of inflammation and have also been reported to play a part in neuromodulation (Proost et al., 1996; Mélik-Parsadaniantz and Rostène, 2008). Contrary to all of the above factors, intravenous administration of IgG has been shown to induce anti-inflammatory action *in vivo* (Nimmerjahn and Ravetch, 2008) and could be beneficial in the treatment of AD (Dodel et al., 2004) by inhibiting the neurotoxic effects of amyloid-β (Aβ).

Although it was originally thought that the blood–brain barrier (BBB) provides an immune privileged status to the brain, RCTs in rodents have shown that freshly activated T cells migrate across the BBB during neuroinflammation, and along with macrophages/monocytes, are present at all times in the brain for immune surveillance (Hickey et al., 1991; Engelhardt, 2006). It is, however, important to note that T cells, particularly the Th1 and Th2 phenotypes, secrete various antagonistic cytokines (Th1 elicits pro-inflammatory response and Th2 elicits anti-inflammatory response) and thereby also control neuro-humoral immune responses during psychiatric disorders (Schwarz et al., 2001). The role of NK cells in various brain disorders such as depression, AD and PD has also recently been reviewed and validated by some researchers (Poli et al., 2013). While exchange of B cells across the BBB has been reported in patients with multiple sclerosis and associated with the development of autoimmunity in the CNS (von Büdingen et al., 2012), their role in psychiatric illnesses such as depression has not been studied in detail so far.

## ROLE OF GLIAL CELLS IN NEURO-IMMUNOMODULATION

Glial cells, microglia and astrocytes, are the primary immune effector cells and express various cytokines in the CNS (Rothwell et al., 1996; Hanisch, 2002). However, the source of cytokines in the brain can be central (via microglia and astrocytes), as well as peripheral (via monocytes, macrophages, Th17 cells, and other T cells) and certain cytokine signals reach the brain parenchyma through humoral, neural, and cellular pathways (see review by Capuron and Miller, 2011 for more information about these pathways).

Microglia are specialized macrophages and are considered the principal immune cells in the brain. They carry phenotypic markers for blood monocytes and tissue macrophages (McGeer et al., 1993) and are shown to be involved in immuno-surveillance and neuroprotection (Conde and Streit, 2006). In particular, microglia are known for the production of cytokines in the CNS and protecting it from numerous pathologies such as infectious diseases, trauma, ischemia, brain tumors, neuroinflammation, and neurodegeneration (Kreutzberg, 1996). A RCT on rodents has shown that microglia in association with cytotoxic T cells are important for neurogenesis, adult brain plasticity, and spatial memory (Ziv et al., 2006). Though microglia are neuroprotective, their overexpression or sustained stimulation can result in enhanced production of cytokines (e.g., IL-1β and TNF-α; Sawada et al., 1989; Hanisch, 2002), as well as in the expression of class I and II major histocompatibility complex antigens as seen in a RCT in rodents and in the post-mortem brain tissues of AD and age-matched control cases (Tooyama et al., 1990), respectively. This overexpression of microglia may lead to severe neuroinflammation, neurodegeneration, and subsequent cognitive dysfunction.

In the presence of an activating stimulus, microglia modulate the immune response by producing pro-inflammatory cytokines. This in turn recruits more microglia to the site, as well as attracts immune cells from the peripheral blood. Likewise, when the stimulus wanes, microglia participate in switching off of the immune response by producing anti-inflammatory cytokines that also causes their own apoptosis (Garden and Möller, 2006). Schwartz et al. (2006) suggested that activation of microglia into either of these forms is determined by the type of stimulus, its duration and its preceding, concomitant and subsequent stimuli.

The role and functions of microglia have been reviewed by many researchers in the past (Mrak and Griffin, 2005; Streit, 2005; Dilger and Johnson, 2008). These reviews report that microglia are primed with aging, become increasingly dysfunctional, lose their neuroprotective properties and upon secondary stimulation release excessive quantities of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6. This in association with genetic factors and acquired environmental risks, predisposes the brain to development of neurodegenerative disorders. Activated microglia and the released cytokines have also been reported to play a role in the formation of amyloid plaques and for the onset of neurodegeneration during aging that leads to AD (Licastro and Chiappelli, 2003; Streit, 2004). Interestingly, the activation of glial cells and expression of pro-inflammatory cytokines like IL-1α, IL-1β, and IL-6 with aging has been observed to be region specific in an *in vitro* model of primary glia cultured from brain regions of male Fisher 344 rats sampled across the life span, occurring more prominently in the hippocampus than in the cerebral cortex (Xie et al., 2003). This might cause neurodegenerative changes in brain regions like the hippocampus, with subsequent effects on cognition.

The discussion above, as well as some reviews (Liu and Hong, 2003; Glezer et al., 2007; Ekdahl et al., 2009), suggest that microglia can display both neuroprotective and neurotoxic effects depending on the extent of their cytokine expression, which therefore makes them the potential target for the treatment of neurological diseases.

Like microglia, astrocytes have neuroprotective functions in the normal brain but could be responsible for neurological diseases as well. They can repair damaged neural tissue, guide neuronal migration during development, mediate synaptic plasticity, act as antigen presenting cells and maintain the structural and functional integrity of the BBB (Montgomery, 1994). Similar to microglia, they are immune effector cells, expressing cytokines (IL-1, IL-6, IL-10, IFN-α and -β, TNF-α and -β) and chemokines, and mediating inflammation and immune reactivity in the brain. The under-expression or overexpression of astrocytes has been reported to cause neuroinflammation with resultant neurodegeneration (Dong and Benveniste, 2001), which emphasizes them as the second most important target in the brain, after microglia, for cytokine-modulation-based paradigms, such as EE.

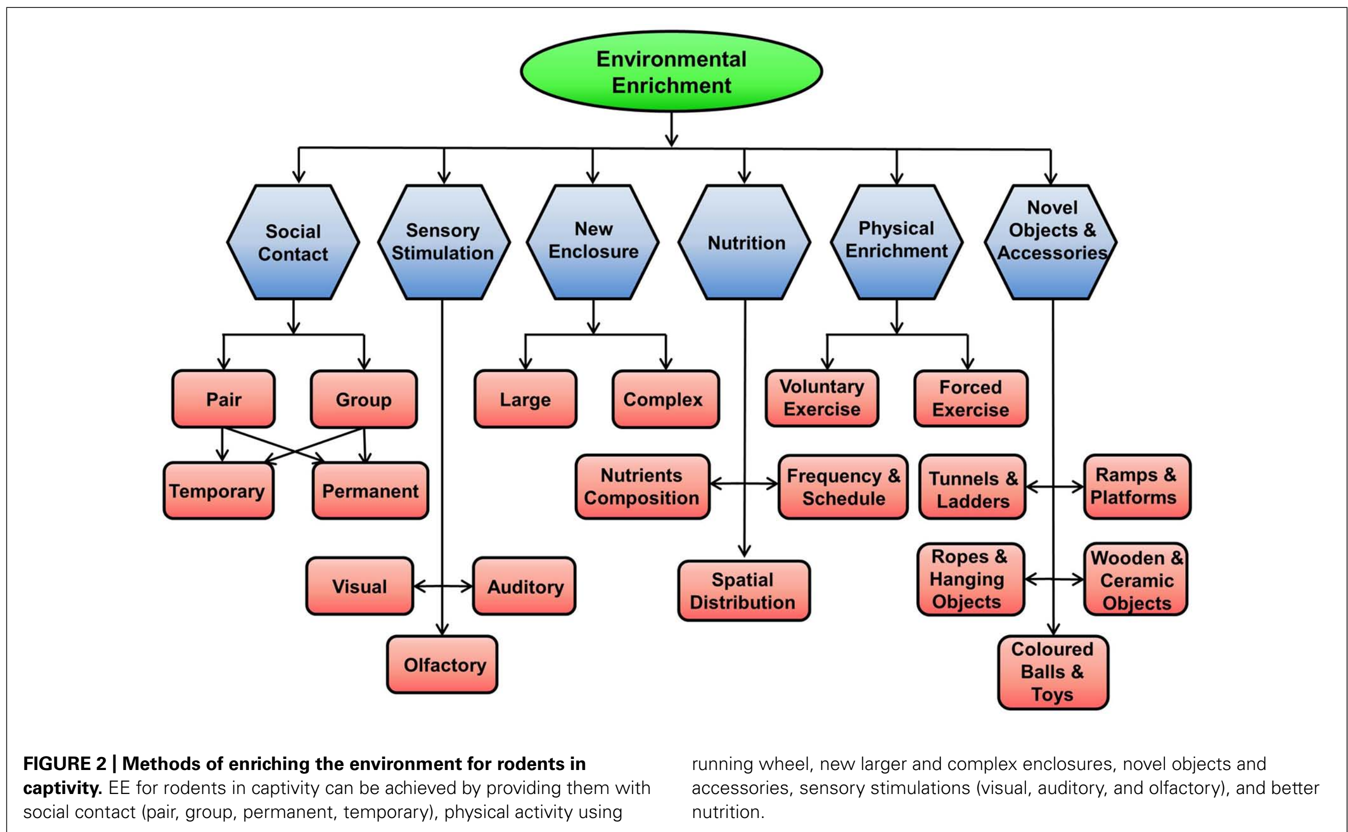



**FIGURE 2 | Methods of enriching the environment for rodents in captivity.** EE for rodents in captivity can be achieved by providing them with social contact (pair, group, permanent, temporary), physical activity using running wheel, new larger and complex enclosures, novel objects and accessories, sensory stimulations (visual, auditory, and olfactory), and better nutrition.

## ENVIRONMENTAL ENRICHMENT

The environment for rodents can be enriched in many ways, as shown in **Figure 2**.

### EFFECTS OF VARIOUS EE METHODS ON NEUROBIOLOGY AND BEHAVIOR

#### *Physical exercise*

***Rodent studies.*** This effectiveness of PE in enhancing neuroplasticity and improving brain function has been utilized in rodent studies for the purpose of enrichment of surroundings in several EE paradigms. RCTs on rodents have shown enhancement in hippocampal neurogenesis, improvement in cognition, learning, and memory performances with PE (Ahmadiasl et al., 2003; Van Praag et al., 2005; Nichol et al., 2009). Similarly, exercise on a running wheel promoted memory acquisition, memory retention, and reversal learning in rodents tested on a Y maze post-exercise treatment (Van der Borght et al., 2007). PE has also been shown to affect behavior in rodents. Indeed, long-term voluntary exercise has been shown to reduce anxiety- (Benaroya-Milshtein et al., 2004; Binder et al., 2004; Duman et al., 2008) and depressive-like behavior (Zheng et al., 2006; Duman et al., 2008; Marais et al., 2009).

Physical exercise on a running wheel when used in conjunction with toys and accessories (e.g., tunnels, ramps, bells, etc.) improved the tissue integrity and cognitive performances of rodents following severe traumatic brain injury (Hamm et al., 1996; Passineau et al., 2001). This combination has also been shown to induce a fivefold increase in hippocampal neurogenesis, enhancing learning, exploratory behavior and locomotor activity in aged mice (Kempermann et al., 2002). Enhanced spatial memory and an increase in dendritic arborization were seen in rats housed in groups and provided with a running wheel, shelter, and toys (Leggio et al., 2005), suggesting a possible beneficial effect of PE on dendritic morphology. In addition, PE has been shown to promote neurogenesis in the dentate gyrus of the hippocampus and modulate neural transmission across the synapses in the hippocampal region by modifying the extracellular concentration of neurotransmitters glutamate and gamma-aminobutyric acid (GABA) in the CA3 area of the hippocampus (Segovia et al., 2006). EE utilizing novel objects and accessories, with PE, ameliorated cognitive deficits in a mouse model of AD (Jankowsky et al., 2005), which could be due to the reduction of Aβ after enrichment (Lazarov et al., 2005). In another study, PE with social enrichment (five to eight mice in a cage) and novel objects and accessories improved spatial memory in mice (Jurgens and Johnson, 2012). The evidence above suggests a potential mechanism of PE, when used alone or in combination with other enrichment methods, in modulating neurobiology and behavior in rodents.

***Human studies.*** Recent reviews have upheld the long-standing view that aerobic exercise modulates neuroplasticity, and improves responsiveness to new challenges and psychosocial functions in humans (Hotting and Roder, 2013; Lees and Hopkins, 2013). In contrast, a recent meta-analytic study on random controlled trials of the effects of exercise on cognitive outcomes in adults

aged over 65 years with mild cognitive impairment has rejected these claims stating that published results have very low statistical power and therefore are inconclusive (Gates et al., 2013). However, this may relate to the age of the subjects included within the analysis, as this limits the number and scope of articles for study. Moreover, the control cohort in the study included conditions like education, and stretching and aerobic exercise which may prove beneficial themselves, therefore preventing a positive finding for PE. Further, the intensity of exercise and external environmental conditions during PE can modulate its effects on brain (discussed later in this review), which were not considered in this review. Nonetheless these disparate findings show the complex nature of exercise physiology in human and animal interventions.

Physical exercise has been considered as an established and effective first-line treatment in mild to moderate depression. The role of PE in depression has been critically reviewed recently which suggests that the efficacy of PE in depression is classically attributed to its impact on changing certain neurobiological mechanisms including monoamine metabolism, HPA axis function, neurotrophic factors, neurogenesis, and neuroinflammation (Eyre and Baune, 2012). Like depression, individuals who were at risk of AD and dementia showed improved cognition after modest PE (Gleeson et al., 2011). Due to these beneficial effects of PE in psychiatric conditions, a suggestion that PE can be used as a non-pharmacological therapy for providing protection from neurodegenerative diseases, stress, and depression has been made by some authors in their reviews (Cotman et al., 2007; Hillman et al., 2008).

A RCT in human volunteers that underwent graded aerobic exercise training showed improvement in their spatial memory (Erickson et al., 2011), suggestive of a constructive role of moderate exercise in enhancing memory in humans. PE has also been shown to enhance cognition in healthy adult males after performing single acute bouts of moderately intense exercise, however, the authors observed that the effects of single acute bouts of moderately intense exercise improves only some aspects of cognition (primarily memory, reasoning, and planning) in healthy young individuals (Nanda et al., 2013). Nevertheless, a test of the effects of a single bout of moderate-intensity exercise on cognition may not be a reliable determination of its long-term effects (see review by Tomporowski, 2003). Interestingly, in a human intervention correlative study, it has been shown that these results can vary based on the pre-exercise performance of participants in the given task; post-exercise low performing participants perform better in the tests of cognition than high performing participants (Drollette et al., 2013). However, the authors of this study categorized preadolescent children into high performing and low performing children while resting, based on flanker task performance for incongruent trials. It is possible that high performing participants while resting showed performance relatively near to their peak levels, while performance of low-performing participants may have been low due to the lack of attention or other confounding variables. A similar paradigm in rodents where mice or rats are tested for cognition before and after exercise could be useful to accept the validity of results from this study.

***Summary of the role of physical exercise in EE studies.*** Overall, it is evident that the role of PE has been widely studied in both rodents and humans. It appears that PE modulates neurobiology and behavior via similar mechanisms in rodents and humans, particularly by enhancing the neuroplasticity of the dentate gyrus of the hippocampus, as seen in rodents (Segovia et al., 2006), and improves spatial memory, behavior, and cognition (Ahmadiasl et al., 2003; Van Praag et al., 2005; Duman et al., 2008; Nichol et al., 2009; Erickson et al., 2011; Nanda et al., 2013). PE is also known to enhance the levels of neurotrophins (Griffin et al., 2009) and modify the extracellular concentration of neurotransmitters (Lazarov et al., 2005) in the hippocampus, in addition to modulating neuroimmune mechanisms (Eyre and Baune, 2012; Speisman et al., 2013; Eyre et al., 2013) which are discussed later in this review. Moreover, the effects of PE on the brain are dependent on its duration and intensity (Mathur and Pedersen, 2008).

#### *Social and cage enrichment*

***Rodent studies.*** Profound effects on the behavior of captive animals when housed in groups could influence the methodological framework and validity of results in EE studies. A study suggests that male mice prefer sleeping in close proximity to a familiar mouse (Van Loo et al., 2004), which represents their preference for social environment. On the other hand, mice that were devoid of social contact showed signs of increased anxiety and depressive-like behavior with greater tendencies to attack other mice (Ma et al., 2011).

Further research has shown the evolution of two kinds of populations in laboratory rodents within a socially enriched environment: "dominant" and "subordinate," both with different physiological and behavioral profiles (Bartolomucci et al., 2001; Cacho et al., 2003). While this has been shown to improve the performance of dominant animals, submissive or socially low ranked animals can suffer from social stress, may show immunosuppression and are usually more susceptible to viral infections and formation of tumors (de Groot et al., 1999; Azpiroz et al., 2003). The adverse effects of social stress on neuronal structure and neurochemical transmission (Blanchard et al., 2001), as well as on the morphology of hippocampal neurons, which are vital for learning and memory (Buwalda et al., 2005) has been suggested, indicating that social stress in a socially enriched environment could alter the findings of investigation on brain function. Indeed, from other studies, it appears that these two populations also exhibit distinct differences in anxiety-like behavior, with dominant mice showing anxiolytic behavior after repeated victory (Haller and Halasz, 2000), while subordinate mice showing anxiogenic-like and decreased exploratory behavior (Keeney and Hogg, 1999). The development of this hierarchy in the population of rodents poses a serious threat to EE paradigms which are based on the principles of enhancement and not impoverishment of the external environment.

These adverse behavioral and physiological changes due to social defeat (i.e., losing a confrontation among animals of same species) can be long lasting. However, mice that were housed together after social defeat showed improvement in behavior (Ruis et al., 1999). Similarly, a study in rats revealed that social housing after social defeat reverses the reduction in heart rate, temperature

and locomotor activity caused by social defeat (de Jong et al., 2005). It appears that while social conflict may be harmful, social enrichment in the absence of these conflicts (e.g., when submissive rodents are housed together) is beneficial for neurobiology and behavior.

Findings from RCTs on rodents suggest that access to nesting material and nest boxes, increased complexity of the environment in cages and bedding material consisting of large particles (e.g., wood shavings) and fibers (e.g., shredded paper) are the natural preferences of rodents (Blom et al., 1996; Van de Weerd et al., 1998a,b; Olsson and Dahlborn, 2002). However, access to the preferred material can incite the expression of territorial behavior, for example, aggression, in rodents. This hypothesis was confirmed when mice provided with a bigger and more complex cage and more objects to explore were more aggressive, but interestingly this aggression was reduced when they were given access to nesting material (Van Loo et al., 2002). A plausible explanation for this could be that nesting material diverts the attention of rodents in cages from their conspecifics. Similar to this, another RCT showed that bedding material can partly be used to compensate for the deprivation of social contact (Van Loo et al., 2004). Notably, Fano et al. (2001) conducted an experiment to investigate agonistic behavior in male mice and reported that these paradigms of aggression are dependent on the intensity and duration of agonistic behavior and the interaction experience accumulated.

***Human studies.*** It is difficult to compare social and cage enrichment in rodents to humans. In humans, a possible equivalent could be active social behavior. An excellent review on human social behavior details the social psychology of humans and associated neurobiology (Adolphs, 2003).

***Summary of the role of social enrichment in EE studies.*** All of the findings above suggest that a subtle balance between social and cage enrichment is vital during EE studies in rodents. This could be achieved through meticulous planning while designing EE models. Limited research has been conducted on the neuromodulatory effects of social and cage enrichment methods on rodents at this stage.

Social enrichment in humans is a more complicated phenomenon and comprises a myriad of disciplines, such as social neuroscience, cognitive science, sociobiology, evolutionary psychology, and social psychology, converging together. Given these complex interactions, it is outside the scope of this review. It should be noted, however, that rodent models of social enrichment may not be ideal for investigating the effects of enrichment with social environmental stimuli in humans.

#### Enrichment with novel objects and accessories

***Rodent studies.*** The environment of rodents can be enriched with novel objects, puzzles (mazes, plastic tubes in different configurations) and accessories (toys, ropes, ladders, tunnels, hanging objects, house, ramps, and platforms) to stimulate their attention and engagement in the environment. Mice given access to novel objects and accessories with or without PE exhibited higher visuo-spatial attention and improved spatial memory when tested on the Morris water maze test (Tees, 1999; Williams et al., 2001; Bennett et al., 2006; Harati et al., 2011), which could be a function of increased hippocampal integrity and levels of neurotrophins in the hippocampus (Pham et al., 1999b; Gobbo and O'Mara, 2004). The reduction in cytochrome *c* oxidase levels in brain regions such as the infralimbic cortex, the paraventricular thalamic and hypothalamic nucleus, the basolateral amygdala, and the ventral hippocampus (Sampedro-Piquero et al., 2013) after enrichment with various objects and accessories could be another possible mechanism for this effect.

Enrichment in large cages furnished with various toys and accessories has been shown to restore the age-related loss of synaptophysin in aged mice. However, the authors observed no change in the number of synapses after enrichment, suggesting that enrichment improves synaptic plasticity by strengthening the synapses, not by formation of new synapses (Nakamura et al., 1999). An increase in play behavior, aggression and locomotor activity was observed in studies primarily investigating immunological alterations in mice after enriching them in complex cages furnished with a variety of items (Marashi et al., 2003, 2004). This method of enrichment has primarily been used in combination with PE, with further studies needed to investigate the effects of stimulation with novel objects alone.

***Human studies.*** Similar experimental paradigms in humans, i.e., activities that can stimulate attention and engagement in the environment, involve the provision of cognitively stimulating lessons (e.g., connecting dots to make an umbrella, naming easily identifiable objects after showing their pictures; Breuil et al., 1994), cognitive training (e.g., verbal episodic memory, reasoning, and visual search and identification; Willis et al., 2006), and cognitive rehabilitation (Clare et al., 2003). Studies on cognitive stimulation reported improvement in the tests of cognition (Breuil et al., 1994) and quality of life (Spector et al., 2003) by the participants. Likewise, improvement in memory and cognition of healthy volunteers, patients in the early stages of AD and vascular dementia, as well as major depressive disorder patients after cognitive training (Willis et al., 2006), cognitive rehabilitation (Clare et al., 2003), and cognitive remediation (Bowie et al., 2013), respectively suggests that these treatments can affect cognition in the healthy brain as well as in neuropathological conditions. These findings of the effects of cognitively enriched environment on cognition are further validated by meta-analytic studies on cognitive remediation for schizophrenic patients (McGurk et al., 2007; Wykes et al., 2011). Although these treatments, different from the method of providing EE with novel objects and accessories in rodents, they do promote a similar improvement in memory and cognition.

***Summary of the role of enrichment with novel objects and accessories in EE studies.*** This is the most common method of EE in rodents, after PE, and has been used extensively either as a standalone treatment or in conjunction with PE and social enrichment. EE with novel objects and accessories enhances memory and cognitive functions in rodents. Similar effects on cognition have been reported in humans in response to cognitive stimulation activities. The biggest question here is whether these treatments in

rodents and humans are mechanistically similar. The perception of novelty for rodents in existing EE studies is "anything new" that rodents have not been exposed to so far. The same may not be applicable to humans. Novelty detection in humans has been shown to be a function of hippocampal (Knight, 1996) and amygdala (Blackford et al., 2010) activity and could depend on the participants within the study. This indicates that formulating a protocol with novel objects and activities according to the interests and likes of human participants, for example, sports equipment, magazines, or movies could be more appropriate method of translating EE paradigms in rodents to human studies.

#### *Sensory enrichment*

***Rodent studies.*** The effects of sensory enrichment on the activity of sensory organs (visual, auditory, and olfactory) and brain functions such as cognition and behavior in various captive animals have been reviewed and validated by Wells (2009).

*Visual stimulation.* Enrichment with objects that stimulate the visual cortex such as toys of different color and sizes, leafy plants, tree branches, and scattered food (to explore) has been shown to increase the thickness, number, and length of neurons, dendritic complexity, and spine density of the occipital cortex and improve visual processing activity in rodents (Venable et al., 1989; Piche et al., 2004; Rasin et al., 2011). This could be due to the enhanced levels of neurotrophins as observed in the visual cortex of newborn rats on exposure to light (Castren et al., 1992). Indeed, studies have shown that neurotrophins increase the length and complexity of dendrites (McAllister et al., 1995), potentiate excitatory synaptic transmission (Carmignoto et al., 1997) and enhance long-term potentiation in the visual cortex of rodents (Akaneya et al., 1997). Conversely, studies that investigated the effects of different light intensity and colors on visual stimulation suggested that mirrors (Sherwin, 2004) and some colors such as red (Sherwin and Glen, 2003) can be aversive and may affect emotionality and performance of mice.

*Auditory stimulation.* RCTs on rodents have shown that pure tone bursts (of different frequencies and intensities) and/or different tones (from hanging chains, wind chimes and bells) enhanced neuroplasticity, number of neurons, basal dendritic length, and spine density in the auditory cortex. This in turn improved directional sensitivity and increased response strength, threshold, selectivity, latency of auditory cortical neurons, and reorganization in the processing of spectral and temporal input in the posterior auditory field (Dinse, 2004; Engineer et al., 2004; Cai et al., 2009; Zhang et al., 2009; Bose et al., 2010; Jakkamsetti et al., 2012). According to some authors, auditory experience during early life can define the functional organization of the auditory cortex and enhance its processing capabilities to discriminate various auditory stimuli (Zhang et al., 2001; Xu et al., 2009).

In studies where adult mice were exposed to music with a slow rhythm, the authors observed enhanced learning performance and higher brain-derived neurotrophic factor (BDNF) levels in the hippocampus (Angelucci et al., 2007a) and hypothalamus (Angelucci et al., 2007b) of mice. The modulating effect of music on BDNF signaling has also been seen in the brain of mice exposed to Mozart's piano sonata for approximately 7 days while in uterus and 60 days postpartum. However, these authors observed a decrease in BDNF levels in the auditory cortex though it increased in the cerebellum (Chikahisa et al., 2006). These results were unexpected, with findings from other studies suggesting that the neurotrophins NT-3 and BDNF can prevent the loss of auditory neurons (Staecker et al., 1996) and that BDNF signaling is important for shaping off of the experience-dependent plasticity in the auditory cortex during early postnatal life (Anomal et al., 2013). However, the authors only tested one type of music and the influence of the mother on the pups was not considered. This gives rise to the possibility that music like Mozart's piano sonata might not be a favorable type of music to utilize in mice and the presence of the mother during music sessions could have affected the development of auditory acuity in new born pups. A hypothesis that different kinds of music induce distinct change in brain functions in rodents could therefore be explored.

*Olfactory stimulation.* A relation between olfactory stimulation and the brain is well documented, and is supported by the findings that olfactory bulbectomized rats show depression-like behavior (Kelly et al., 1997). Different odors stimulate the olfactory bulb which directly communicates with the olfactory cortex, hippocampus, amygdala, and hypothalamus in the brain and can induce behavioral changes. Enriched olfactory experiences in early life have been shown to enhance the functions of the adult olfactory bulb (Rabin, 1988; Rosselli-Austin and Williams, 1990), while odor deprivation in neonates reduced neurogenesis and the survival of the neurons (Corotto et al., 1994), as well as increased apoptosis in the olfactory bulbs of adult rodents (Najbauer and Leon, 1995). A study using an experimental paradigm that investigated the effects of enriched odor on 2-month-old mouse brains reported no effect on hippocampal neurogenesis or spatial memory on exposure to enriched odors; but an increase in the number of neurons in the olfactory bulb and improvement in odor memory were seen (Rochefort et al., 2002). While this study is suggestive of no effects of enriched odors on cognition and memory, it is unlikely that stressful odors such as smell of rotten food or injuries will also have no effect on behavior, memory, and cognition. Indeed, avoidance response has been shown in healthy rats to sickness-related odor cues (Arakawa et al., 2010). Moreover, novelty in odor determines the extent of improvement in short-term odor memory and neurogenesis in the olfactory bulb, which has been shown to be mediated by nor-adrenergic mechanisms (Veyrac et al., 2008). Further investigation on olfactory stimuli could clarify the significance of different odors in EE studies.

***Human studies***

*Visual stimulation.* Depending on the nature of external visual stimuli (favorable or aversive), contrasting neurobiological and behavioral outcomes are plausible. Distinct arousal of emotions and enhanced episodic recognition memory have been observed in response to pleasant and aversive visual stimuli, and found to be related to amygdala activity (Hamann et al., 1999). The role of different visual stimuli in evoking emotions and behavior in day-to-day human life is evident. However, a study in humans has shown that 1 min of exposure to blue light can trigger stimulation

of cognitive brain activity in visually blind individuals (Vandewalle et al., 2013), suggesting that photoreception can modulate brain functions even in the absence of image formation. On the other hand, other sensory modalities such as tactile tasks could also activate the visual cortex, as seen in response to braille reading in blind subjects (Sadato et al., 1996). Indeed, activities like cognitive training involving visual tasks (e.g., recalling pictures after seeing them briefly; Breuil et al., 1994) could also provide visual stimulation to participants.

*Auditory stimulation.* While daily activities involve listening to various sounds, auditory enrichment for humans primarily involves listening to music of your own choice. Beneficial effects of music on the well-being, mood, learning performance, and cognitive development in humans are well known (McCraty et al., 1998; Kemper and Danhauer, 2005; Rickard et al., 2005; Hars et al., 2014). Music therapy has been shown to reduce irritability and depression (Hanser and Thompson, 1994; Ragneskog et al., 1996; Maratos et al., 2008), as well as improve emotional and behavioral responses in dementia patients (Sherratt et al., 2004), suggestive of its significance for the treatment of psychiatric conditions. Indeed, music could even be more potent in reducing depression than psychotherapy (Castillo-Pérez et al., 2010). Music has also been shown to enhance cognitive ability. In an experiment on patients with a left or right hemisphere middle cerebral artery stroke, listening to self-selected music for 2 months improved mood and enhanced cognitive recovery (Särkämö et al., 2008). Some authors have proposed that music therapy can be used as an alternate therapy in psychiatric conditions like depression and schizophrenia (Lin et al., 2011). Moreover, a review suggested that learning mechanisms mitigating effects of auditory stimuli on the brain could be applied to better understand the biology underlying everyday learning (Strait and Kraus, 2014). However, it is possible that different kinds of music could correlate to differential effects on the brain of psychiatric patients, as seen in healthy volunteers (Möckel et al., 1994; Blood et al., 1999).

*Olfactory stimulation.* Olfactory stimulation with a pleasant odor could improve learning and behavior in humans and indeed has a role to play from the first week after birth. A review by Schaal (1988) suggests that olfactory cues activate the olfactory bulb and help infants in the first postnatal week to bond with their mother and differentiate familiar from unfamiliar individuals. In his review, Herz (2009) analyzed the effects of various odors on behavior in humans and suggested that certain odors such as that of sandalwood or those self-selected by participants as pleasant could be used for the treatment of anxiety, depression, and insomnia. It has been shown that olfactory deficits may predict AD or PD in the patient (Mesholam et al., 1998; Devanand et al., 2000). Indeed, neurodegenerative and psychiatric diseases have been shown to reduce olfactory bulb neurogenesis in humans (Turetsky et al., 2000; Winner et al., 2011). Moreover, an aversive olfactory stimuli, for example, odor of a mixture of sulfide gasses, could even initiate emotions by activating the amygdala (Zald and Pardo, 1997). These findings clearly suggest that olfactory cues might have a role to play in psychiatric conditions.

***Summary of the role of sensory enrichment in EE studies.*** Taken together, studies on sensory enrichment have shown prominent effects on the neurobiology and behavior of rodents, with analogous evidence also evident within human literature. A similarity between sensory enrichment and enrichment with novel objects and accessories in rodent studies can be seen, since the standard environment of rodents in captivity is devoid of any special object or sensation. As such, a new sensation can be a novel input for effecting changes to brain function, such as cognition, in rodents. It is likely that the sights of aversive stimuli such as that of dominant or injured animals and favorable stimuli such as the introduction of a running wheel or novel toys could influence behavior and cognition of rodents in itself. The opposite, that enriched environments can enhance visual processing activity is also possible, as suggested by Cancedda et al. (2004). A possible explanation for this could be enhancement of the levels of neurotrophins in the visual cortex which promote neurogenesis, when environment is enriched with running wheels, and novel objects, toys and accessories (Torasdotter et al., 1998; Pham et al., 1999a).

See **Table 1** for studies detailing effects of different enrichment methods on neurobiology and behavior.

## THE INFLUENCE OF EE ON NEUROBIOLOGY AND BEHAVIOR VIA MODULATION OF CYTOKINES AND IMMUNE CELLS

### *Physical exercise*

***Anti-inflammatory and humoral immune mechanisms of physical exercise.*** Several studies have reported the anti-inflammatory effects of PE during diseases and metabolic disorders which are associated with chronic low-grade systemic inflammation such as cardiovascular disease and type II diabetes mellitus (Petersen and Pedersen, 2005; Wilund, 2007). PE has also been shown to slow down cellular aging which is generally associated with inflammatory conditions, an increased occurrence of circulating autoantibodies and lymphoproliferative disorders and hence greater morbidity and mortality rates (Shinkai et al., 1998; Senchina and Kohut, 2007).

Several mechanisms have been investigated and cited for the anti-inflammatory effects of PE. It is clear that PE affects skeletal muscles which are able to act as an endocrine organ in body as they release myokines/cytokines on contraction thereby influencing metabolism and modifying cytokine production in other tissues and organs (Petersen and Pedersen, 2006). However, PE also increases the secretion of cortisol and adrenaline from the adrenal glands, enhances the production and release of IL-6 and other myokines from working skeletal muscles and reduces the expression of TLRs on monocytes and macrophages. Research suggests that this increase in IL-6 in response to PE is dependent on its intensity, duration, the mass of muscle recruited, and endurance capacity (Petersen and Pedersen, 2005; Mathur and Pedersen, 2008).

The production and release of IL-6 from muscle fibers is important as it enhances lipid turnover by stimulating lipolysis as well as fat oxidation, thereby reducing the production of adipokines including TNF-α, leptin, retinal-binding protein 4, lipocalin 2, IL-6, IL-18, CCL2, and CXCL5 (Petersen and Pedersen, 2005; Eyre and Baune, 2012). Evidently the increase in

**Table 1 | Effects of environmental enrichment on neurobiology and behavior.**

| Study's primary objective | Animal species/strain | EE methods | Frequency of changing EE method | Protein/behavioral parameters investigated | Significant findings | Reference |
|---|---|---|---|---|---|---|
| • Effects of EE on neurodegeneration during AD | • AD11 mice | • Large cages with wire mesh lid, several food hoppers, running wheel and objects of different shapes (tunnels, shelters, stairs, boxes) | • Once per week | • Visual object recognition test<br>• Morris water maze test<br>• IHC | • ↑ Visual object recognition memory and spatial memory<br>• ↓ Aβ deposition in hippocampus<br>• ↓ Progression of neurodegeneration | Berardi et al. (2007) |
| • Effects of long-term EE on hippocampal neurogenesis | • 10 months old female C57BL/6 mice | • Large cages, with re-arrangeable set of plastic tubes, a running wheel, nesting material, and toys | – | • Behavioral testing with Activity chamber, Rotarod, and Water maze<br>• IHC and IF for lipofuscin deposits in neurons | • Fivefold increase in hippocampal neurogenesis in enriched environment<br>• ↑ Learning, exploratory behavior, and locomotor activity<br>• ↓ Lipofuscin deposits in the dentate gyrus | Kempermann et al. (2002) |
| • Effects of environmental complexity on spatial abilities, dendritic arborization, and spine density | • Wistar rat (21 days old) | • Ten rats in a large cage of two levels connected by ramps, containing wood shavings, a running wheel, a shelter, plastic colored toys, and constructions | • Once a week | • Full-baited maze procedure and forced-choice procedure, performed in a radial maze<br>• Morris water maze<br>• *In vivo* Golgi-like filling of the neurons for the visualization of dendritic arborization | • ↑ Performance in the Radial maze and Morris water maze tasks<br>• ↑ Dendritic arborization and spine density in layer-III parietal pyramidal neurons | Leggio et al. (2005) |
| • Influence of EE on neurotrophins levels in the cerebellum | • Adult male Wistar rats | • 10 rats in a large cage with two levels, connected by ramps, contain wood shavings, a running wheel, a shelter, plastic colored toys, and constructions | • Twice per week | • Determination of BDNF and NGF in all brain regions using ELISA | • ↑ BDNF levels in the cerebellum, frontal cortex and hippocampus<br>• ↓ BDNF levels in the striatum<br>• ↑ NGF levels in the cerebellum and striatum<br>• No significant change in NGF levels in the frontal cortex and hippocampus | Angelucci et al. (2009) |

*(Continued)*

**Table 1 | Continued**

| Study's primary objective | Animal species/strain | EE methods | Frequency of changing EE method | Protein/behavioral parameters investigated | Significant findings | Reference |
|---|---|---|---|---|---|---|
| • Effects of EE on the neurogenesis and the extracellular concentrations of glutamate and GABA in the hippocampus | • Male Wistar rats of 2 and 25 months | • Two running wheels, a re-arrangeable set of plastic tunnels, an elevated platform, toys | • Every 3–4 days | • Water maze test<br>• Neurogenesis in the dentate gyrus of hippocampus using BrdU labeling<br>• Glutamate and GABA concentration in CA3 region of hippocampus using microdialysis probes | • ↑ Spatial memory performance<br>• ↑ Hippocampal neurogenesis in both young and aged enriched rats<br>• ↓ Hippocampal neurogenesis in control rats<br>• No effect of EE on basal concentration of Glutamate and GABA in young rats<br>• ↑ Basal glutamate and GABA concentration in old rats | Segovia et al. (2006) |
| • Effects of ageing and EE on synaptic plasticity | • Male Fischer rats | • Large cages furnished with various toys and small constructions | – | • Electron microscopic morphometry for the analyses of density and sizes of synapses | • ↓ Synaptic vesicle density with age<br>• EE restored age-related loss of synaptophysin | Nakamura et al. (1999) |

*EE, environmental enrichment; AD, Alzheimer's disease; IHC, immunohistochemistry; Aβ, amyloid-β; IF, immunofluorescence; BDNF, rain-derived neurotrophic factor; NGF, nerve growth factor; ELISA, enzyme linked immunosorbent assay; BrdU, bromodeoxyuridine; GABA, gamma-amino butyric acid; CA, Cornu Ammonis.*

energy expenditure associated with exercise also assists in promoting lipolysis and reducing production of adipokines (Gleeson et al., 2011). Moreover, IL-10 produced in response to IL-6 acts as an anti-inflammatory molecule and further inhibits the production of IL-1α, IL-1β, and TNF-α as well as the production of chemokines. Additionally, another anti-inflammatory mechanism of PE, where it inhibits monocyte and macrophage infiltration into adipose tissues as well as stimulates phenotype switching within adipose tissue has also been suggested (Eyre and Baune, 2012).

Physical exercise is also likely to suppress TNF-α via IL-6-independent pathways, since a modest decrease of TNF-α after PE was still seen in IL-6 knockout mice (Keller et al., 2004). High levels of cortisol and epinephrine are triggered by PE due to the activation of the HPA axis and the sympathetic nervous system, and this cortisol and epinephrine infusion in turn has been shown to blunt the appearance of TNF-α in response to endotoxin *in vivo* (Petersen and Pedersen, 2005; Gleeson et al., 2011). In a study on resting subjects, endotoxin induced a two- to threefold increase in circulating levels of TNF-α. In contrast, when the subjects performed 3 h of ergometer cycling and received the endotoxin bolus at 2.5 h, the TNF-α response was completely diminished (Petersen and Pedersen, 2005). However, the mechanism whereby cortisol and epinephrine inhibit TNF-αproduction is still not clear. It appears that epinephrine and IL-6 inhibit endotoxin-induced production of TNF-α via independent mechanisms. The possibility exists that, with regular PE, the anti-inflammatory effects of an acute bout of PE will protect against chronic systemic low-grade inflammation, but such a link between the acute effects of PE and the long-term benefits has not yet been proven.

***Cellular immune mechanisms of physical exercise.*** Changes to cellular immunity in response to PE have also been reported by several authors. Leukocytosis is commonly seen during exercise, the extent of which is related to the intensity and duration of exercise. However, the cellular changes post-exercise are determined mainly by the time elapsed since starting exercise and not the work intensity and the total work done (McCarthy and Dale, 1988). A study on eight internationally competitive oarsmen, undergoing 6 min of "all-out" bouts of ergometer rowing over 2 days showed that compared with levels at rest, the first bout of exercise increased the concentration of leukocytes (twofold); neutrophilic granulocytes (twofold); lymphocytes (twofold); monocytes (twofold); the blood mononuclear cell (BMNC) subsets $CD3^+$ (twofold), $CD4^+$ (twofold), $CD8^+$ (threefold), $CDl6^+$ (eightfold), $CDl9^+$ (twofold), and $CDl4^+$ (twofold); the NK cell activity (twofold); and plasma IL-6 (threefold). The increase in leukocytes, neutrophilic granulocytes, lymphocytes, the BMNC subsets $CD4^+$, $CD8^+$, $CD16^+$, $CD19^+$, and CD $14^+$, as well as in the NK cell activity was even higher after the last bout of ergometer rowing by one- to fivefold. More importantly, all above values were at or more than the levels at rest during the recovery period. Indeed, leukocytosis, neutrophilocytosis, lymphocytosis, and higher NK cell activity was observed even on the day after the bout. This study is a good example of how PE can modulate levels of immune cells in the blood and improve cellular immunity (Nielsen et al., 1996). Significant cytological changes post-exercise have also been observed by Nehlsen-Cannarella (1998). The authors observed that immediately after PE, there was an increase in both the circulating leukocyte and neutrophil count, but only a small increase in the monocyte count. This was followed by a further increase in neutrophil numbers, although leukocyte numbers fell below the pre-exercise levels. Accompanying the increase in neutrophil count was the marked release of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1) followed by IL-1 receptor antagonists, the products of monocytes and tissue macrophages, as well as brain glial cells. Moreover, while the number of NK cells show increase after moderate exercise, they could decline after high-intensity PE (Jankowsky et al., 2005). Further, their activity on a per cell basis remains the same (Duman et al., 2008; Marais et al., 2009) or increases (Griffin et al., 2009), depending on the intensity and duration of exercise. In a study where healthy volunteers underwent 60 min of bicycle exercise at 75% maximal oxygen uptake ($Vo_{2max}$), decline in T helper cells ($CD4^+$ cells) and increase in NK cell subset ($CD16^+$) were seen in the blood (Tvede et al., 1989), consistent with the other findings above. Moderate PE has also been shown to affect various functions of neutrophils such as enhancing phagocytosis (Ortega et al., 1993) and production of microbicidal reactive oxygen species (Smith et al., 1990) by neutrophils. Both short-term and chronic PE improve the function of macrophages by enhancing phagocytosis, however, exhaustive PE has been shown to suppress antigen processing by macrophages (Ceddia and Woods, 1999; Ortega et al., 2007). This suggests that moderate exercise could be useful for inducing beneficial immune changes in the body, while exhaustive exercise could be harmful to the immune system.

***Physical exercise modulates cytokines and other humoral and cellular immune factors in the brain.*** Systemic cytokines can cross the BBB and affect various brain regions including the hippocampus, cerebellum, pituitary, and cortex. This has been reviewed in detail by several authors (Banks et al., 1995; Banks and Erickson, 2010), which suggests that any change in the systemic cytokine levels during infections can potentially affect brain function. However, this association of brain function with systemic cytokine levels requires further investigation. In addition, cytokines produced and expressed within the brain can also initiate neuroimmune reactions on their own. For instance, in the study conducted by Tarkowski et al. (2003), the authors observed low levels of TNF-α in the serum compared to the cerebrospinal fluid which provided evidence for local production of TNF-α within the brain rather than in the periphery. The observed presence of high levels of TNF-α in the brain of dementia patients indicates active neuroinflammation with resultant neurodegeneration, which contributes to the pathophysiology of several brain diseases.

Higher levels of complement component C4 and CRP has been observed in the serum of patients with major depression (Berk et al., 1997). Similarly, mRNAs of all components of the classical complement pathway are increased, particularly C1q mRNA by 11- to 80-fold and C9 mRNA by 10- to 27-fold over control levels in the entorhinal cortex, hippocampus, and midtemporal gyrus regions

of the brain in patients with AD (Yasojima et al., 1999). Activated complement components have also been observed in the affected brain regions of patients with PD (McGeer and McGeer, 2004) and age-related macular degeneration (Anderson et al., 2010). These results further confirm that neuroinflammatory and neurodegenerative processes drive the pathophysiology in depression, AD, PD, and other aging-related brain diseases (McGeer and McGeer, 2003; McGeer et al., 2005).

Physical exercise has been shown to reduce inflammation and oxidative stress in the brain. A study by Speisman et al. (2013) in aged rats showed that daily voluntary exercise on a running wheel decreased hippocampal IL-1β and serum monocyte chemoattractant protein-1 (a chemokine that regulates migration and infiltration of monocytes/macrophages from the blood across the vascular endothelium, a key mechanism during inflammation). However, rather surprisingly, the authors also observed increased IL-18 concentration in the hippocampus, which has pro-inflammatory functions. Since, levels of IL-18 correlated with hippocampal neurogenesis, the authors suggested that the pro-angiogenic properties of IL-18 might have improved vascular health and hence stimulated hippocampal neurogenesis. Several other immune pathways have also been proposed for the anti-neuroinflammatory effect of PE (Eyre and Baune, 2012; Eyre et al., 2013). These include (i) increased attraction of macrophages into the CNS and hence enhancement of their regulatory effects on neurotoxic microglia, (ii) upregulation of MKP-1 which plays an essential role in negatively regulating the pro-inflammatory macrophage MAPK activation, and (iii) modulation of hippocampal T cells which are responsible for neuroregeneration and for modulation of microglia. Moreover, certain types of exercise could have greater effects on the immune factors than others and modulate anti-inflammatory mechanisms by influencing several immune factors at the same time. For instance, in a RCT on older adults, aerobic exercise treatment resulted in significant reductions in serum CRP, TNF-α, IL-6, and IL-18 in the participants while flexibility/resistance exercise only caused a decrease in serum TNF-α levels (Kohut et al., 2006). Further, the possible anti-inflammatory and immunomodulatory effects of change in the levels of systemic cytokines and immune cells after PE on the brain cannot be overlooked, although this needs further analysis.

***Summary of the neuro-immunomodulatory role of PE in EE studies.*** In terms of the neuroimmune effects of PE, a similarity is seen in human and rodent studies. Exercise has been shown to reduce levels of TNF-α and IL-1β, as well as certain cellular biomarkers in the brains of rodents and humans. However, human and rodent studies assessing the positive effects of PE on neuroimmune mechanisms are difficult to compare due to the utilization of different types, durations and intensities of the exercise and inconsistencies in the immune markers investigated (Eyre and Baune, 2012). Although, PE can be used either as a stand-alone or adjunctive therapy, and has preventative properties for brain pathologies, monitoring and controlling the external environmental variables could be very important to achieve the desired effects on neuroimmune mechanisms.

It has been shown that frequent bouts of PE with exposure to harsh environments such as extremes of heat, cold and humidity, as well as pathogens and stressors to the immune system including lack of sleep, severe mental stress, malnutrition, and bodyweight loss can precipitate diseases associated with inflammatory conditions (Neiman and Pedersen, 1999), which in many respects mimics the immune reactions observed in clinical sepsis (Shephard and Shek, 1998). This indicates that exhaustive exercise or acute bouts of exercise in adverse environmental conditions may act as a deterrent to the normal functioning of the immune system, inducing immunosuppression and increased susceptibility to infections. Some authors have also reported a correlation between PE, external environment temperature and immune changes in the body. Brenner et al. (1999) observed greater elevation in the number of immune cells, such as leucocytes, neutrophils, and NK cells after PE in a hot environment. Prior exercise has been shown to significantly augment leukocyte, granulocyte, and monocyte response to cold exposure (Brenner et al., 1999). This suggests an association between PE, external environmental conditions, and immunological changes in the body and indicates that several external environmental variables can interfere with the results in exercise-based paradigms.

**Figure 3** shows the immunomodulatory mechanism of physical exercise in producing beneficial neurobiological and behavioral effects.

#### *Other forms of environmental enrichment*

***Social enrichment.*** Noticeable alterations in the humoral and cellular immune responses have been observed in animals reared in a socially enriched environment, particularly in submissive animals. These include reduction in the proliferation of splenocytes, and the production of some cytokines (IL-4 and IL-10) and serum antibodies in subordinated animals (Fleshner et al., 1989; Bartolomucci et al., 2001). Similarly, a decrease in T cell proliferation and IL-2 production in submissive animals (Hardy et al., 1990) and a reduction in the number and activity of T cells and NK cells (Stefanski and Engler, 1999; Stefanski, 2001) have been reported after severe social stress. In another experiment, 2 h of social confrontation led to an increase in the number of granulocytes, a decrease in lymphocyte numbers and elevated CD4/CD8 and T cells/B cells ratio in defeated animals (Stefanski and Engler, 1998). These findings clearly suggest that immunosuppression in subordinate animals is caused by the impairment of both humoral and cellular immunity, possibly from social stress as mentioned earlier in this review. The decrease in lymphocytes has also been seen in a similar paradigm in female rhesus macaque, where low social ranking animals showed a reduced proportion of CD8 cytotoxic cells than higher social ranking animals (Tung et al., 2012). This study holds importance as it is suggestive of similar possible consequences in human populations.

***Enrichment with novel objects and accessories.*** Though not studied as extensively as PE for immunomodulatory effects, enrichment with novel objects and accessories has been reported to primarily modify cytokine levels within the brain. Jurgens and Johnson (2012) observed reduced expression of IL-1β and TNF-α within the hippocampus, following an experimental paradigm involving exposure to novel objects and PE. This study was unable to be determined whether PE and enrichment with novel

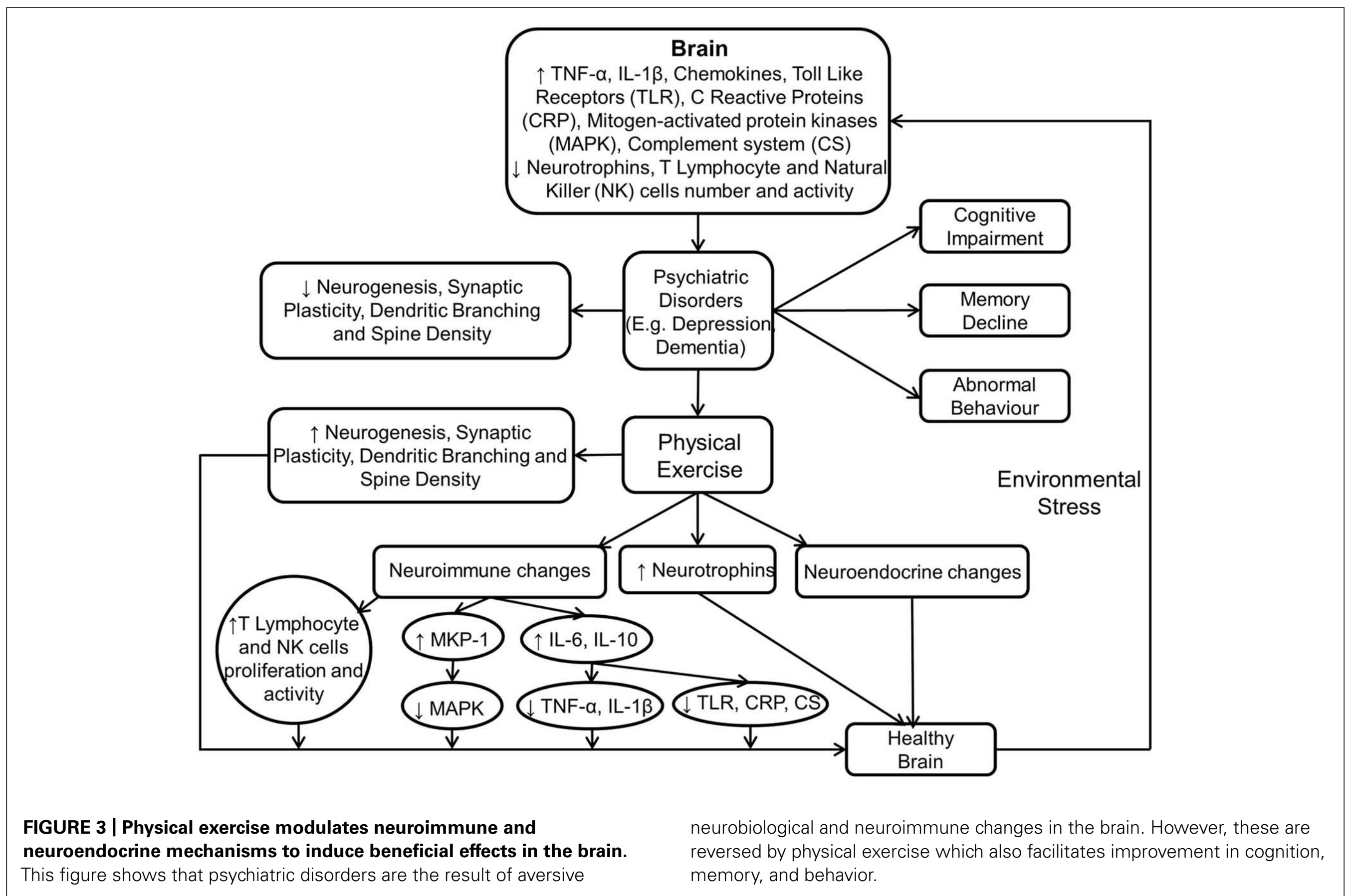



**FIGURE 3 | Physical exercise modulates neuroimmune and neuroendocrine mechanisms to induce beneficial effects in the brain.** This figure shows that psychiatric disorders are the result of aversive neurobiological and neuroimmune changes in the brain. However, these are reversed by physical exercise which also facilitates improvement in cognition, memory, and behavior.

objects showed synergistic or independent effects, since the anti-inflammatory role of PE, as mentioned previously, has been well established. An independent comparative study on PE and enrichment with novel objects and accessories in the future could answer this question. In another experimental study on aged rodents, enrichment with novel objects produced a significant increase in IL-2 and TNF-α levels in the cultured supernatants of peritoneal leucocytes (Arranz et al., 2010). The authors suggested that this increase may have compensated for the age-related loss of these cytokines. However, unlike other EE studies where a number of objects, toys, and accessories were used in different combinations and changed once or twice a week, the authors in this study used only two objects at a time to maintain novelty and changed every 48 h, which could have led to handling stress and low levels of enrichment in cages. Modulation of cellular immune factors such as CD4 and CD8 T lymphocytes, and cytokines IL-2 and IL-1β by enrichment with novel objects and accessories after stressed pregnancies has also been shown in adolescent rats (Laviola et al., 2004), which suggests that novel and cognitively demanding environments can relieve stress through modulation of humoral and cellular immune factors.

***Sensory enrichment.*** Environmental enrichment studies that used sensory stimulation have not yet investigated potential immunomodulatory effects. However, research indicates that sensory stimulation can have prominent effects on the immune system. Researchers have observed enhancement in the systemic proliferation of T lymphocytes (CD4 and CD8 cells) in response to visible light passing through the eye (Roberts, 1995, 2000). A randomized trial with two experimental conditions, first watching a neutral slide show and then a disease slide show, in humans participants has shown that mere visual perception of other people's disease symptoms can boost the immune response to microbial stimuli and increased levels of IL-6 in whole blood (Schaller et al., 2010). Visual stimuli can therefore have an important role in modulating cytokine levels in the brain, however, further research is required to establish its role in EE studies.

Immunomodulatory effects of auditory stimulation have been reported particularly in response to music in humans. Music exposure has been shown to enhance lymphocyte function in the brain, thereby reversing stress induced immunosuppression of rats during a controlled trial with two treatments, music and auditory stress (Núñez et al., 2002). Like music, group drumming therapy in age- and sex-matched human volunteers has been shown to enhance cellular immunity by increasing lymphocyte activated NK cell activity during a single trial experimental intervention with control groups (Bittman et al., 2001). Though these immunomodulatory effects in response to auditory enrichment suggest the possible association of the latter with cytokines, conclusive evidence for this association in rodent based EE studies is still not available.

Neuroimmune mechanisms associated with olfactory stimulation used as enrichment are again poorly studied, although a relationship between olfaction, autoimmunity and brain does exist and has been reviewed in detail by Strous and Shoenfeld (2006). However, the relationship between olfactory stimuli used as enrichment and immune factors in brain is not clear.

***Summary of the neuro-immunomodulatory role of other forms of EE.*** It is evident that less research has been conducted on the immunomodulatory roles of EE methods other than PE. While social enrichment could lead to immunosuppression in some rodents (Fleshner et al., 1989; Hardy et al., 1990; Stefanski and Engler, 1999; Bartolomucci et al., 2001; Stefanski, 2001), enrichment with novel objects and accessories could potentially decrease inflammation with in the brain (Jurgens and Johnson, 2012). Little work has been conducted on the immunomodulatory effects of sensory stimulation in EE studies. It is possible that a combination of different enrichment methods could provide greater enrichment to rodents and eliminate the limitations of any single enrichment method. For example, it is possible that a combination of enrichment with novel objects and accessories, and some favorable sensory stimuli could prevent immunosuppression due to social stress in a socially enriched environment.

**Table 2** presents studies that investigated the effects of EE on various cytokines and other immune factors.

### EFFECTS OF EE ON GLIAL CELLS

Numbers of microglia and astrocytes, have been shown to be increased in certain regions such as the cortex and amygdala (Ehninger and Kempermann, 2003; Okuda et al., 2009) in the brains of enriched rodents during RCTs. These glial cells are known to express various cytokines and modulate the production of neurotrophins, mainly BDNF (Ferrini and De Koninck, 2013), a protein known for regulating neurogenesis in the dentate gyrus of the hippocampus (Rossi et al., 2006; Fan et al., 2007) and enhancing dendritic branching (McAllister et al., 1995; Horch and Katz, 2002; Horch, 2004). BDNF is indeed shown to enhance hippocampal neurogenesis in mice enriched with a running wheel and differently shaped objects (Rossi et al., 2006). Several other neuroglial changes have also been reported in rodents kept in an environment enriched with different methods. These include differentiation of oligodendrocyte progenitor cells into astrocytes in the amygdala of mice enriched with running wheels, tunnels and shelters (Okuda et al., 2009) and prevention of astroglial pathological changes in mice enriched with toys, nesting material, plastic houses, and tubes (Beauquis et al., 2013). Researchers also observed an increase in the expression of astrocyte GFAP (glial fibrillary acidic protein) and microglial IBA1 (ionized calcium-binding adapter molecule 1) in the dentate gyrus of rats provided with a running wheel, a polyvinyl chloride (PVC) tube and various small objects and toys (Williamson et al., 2012), and inhibition of age induced gliosis in the hippocampus of rats reared in two series of three large interconnected wire mesh cages containing various objects such as toys, balls ladders, and footbridges to play with (Soffié et al., 1999). All these changes are suggestive of the vital impact that EE has on glial cells which may in turn modulate glia-based neuroimmune mechanisms. Indeed, EE for rodents in large cages with toys and accessories and/or running wheels has shown beneficial effects in models of several brain diseases such as AD (Beauquis et al., 2013), and schizophrenia and depression (Laviola et al., 2008), which are generally associated with abnormalities in glial cells morphology and functioning (Cotter et al., 2001; Nagele et al., 2004).

**Table 3** presents studies that investigated the effects of EE on glial cells.

## DISCUSSION

### IMMUNOMODULATION BY DIFFERENT METHODS OF ENVIRONMENTAL ENRICHMENT

Provision of voluntary wheel running, social housing, cognitive training, and sensory stimulation may act as mild stressors in the initial stages but a number of studies have shown constructive neurobiological and behavioral variations in response to these enrichment methods, particularly to PE and enrichment with novel objects and accessories. Ironically, the same causal factors for stress when altered finely can become favorable for living and can be used for enrichment during psychiatric disorders. See **Figure 4** for more details.

The neuroimmunomodulatory role of PE has been extensively studied and appears to be the strongest form of enrichment when used alone in both rodents and human studies. PE can modulate a number of brain regions which may in turn result in varied functional outcomes, such as improvement in memory (Erickson et al., 2011), learning (Van Praag et al., 2005), anxiety- and depressive-like behaviors (Binder et al., 2004; Zheng et al., 2006; Duman et al., 2008; Marais et al., 2009), cognition (Griffin et al., 2009; Nichol et al., 2009), and motor activity (Biernaskie and Corbett, 2001), and this has been highly regarded by researchers in their publications. PE mostly affects the humoral immune system; however, its role in the modulation of cellular immune system cannot be ignored. Post-exercise, production and expression of anti-inflammatory factors, particularly anti-inflammatory cytokines (e.g., IL-6, IL-10) are enhanced in both the systemic circulation as well as within the brain. This subsequently reduces the level of pro-inflammatory factors, such as the cytokines TNF-α and IL-1β (Eyre and Baune, 2012), chemokines (Ostrowski et al., 2001), TLRs (Gleeson et al., 2006), and CRP (Koletzko, 2003), helping in alleviating both systemic and neuroinflammation, the latter being the causal factor for most psychiatric disorders. An increase in the number of T lymphocytes and NK cells after PE (Kaufman et al., 1994) strengthens adaptive immunity. Modulation of glial cells, T cells, and macrophages in the brain by PE also helps in reducing the neurotoxic effects and enhances neurogenesis in the brain, particularly in the hippocampus (Eyre and Baune, 2012). Though moderate PE has been reported to induce beneficial effects, exhaustive PE has been shown to result in immunosuppression in human participants (Mars et al., 1998; Tuan et al., 2008) which suggests that voluntary wheel running is probably more useful for inducing favorable neuroimmune changes than forced exercise on a treadmill. The latter could cause stress to rodents in EE studies. Further, external environmental conditions (e.g., heat, cold, humidity) could play a role in the immunomodulatory effects of PE on the brain, as stated earlier in this review. While rodents are reared in standard environmental conditions with all

**Table 2 | Effects of environmental enrichment on various cytokines and other immune parameters.**

| Study's primary objective | Animal species/strain | EE methods | Frequency of changing EE method | Immune markers/behavior investigated | Significant findings | Reference |
|---|---|---|---|---|---|---|
| • Effect of different forms of EE on behavioral, endocrinological, and immunological parameters in male mice | • Congenic mice strain CS of the inbred strain ABG | • EH and SEH<br>• EH: Standard cages with a plastic inset and wooden scaffolding<br>• SEH: Spacious glass terraria with passable enriched cage, extra plains, plastic stairs, wooden footpaths, hemp ropes and a climbing tree. Food and water available at two places | • Once a week | • Spontaneous behavior in home cage<br>• Immunological parameters: $CD4^+$ and $CD8^+$ cells, cytokines (IL-2, IL-4, IL-10, and IFN-γ), IgG1 and IgG2a | • ↑ Aggressive behavior in EH and SEH mice<br>• ↑ Play behavior in SEH mice. No significant differences between controls and EH mice<br>• ↓ IgG1 in serum<br>• ↑ IgG2a/IgG1<br>• ↓ IFN-γ/IL-10 and IL-2/IL-10 | Marashi et al. (2003, 2004) |
| • Effect of EE on the negative effects of influenza infection on hippocampus and spatial cognition | • 6 weeks old male Balb/c mice | • Social enrichment (five to eight per cage), toys, tunnels, ladders, housing chamber, nesting material, running wheel | • Three to four times per week | • Behavioral testing – Morris water maze<br>• Hippocampal cytokines (IL-1β, TNF-α, IL-6), chemokines (CX3CL), interferons (IFN-α and -β), and neurotrophins (BDNF and NGF) | • Improved spatial learning<br>• ↓ Expression of IL-1β and TNF-α in the hippocampus<br>• ↓ Hippocampal inflammation and cognitive deficit<br>• ↑ Hippocampal BDNF and CX3CL1 (anti-inflammatory chemokine) expression | Jurgens and Johnson (2012) |
| • Effect of PE on protective immunity against infection | • Sprague-Dawley male rats | • Training for swimming given to rats. At the end of training period, rats were exercised by subjecting them to an exhaustive swim | – | • ELISA test for antibody response<br>• Immunofluorescent lymphocyte subtyping | • ↑ IgG and IgM production, T suppressor cells, and NK cells<br>• ↓ T helper cells and T helper/T suppressor ratio | Kaufman et al. (1994) |

*(Continued)*

**Table 2 | Continued**

| Study's primary objective | Animal species/strain | EE methods | Frequency of changing EE method | Immune markers/behavior investigated | Significant findings | Reference |
|---|---|---|---|---|---|---|
| • Effects of EE on several functions and oxidative stress parameters of peritoneal leucocytes in mice at different ages | • Female ICR/CD-1 mice | • Two different objects in the cages at a time<br>• These objects include orange bucket, jolly ball, hoop, holed ball, yellow tunnel, rough red object, yellow billiard ball, and silver ball<br>• A red kennel maintained permanently inside the cage | • Every 2 days at 08:00 hours | • Flow cytometry to analyze leukocyte differentiation antigens (CD11b, CD11c, CD4, CD19) and membrane expression of TLR-2 and -4<br>• Evaluation of macrophage chemotaxis, and phagocytosis<br>• Analysis of lympho-proliferation and cytotoxicity<br>• ELISA for analysis of IL-2 and TNF-α | • Higher macrophage chemotactic activity and phagocytosis<br>• ↑ Basal lymphocyte proliferation and chemotactic activity<br>• EE prevented age-related decline of IL-2 and TNF-α levels in old enriched mice<br>• ↓ Expression of TLR-2 and 4 on CD4 and CD8 cells | Arranz et al. (2010) |
| • Effects of EE on NK cell activity, psychological stress response, and behavioral parameters | • 1 month old male C3H/eB mice | • A variety of stimuli – ladders, tunnels, and running wheels | • Once a week | • Behavioral test: Grip strength test, Elevated plus maze, and Staircase test.<br>• Measurement of NK activity by a standard chromium release assay | • Decreased anxiety-like behavior and increased activity in Elevated plus maze and Staircase test, respectively<br>• Higher NK cell activity in spleen | Benaroya-Milshtein et al. (2004) |

*EE, environmental enrichment; EH, enriched housing; SEH, super enriched housing; CD, cluster of differentiation 4; IL, interleukin; IFN, interferon; IgG, immunoglobulin G; TNF-α, tumor necrosis factor-α; CX3CL, chemokine (C-X3-C motif) ligand; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; NK, natural killer; TLR, Toll-like receptor; ROS, reactive oxygen species; ELISA, enzyme linked immunosorbent assay.*

**Table 3 | Effects of environmental enrichment on glial cells.**

| Study's primary objective | Animal species/strain | EE methods | Frequency of changing EE method | Proteins/behavior investigated | Significant findings | Reference |
|---|---|---|---|---|---|---|
| • Modulation of glial cells with EE in an animal model of AD | • PDAPP-J20 crossed with C57BL/6J mice | • Large cages with toys, extra nesting material, small plastic houses, and tubes. No running wheel | • Every 2 days | • Aβ peptides<br>• Amyloid plaques<br>• GFAP$^{+}$ glial cells | • ↓ Astrocytes association with Aβ plaques<br>• ↓ Aβ peptides | Beauquis et al. (2013) |
| • Effect of EE on glial cells within the hippocampus | • Adult male Sprague-Dawley rats | • A running wheel, a PVC tube, various small objects, and toys | • EE for 12 h each day in a separate cage | • Expression of various cytokines, chemokines, GFAP, and IBA1<br>• Expression of growth factors, BDNF and GDNF in the hippocampus. | • ↑ Astrocytes and microglia antigens expression in the hippocampus but not in CA1, CA3, and cortex<br>• ↓ Expression of TNF, IL-1β, and chemokines Cc12, Cc13, and Cxc12 in the hippocampus<br>• ↑ Hippocampal BDNF mRNA | Williamson et al. (2012) |
| • Effect of EE on the short-term memory for event durations and on the astrocytes percentage in hippocampus, frontal cortex, and corpus callosum | • Naive male Wistar derived rats 5 and 21 months of age | • Three large wire mesh cages interconnected with two tunnels, wooden and metallic objects, toys and balls, ladders, footbridges, and papers | • Daily | • Delayed symbolic matching to sample task test<br>• Estimation of astrocytes by evaluating GFAP percentage | • Behavioral testing resulted in ↑ in the astrocytes number and size and GFAP % in the hippocampus and corpus callosum of young rats<br>• Conversely, Behavioral testing resulted in ↓ in the astrocytes number and size and GFAP % in the hippocampus and corpus callosum of old rats<br>• ↓ Astrocytes number and size in the hippocampus and corpus callosum of old enriched rats<br>• Reduction in memory deficit with age in old enriched rats but no total reversal of age-related impairment | Soffié et al. (1999) |

*EE, environmental enrichment; AD, Alzheimer's disease; Aβ, amyloid-β; GFAP, glial fibrillary acidic protein; PVC, polyvinyl chloride; IBA1, ionized calcium-binding adapter molecule 1; BDNF, brain-derived neurotrophic factor; GDNF, glial cell-derived neurotrophic factor; CA, Cornu Ammonis; Cc12, (C–C motif) ligand 12; Cc13, (C–C motif) ligand 13; Cxc12, (C–X–C motif) ligand 12; mRNA, messenger ribonucleic acid.*

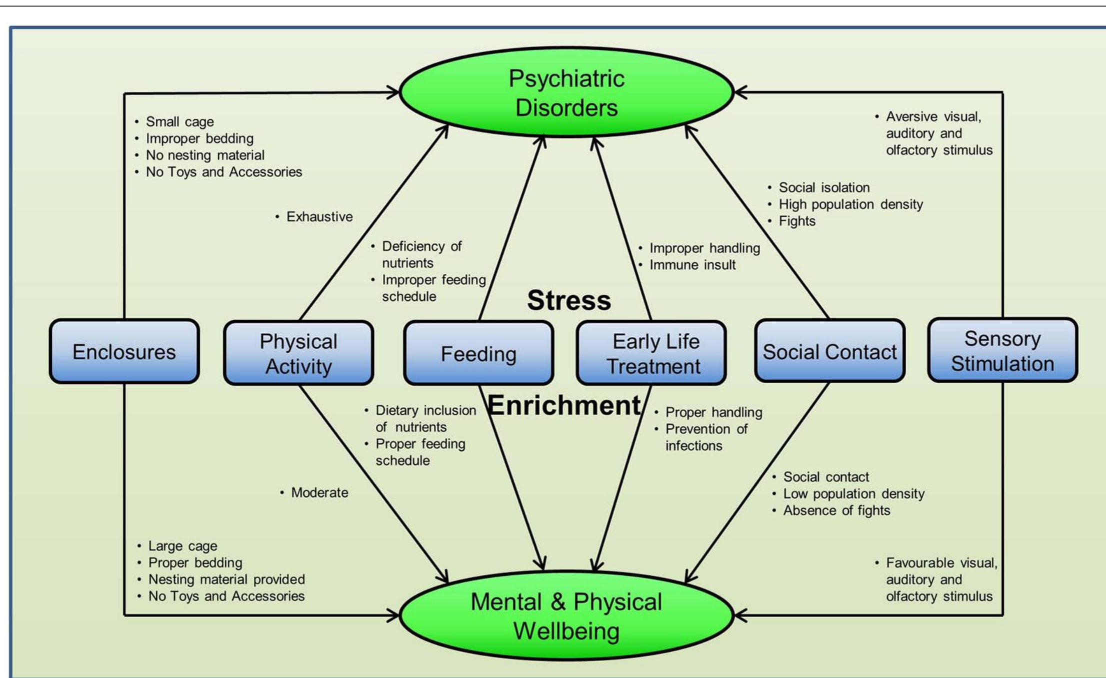



**FIGURE 4 | Differential effects of enrichment methods in rodents.** The same stressful factor when subtly modified can become enrichment for the animals in captivity. This suggests that environment for rodents can be enriched by changing the existing arrangement of different factors around them and no special efforts are required.

variables controlled throughout the life span of rodents, the same may not be applicable to humans.

Social and cage enrichment are the simplest avenues to modulate behavior, however, formation of dominant and subordinate populations can affect the response, with detrimental effects seen mainly in subordinate animals. The latter have shown signs of immunosuppression and depression in enrichment studies which clash with the principles of enrichment, i.e., making the environment favorable for living. In fact, cellular analyses have revealed loss in the number and function of splenocytes, decreased anti-inflammatory cytokines (e.g., IL-10), T cells, NK cells, and serum antibodies in subordinate animals (Fleshner et al., 1989; Bartolomucci et al., 2001). Several EE studies have used social enrichment for rodents (Angelucci et al., 2009; Jurgens and Johnson, 2012); however, they have not reported on the presence or absence of dominant and subordinate, which might have confounded the findings from these studies.

"Novelty seeking" behavior is the inherent tendency to explore novel objects and accessories, and has been investigated in many enrichment studies. Novel objects and accessories used in conjunction with PE have been reported to reduce the expression of IL-1β and TNF-α in the hippocampus (Jurgens and Johnson, 2012) suggestive of anti-inflammatory effects. This, however, makes it difficult to conclude whether these anti-inflammatory effects were seen in response to PE and/or to the novel objects and accessories. Nevertheless, the role of novel objects in cell-mediated immunity cannot be disregarded as improvement in macrophage chemotaxis and phagocytosis, lymphocyte chemotaxis, and NK with two novel objects at a time (Arranz et al., 2010). It is possible that changes in the methods of enrichment and rearrangement of objects in space and time are required for the sustained beneficial effects on the brain of the complex environment devoid of running wheels; but substantial evidence is still required to establish this hypothesis. Some EE studies failed to mention whether the objects were changed (Nakamura et al., 1999; Kempermann et al., 2002; Lazarov et al., 2005). If the objects were not replaced regularly to maintain novelty, it may have affected the immune response and behavior of rodents during study. Furthermore, few studies have investigated the immunomodulatory effects of enrichment with novel objects, and no meta-analysis is available to verify the results at this stage, thus making it essential to validate these findings with more extensive research. In terms of the human environments, a subject receives several kinds of stimulus in addition to PE, which could have confounding effects on modulation of brain function.

The immunomodulatory mechanisms associated with sensory enrichment have not been investigated in EE studies. Sensory enrichments have been shown to enhance sensory functions (visual, auditory, or olfactory), as well as improve cognition and behavior (see review by Wells, 2009), although the neuroimmune mechanisms accountable for this improvement in brain functions are not fully described and therefore this needs further attention in future studies.

It appears that EE is a very complex process and that a standard rodent environment may also involve all methods of enrichment at the same time to some extent. For instance, rodents climbing the cage walls/grid could be an example of resistance exercise which is seen even in the absence of a running wheel in cages and has been

reported to improve spatial memory (Cassilhas et al., 2012). Similarly, resistance exercise in healthy humans (knee extension under alternating concentric and eccentric conditions for muscle work), in the absence of any endurance exercise, improved serum concentrations of insulin-like growth factor 1 (IGF-1), which has been shown to mediate positive effects of exercise on brain functions (Carro et al., 2000; Vega et al., 2010). Further, social enrichment through housing in pairs could lead to the formation of a dominant and submissive mouse (Malatynska and Knapp, 2005). In addition, it is possible that the sight of a novel object, auditory stimulus during handling or changing cages, and olfactory cues from handler or an injured mouse in a social environment could modify immune parameters in the brain, and in turn neurobiology and behavior. Likewise, any new nesting material or sensory stimuli could also possibly stimulate "novelty seeking" behavior of rodents. Therefore, this necessitates thorough examination of all external environmental variables in EE paradigms.

No research to date has used standardized enrichment techniques and different methods have been used randomly. This standardization of enrichment techniques is necessary since different methods of EE can elicit diverse effects on neurobiology, behavior, and neuroimmune mechanisms. Moreover, a standard human environment is similar to an enriched environment for rodents. The results from enrichment through PE in rodents can successfully be translated to human intervention for psychiatric illnesses; however, findings from other enrichment methods in rodents may be of less value in translating the effects to humans. An intense examination of human nature and its application while formulating an environment enriched with cognitively stimulating activities is essential for similar interventions in humans.

## A NOTE OF CAUTION ON THE USE OF NUTRITIONAL ENRICHMENT

While some researchers have advocated the use of food for enrichment to improve behavior (Harris et al., 2001; Brown, 2009), others have avoided using nutritional enrichment apparently due to the confounding effects of various nutrients on neurobiology and behavior when used in conjunction with other enrichment methods. Evidently, diets rich in essential fatty acids (EFA), such as omega-3, normalize the levels of brain proteins, reduce oxidative stress, maintain neuronal plasticity, improve mood, and enhance learning and cognitive abilities (Richardson, 2003; Wu et al., 2004) in both rodents and humans. Such subjects are also less susceptible to stress and show improved behavior and enhanced memory (Wainwright et al., 1994; Wainwright, 2002; Fedorova and Salem, 2006; Uauy and Dangour, 2006). Similarly, a high protein and glucose diet has been shown to enhance the growth of the brain and its functions (Diamond, 2001). The effects of anti-oxidants, anti-inflammatory components, vitamins, and minerals in food on behavior, learning, and cognition have been studied and reviewed extensively in the past (Wasantwisut, 1996; Beard, 2003; Navarro et al., 2005; Buell and Dawson-Hughes, 2008; Joseph et al., 2009; Ford et al., 2010; Kennedy and Haskell, 2011). Yet, the effects of food variety are difficult to distinguish from the intrinsic nutritional effects of the specific food that is used for enrichment. It is likely that food used in combination with other enrichment methods could have confounded the neurobiological and behavioral effects depending on its nutrient composition in EE studies.

## TIME OF ENRICHMENT IN THE LIFE OF AN ANIMAL

Besides different methods of EE, the stage of life when enrichment has been given can potentially affect results. During neonatal and early prenatal periods, the brain develops rapidly, large numbers of new synapses are formed and growth and differentiation of the cerebro-cortical region takes place. Any changes in the brain induced during this period can persist throughout life. Environmental conditions during prenatal and early stages of the life cycle can have distinct effects on neurobiology and behavior (Chapillon et al., 2002; Gutman and Nemeroff, 2002). Moreover, studies have shown that immune insult during pregnancy can affect growth and behavior of offspring and makes them susceptible to mental disorders such as AD, schizophrenia, and autism (Shi et al., 2003; Bakos et al., 2004; Brown, 2006).

While prenatal stress has been shown to suppress NK cell cytotoxicity and reduce B cell proliferation in an experiment on rats (Kay et al., 1998), a review suggests that perinatal infection can cause long-term alteration in cytokine production and brain glial cell function and is generally manifested by marked cognitive and behavioral changes throughout the lifespan (Bilbo and Schwarz, 2009). When exposed to perinatal infection and neonatal maternal separation in controlled trials, rodents displayed marked cognitive and behavioral changes, and impaired learning and memory which persisted throughout their lifespan (Bilbo and Schwarz, 2009; Vivinetto et al., 2013). Loss of hippocampal plasticity (Mirescu et al., 2004) and sex-specific changes in hippocampal dendritic complexity and dendritic spine density (Bock et al., 2011) has also been reported in adult rats exposed to early life stressful experiences. This suggests that providing enrichment during early stages of the life cycle can help to safeguard against psychiatric disorders in later life. Indeed, neonatal handling with an enriched environment can reduce the signs of emotionality and anxiety, augment novelty seeking behavior, and can have preventative actions on age-related learning impairments and hippocampal neuronal atrophy in rodents (see review by Fernandez-Teruel et al., 1997). Likewise, enrichment with frequently changed toys after weaning provided a beneficial intervention for reversing the harmful effects of maternal separation in rats (Francis et al., 2002). This decreased reactivity to stressful stimuli, however, was later found to be the function of a less sensitive HPA axis (Anisman et al., 1998; Welberg et al., 2006). Contrary to these findings, Papaioannou et al. (2002) observed sexual differences and suggested that neonatal handling increases the capacity of male Wistar rats to face chronic stressors, and increases the susceptibility to express "depressive" behavior in female. The authors have attributed this discrepancy between the two sexes to the combination of decreased serotonergic activity with high circulating corticosterone levels in female rats.

Controlled trials on Sprague-Dawley rats have established that enrichment in early life increases T cell numbers, enhances production of anti-inflammatory cytokines (e.g., IL-2, IL-10) and lowers production of the pro-inflammatory cytokine IL-1β in various brain regions such as the hypothalamus and frontal cortex (Laviola et al., 2004; Bilbo et al., 2007), suggesting attenuating effects of early life enrichment on neuroinflammation. This suggests that requisite neurological and behavioral enhancements can be more readily achieved by enriching the environment of an

animal at the prenatal stage and preserving it later in life with regular novel and enriching inputs.

### SIGNIFICANCE OF IMMUNE AND NON-IMMUNE FACTORS IN ENVIRONMENTAL ENRICHMENT PARADIGMS

It is evident that overexpression of pro-inflammatory cytokines and chemokines in the brain, in addition to decreases in cytotoxic T cell proliferation and activity may result in diminished cognitive performance and development of neuropathology (Arvin et al., 1996; Hawkley and Cacioppo, 2004; Baune et al., 2008). These cytokines are primarily expressed by glial cells in the brain whose levels increase with aging. However, the neuronal aging slows down in mice raised in enriched environment, and is characterized by sustained neurogenesis and reduced neuronal damage in the cellular microenvironment of the dentate gyrus (Nilsson et al., 1999; Gould et al., 2000; Kempermann et al., 2002). Indeed, EE has been shown to improve the plasticity of cognitive functions and learning performance, and reduce the impairment of spatial memory in aged rodents (Kobayashi et al., 2002; Frick and Fernandez, 2003; Frick et al., 2003). In addition, the role of cellular immunity is important when studying immune effects of external environmental stimuli (Stefanski and Engler, 1999; Stefanski, 2001; Benaroya-Milshtein et al., 2004).

Several non-immune factors such as neurotransmitters and neurotrophins have also been implicated in the neural changes within the enriched environment. It has been suggested that neurotransmitters, such as dopamine, serotonin, and GABA, mediate communication between the nervous system and immune system (Mössner and Lesch, 1998; Basu and Dasgupta, 2000; Felten, 2008; Bhat et al., 2010) and their levels change in different brain regions of rodents, such as the hippocampus and prefrontal cortex, in response to an enriched environment (Mora et al., 2007). Indeed, in a study on aged rats kept in an environment enriched with running wheels, a re-arrangeable set of plastic tunnels, an elevated platform and toys, the extracellular levels of the neurotransmitters GABA and glutamate showed an increase in the CA3 area of the hippocampus (Segovia et al., 2006). Likewise, levels of neurotrophins, nerve growth factor, BDNF, and neurotrophin-3, have been shown to be increased in different brain region of rats, such as the cerebral cortex, hippocampus, and forebrain after treatment with EE comprising of running wheels, toys, and novel objects (Ickes et al., 2000; Pham et al., 2002; Gobbo and O'Mara, 2004; Angelucci et al., 2009). Indeed, the increase in hippocampal expression of BDNF in response to voluntary wheel-running (Duman et al., 2008) and 1-week forced treadmill exercise (Griffin et al., 2009) in rodents has been attributed to improvement in cognitive functions and reduction in anxiety- and depressive-like behaviors by some authors.

Taken together, the finding in this review, suggest that improvement and development of the environment is beneficial to preserve and enhance species-typical behavioral aspects by altering immune parameters in association with genes and neurochemicals. However, the immunomodulatory roles of enrichment methods other than PE have received less attention and a better understanding is required.

## LIMITATIONS OF THIS REVIEW

The primary aim of this review is to discuss the neuro-immunomodulatory mechanisms that govern effects of various EE methods on brain functions such as cognition and memory. Since most EE studies have been conducted on rodents, the studies included in this review investigated the role of EE in modulating neurobiology and behavior, and associated immune mechanisms in rats and mice. As such, all evidence reported may not provide similar results in other animals or in humans. However, this review provides the foundation to model similar or equivalent enrichment techniques in other species of animal, as well as portray the possible consequences of enriching the existing environment for humans, though the outcome may vary depending on the existing circumstances of each individual. This is contrary to rodents in cages when all animals are in similar environmental conditions during study. Moreover, the limited number of studies on the immunomodulatory effects of EE methods in rodents, other than exercise, also limited our efforts to include all immune factors, for example, CRP, MAPK, and the complement system, under consideration for each enrichment method and compare them with similar results in human intervention. This suggests that though this review provides a comprehensive account for EE effects of neuroimmune modulation, extensive research is still needed to establish that EE provides beneficial intervention for psychiatric disorders and neuropathological conditions via modulation of cytokines and other humoral and cellular immune factors.

## CONCLUDING REMARKS

There are many complexities involved in EE paradigms, as clear from our discussion in this review. Although it is widely accepted that enriching the environment exerts distinct beneficial effects on the learning and memory competence of an animal and therefore, considering various environmental factors is vital while formulating EE methodology for studies on its effects on brain functions. Substantial evidence confirms that PE alleviates psychiatric disorders via modulation of neuroimmune mechanisms (Kaufman et al., 1994; Pedersen and Hoffman-Goetz, 2000; Eyre and Baune, 2012), however, the same cannot be said conclusively for other enrichment methods. While the immunomodulatory mechanisms of EE in controlling brain diseases and cognitive disorders in rodents have received much attention in the last decade, similar studies in humans to investigate the immunomodulatory effects of analogous EE methods (as pointed out earlier in this review) in psychiatric disorders need to be conducted. Since physical activity is a form of EE in rodents and reduces cognitive and memory deficits, the hypothesis that a combination of PE and cognitive training will have a preventative and therapeutic effect on human brain disorders via anti-neuroinflammatory and anti-neurodegenerative mechanisms, still needs to be investigated. Additionally, a study on combined effect of PE with other EE methods and/or pharmacological drugs on a long-term and short-term basis in rodents will be helpful to develop new, and optimize current, immunomodulatory preventative and treatment therapies for cognitive dysfunction and associated brain disorders.

## ACKNOWLEDGMENTS

The presented work is supported by the National Health and Medical Research Council Australia (APP 1043771 to Bernhard T. Baune). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## REFERENCES

Adolphs, R. (2003). Cognitive neuroscience of human social behaviour. *Nat. Rev. Neurosci.* 4, 165–178. doi: 10.1038/nrn1056

Ahmadiasl, N., Alaei, H., and Hanninen, O. (2003). Effect of exercise on learning, memory and levels of epinephrine in rats' hippocampus. *J. Sports Sci. Med.* 2, 106–109.

Akaneya, Y., Tsumoto, T., Kinoshita, S., and Hatanaka, H. (1997). Brain-derived neurotrophic factor enhances long-term potentiation in rat visual cortex. *J. Neurosci.* 17, 6707–6716.

Anderson, D. H., Radeke, M. J., Gallo, N. B., Chapin, E. A., Johnson, P. T., Curletti, C. R., et al. (2010). The pivotal role of the complement system in aging and age-related macular degeneration: hypothesis re-visited. *Prog. Retin. Eye Res.* 29, 95–112. doi: 10.1016/j.preteyeres.2009.11.003

Angelucci, F., De Bartolo, P., Gelfo, F., Foti, F., Cutuli, D., Bossu, P., et al. (2009). Increased concentrations of nerve growth factor and brain-derived neurotrophic factor in the rat cerebellum after exposure to environmental enrichment. *Cerebellum* 8, 499–506. doi: 10.1007/s12311-009-0129-1

Angelucci, F., Fiore, M., Ricci, E., Padua, L., Sabino, A., and Tonali, P. A. (2007a). Investigating the neurobiology of music: brain-derived neurotrophic factor modulation in the hippocampus of young adult mice. *Behav. Pharmacol.* 18, 491–496. doi: 10.1097/FBP.0b013e3282d28f50

Angelucci, F., Ricci, E., Padua, L., Sabino, A., and Tonali, P. A. (2007b). Music exposure differentially alters the levels of brain-derived neurotrophic factor and nerve growth factor in the mouse hypothalamus. *Neurosci. Lett.* 429, 152–155. doi: 10.1016/j.neulet.2007.10.005

Anisman, H., Zaharia, M. D., Meaney, M. J., and Merali, Z. (1998). Do early-life events permanently alter behavioral and hormonal responses to stressors? *Int. J. Dev. Neurosci.* 16, 149–164. doi: 10.1016/S0736-5748(98)00025-2

Anomal, R., de Villers-Sidani, E., Merzenich, M. M., and Panizzutti, R. (2013). Manipulation of BDNF signaling modifies the experience-dependent plasticity induced by pure tone exposure during the critical period in the primary auditory cortex. *PLoS ONE* 8:e64208. doi: 10.1371/journal.pone.0064208

Arakawa, H., Arakawa, K., and Deak, T. (2010). Sickness-related odor communication signals as determinants of social behavior in rat: a role for inflammatory processes. *Horm. Behav.* 57, 330–341. doi: 10.1016/j.yhbeh.2010.01.002

Arranz, L., De Castro, N. M., Baeza, I., Mate, I., Viveros, M. P., and De la Fuente, M. (2010). Environmental enrichment improves age-related immune system impairment: long-term exposure since adulthood increases life span in mice. *Rejuvenation Res.* 13, 415–428. doi: 10.1089/rej.2009.0989

Arvin, B., Neville, L. F., Barone, F. C., and Feuerstein, G. Z. (1996). The role of inflammation and cytokines in brain injury. *Neurosci. Biobehav. Rev.* 20, 445–452. doi: 10.1016/0149-7634(95)00026-7

Avitsur, R., Kavelaars, A., Heijnen, C., and Sheridan, J. F. (2005). Social stress and the regulation of tumor necrosis factor-α secretion. *Brain Behav. Immun.* 19, 311–317. doi: 10.1016/j.bbi.2004.09.005

Azpiroz, A., Garmendia, L., Fano, E., and Sanchez-Martin, J. R. (2003). Relations between aggressive behavior, immune activity, and disease susceptibility. *Aggress. Violent Behav.* 8, 433–453. doi: 10.1016/S1359-1789(02)00066-6

Bakos, J., Duncko, R., Makatsori, A., Pirnik, Z., Kiss, A., and Jezova, D. (2004). Prenatal immune challenge affects growth, behavior, and brain dopamine in offspring. *Ann. N. Y. Acad. Sci.* 1018, 281–287. doi: 10.1196/annals.1296.033

Banks, W., Kastin, A., and Broadwell, R. (1995). Passage of cytokines across the blood–brain barrier. *Neuroimmunomodulation* 2, 241–248. doi: 10.1159/000097202

Banks, W. A., and Erickson, M. A. (2010). The blood–brain barrier and immune function and dysfunction. *Neurobiol. Dis.* 37, 26–32. doi: 10.1016/j.nbd.2009.07.031

Bartolomucci, A., Palanza, P., Gaspani, L., Limiroli, E., Panerai, A. E., Ceresini, G., et al. (2001). Social status in mice: behavioral, endocrine and immune changes are context dependent. *Physiol. Behav.* 73, 401–410. doi: 10.1016/S0031-9384(01)00453-X

Basu, S., and Dasgupta, P. S. (2000). Dopamine, a neurotransmitter, influences the immune system. *J. Neuroimmunol.* 102, 113–124. doi: 10.1016/S0165-5728(99)00176-9

Batzina, A., Dalla, C., Papadopoulou-Daifoti, Z., and Karakatsouli, N. (2014). Effects of environmental enrichment on growth, aggressive behaviour and brain monoamines of gilthead seabream *Sparus aurata* reared under different social conditions. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 169, 25–32. doi: 10.1016/j.cbpa.2013.12.001

Baumans, V. (2005). Environmental enrichment for laboratory rodents and rabbits: requirements of rodents, rabbits, and research. *ILAR J.* 46, 162–170. doi: 10.1093/ilar.46.2.162

Baune, B. T., Konrad, C., Grotegerd, D., Suslow, T., Ohrmann, P., Bauer, J., et al. (2012). Tumor necrosis factor gene variation predicts hippocampus volume in healthy individuals. *Biol. Psychiatry* 72, 655–662. doi: 10.1016/j.biopsych.2012.04.002

Baune, B. T., Wiede, F., Braun, A., Golledge, J., Arolt, V., and Koerner, H. (2008). Cognitive dysfunction in mice deficient for TNF- and its receptors. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 147B, 1056–1064. doi: 10.1002/ajmg.b.30712

Beard, J. (2003). Iron deficiency alters brain development and functioning. *J. Nutr.* 133, 1468S–1472S.

Beauquis, J., Pavia, P., Pomilio, C., Vinuesa, A., Podlutskaya, N., Galvan, V., et al. (2013). Environmental enrichment prevents astroglial pathological changes in the hippocampus of APP transgenic mice, model of Alzheimer's disease. *Exp. Neurol.* 239, 28–37. doi: 10.1016/j.expneurol.2012.09.009

Benaroya-Milshtein, N., Hollander, N., Apter, A., Kukulansky, T., Raz, N., Wilf, A., et al. (2004). Environmental enrichment in mice decreases anxiety, attenuates stress responses and enhances natural killer cell activity. *Eur. J. Neurosci.* 20, 1341–1347. doi: 10.1111/j.1460-9568.2004.03587.x

Bennett, J. C., McRae, P. A., Levy, L. J., and Frick, K. M. (2006). Long-term continuous, but not daily, environmental enrichment reduces spatial memory decline in aged male mice. *Neurobiol. Learn. Mem.* 85, 139–152. doi: 10.1016/j.nlm.2005.09.003

Berardi, N., Braschi, C., Capsoni, S., Cattaneo, A., and Maffei, L. (2007). Environmental enrichment delays the onset of memory deficits and reduces neuropathological hallmarks in a mouse model of Alzheimer-like neurodegeneration. *J. Alzheimers Dis.* 11, 359–370.

Berk, M., Wadee, A., Kuschke, R., and O'Neill-Kerr, A. (1997). Acute phase proteins in major depression. *J. Psychosom. Res.* 43, 529–534. doi: 10.1016/S0022-3999(97)00139-6

Bhat, R., Axtell, R., Mitra, A., Miranda, M., Lock, C., Tsien, R. W., et al. (2010). Inhibitory role for GABA in autoimmune inflammation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2580–2585. doi: 10.1073/pnas.0915139107

Biernaskie, J., and Corbett, D. (2001). Enriched rehabilitative training promotes improved forelimb motor function and enhanced dendritic growth after focal ischemic injury. *J. Neurosci.* 21, 5272–5280.

Bilbo, S. D., Newsum, N. J., Sprunger, D. B., Watkins, L. R., Rudy, J. W., and Maier, S. F. (2007). Differential effects of neonatal handling on early life infection-induced alterations in cognition in adulthood. *Brain Behav. Immun.* 21, 332–342. doi: 10.1016/j.bbi.2006.10.005

Bilbo, S. D., and Schwarz, J. M. (2009). Early-life programming of later-life brain and behavior: a critical role for the immune system. *Front. Behav. Neurosci.* 3:14. doi: 10.3389/neuro.08.014.2009

Binder, E., Droste, S. K., Ohl, F., and Reul, J. M. (2004). Regular voluntary exercise reduces anxiety-related behaviour and impulsiveness in mice. *Behav. Brain Res.* 155, 197–206. doi: 10.1016/j.bbr.2004.04.017

Bittman, B. B., Berk, L. S., Felten, D. L., Westengard, J., Simonton, O. C., Pappas, J., et al. (2001). Composite effects of group drumming music therapy on modulation of neuroendocrine-immune parameters in normal subjects. *Altern. Ther. Health Med.* 7, 38.

Blackford, J. U., Buckholtz, J. W., Avery, S. N., and Zald, D. H. (2010). A unique role for the human amygdala in novelty detection. *Neuroimage* 50, 1188–1193. doi: 10.1016/j.neuroimage.2009.12.083

Blanchard, R. J., McKittrick, C. R., and Blanchard, D. C. (2001). Animal models of social stress: effects on behavior and brain neurochemical systems. *Physiol. Behav.* 73, 261–271. doi: 10.1016/S0031-9384(01)00449-8

Blom, H., Van Tintelen, G., Van Vorstenbosch, C., Baumans, V., and Beynen, A. (1996). Preferences of mice and rats for types of bedding material. *Lab. Anim.* 30, 234–244. doi: 10.1258/002367796780684890

Blood, A. J., Zatorre, R. J., Bermudez, P., and Evans, A. C. (1999). Emotional responses to pleasant and unpleasant music correlate with activity in paralimbic brain regions. *Nat. Neurosci.* 2, 382–387. doi: 10.1038/7299

Bock, J., Murmu, M. S., Biala, Y., Weinstock, M., and Braun, K. (2011). Prenatal stress and neonatal handling induce sex-specific changes in dendritic complexity and dendritic spine density in hippocampal subregions of prepubertal rats. *Neuroscience* 193, 34–43. doi: 10.1016/j.neuroscience.2011.07.048

Bose, M., Munoz-Llancao, P., Roychowdhury, S., Nichols, J. A., Jakkamsetti, V., Porter, B., et al. (2010). Effect of the environment on the dendritic morphology of the rat auditory cortex. *Synapse* 64, 97–110. doi: 10.1002/syn.20710

Bowie, C. R., Gupta, M., Holshausen, K., Jokic, R., Best, M., and Milev, R. (2013). Cognitive remediation for treatment-resistant depression: effects on cognition and functioning and the role of online homework. *J. Nerv. Ment. Dis.* 201, 680–685. doi: 10.1097/NMD.0b013e31829c5030

Boyke, J., Driemeyer, J., Gaser, C., Büchel, C., and May, A. (2008). Training-induced brain structure changes in the elderly. *J. Neurosci.* 28, 7031–7035. doi: 10.1523/JNEUROSCI.0742-08.2008

Brenner, I. K., Castellani, J. W., Gabaree, C., Young, A. J., Zamecnik, J., Shephard, R. J., et al. (1999). Immune changes in humans during cold exposure: effects of prior heating and exercise. *J. Appl. Physiol.* 87, 699–710.

Breuil, V., De Rotrou, J., Forette, F., Tortrat, D., Ganansia-Ganem, A., Frambourt, A., et al. (1994). Cognitive stimulation of patients with dementia: preliminary results. *Int. J. Geriatr. Psychiatry* 9, 211–217. doi: 10.1002/gps.930090306

Brown, A. S. (2006). Prenatal infection as a risk factor for schizophrenia. *Schizophr. Bull.* 32, 200–202. doi: 10.1093/schbul/sbj052

Brown, C. (2009). Novel food items as environmental enrichment for rodents and rabbits. *Lab. Anim.* 38, 119–120. doi: 10.1038/laban0409-119

Bsibsi, M., Ravid, R., Gveric, D., and van Noort, J. M. (2002). Broad expression of Toll-like receptors in the human central nervous system. *J. Neuropathol. Exp. Neurol.* 61, 1013–1021.

Buell, J. S., and Dawson-Hughes, B. (2008). Vitamin D and neurocognitive dysfunction: preventing "D"ecline? *Mol. Aspects Med.* 29, 415–422. doi: 10.1016/j.mam.2008.05.001

Buwalda, B., Kole, M. H., Veenema, A. H., Huininga, M., de Boer, S. F., Korte, S. M., et al. (2005). Long-term effects of social stress on brain and behavior: a focus on hippocampal functioning. *Neurosci. Biobehav. Rev.* 29, 83–97. doi: 10.1016/j.neubiorev.2004.05.005

Cacho, R., Fano, E., Areso, P., Garmendia, L., Vegas, O., Brain, P., et al. (2003). Endocrine and lymphoproliferative response changes produced by social stress in mice. *Physiol. Behav.* 78, 505–512. doi: 10.1016/S0031-9384(03)00018-0

Cai, R., Guo, F., Zhang, J., Xu, J., Cui, Y., and Sun, X. (2009). Environmental enrichment improves behavioral performance and auditory spatial representation of primary auditory cortical neurons in rat. *Neurobiol. Learn. Mem.* 91, 366–376. doi: 10.1016/j.nlm.2009.01.005

Campbell, A. (2004). Inflammation, neurodegenerative diseases, and environmental exposures. *Ann. N. Y. Acad. Sci.* 1035, 117–132. doi: 10.1196/annals.1332.008

Cancedda, L., Putignano, E., Sale, A., Viegi, A., Berardi, N., and Maffei, L. (2004). Acceleration of visual system development by environmental enrichment. *J. Neurosci.* 24, 4840–4848. doi: 10.1523/JNEUROSCI.0845-04.2004

Capuron, L., and Miller, A. H. (2011). Immune system to brain signaling: neuropsychopharmacological implications. *Pharmacol. Ther.* 130, 226–238. doi: 10.1016/j.pharmthera.2011.01.014

Carmignoto, G., Pizzorusso, T., Tia, S., and Vicini, S. (1997). Brain-derived neurotrophic factor and nerve growth factor potentiate excitatory synaptic transmission in the rat visual cortex. *J. Physiol.* 498(Pt 1), 153–164.

Carro, E., Nuñez, A., Busiguina, S., and Torres-Aleman, I. (2000). Circulating insulin-like growth factor I mediates effects of exercise on the brain. *J. Neurosci.* 20, 2926–2933.

Caspi, A., and Moffitt, T. E. (2006). Gene–environment interactions in psychiatry: joining forces with neuroscience. *Nat. Rev. Neurosci.* 7, 583–590. doi: 10.1038/nrn1925

Cassilhas, R., Lee, K., Fernandes, J., Oliveira, M., Tufik, S., Meeusen, R., et al. (2012). Spatial memory is improved by aerobic and resistance exercise through divergent molecular mechanisms. *Neuroscience* 202, 309–317. doi: 10.1016/j.neuroscience.2011.11.029

Castillo-Pérez, S., Gómez-Pérez, V., Velasco, M. C., Pérez-Campos, E., and Mayoral, M.-A. (2010). Effects of music therapy on depression compared with psychotherapy. *Arts Psychother.* 37, 387–390. doi: 10.1016/j.aip.2010.07.001

Castren, E., Zafra, F., Thoenen, H., and Lindholm, D. (1992). Light regulates expression of brain-derived neurotrophic factor mRNA in rat visual cortex. *Proc. Natl. Acad. Sci. U.S.A.* 89, 9444–9448. doi: 10.1073/pnas.89.20.9444

Ceddia, M., and Woods, J. (1999). Exercise suppresses macrophage antigen presentation. *J. Appl. Physiol.* 87, 2253–2258.

Chapillon, P., Patin, V., Roy, V., Vincent, A., and Caston, J. (2002). Effects of pre- and postnatal stimulation on developmental, emotional, and cognitive aspects in rodents: a review. *Dev. Psychobiol.* 41, 373–387. doi: 10.1002/dev.10066

Chikahisa, S., Sei, H., Morishima, M., Sano, A., Kitaoka, K., Nakaya, Y., et al. (2006). Exposure to music in the perinatal period enhances learning performance and alters BDNF/TrkB signaling in mice as adults. *Behav. Brain Res.* 169, 312–319. doi: 10.1016/j.bbr.2006.01.021

Clare, L., Woods, R. T., Moniz Cook, E. D., Orrell, M., and Spector, A. (2003). Cognitive rehabilitation and cognitive training for early-stage Alzheimer's disease and vascular dementia. *Cochrane Database Syst. Rev.* doi: 10.1002/14651858.CD003260

Colcombe, S. J., Erickson, K. I., Scalf, P. E., Kim, J. S., Prakash, R., McAuley, E., et al. (2006). Aerobic exercise training increases brain volume in aging humans. *J. Gerontol. A Biol. Sci. Med. Sci.* 61, 1166–1170. doi: 10.1093/gerona/61.11.1166

Conde, J. R., and Streit, W. J. (2006). Microglia in the aging brain. *J. Neuropathol. Exp. Neurol.* 65, 199–203. doi: 10.1097/01.jnen.0000202887.22082.63

Corotto, F., Henegar, J., and Maruniak, J. (1994). Odor deprivation leads to reduced neurogenesis and reduced neuronal survival in the olfactory bulb of the adult mouse. *Neuroscience* 61, 739–744. doi: 10.1016/0306-4522(94)90397-2

Cotman, C. W., Berchtold, N. C., and Christie, L.-A. (2007). Exercise builds brain health: key roles of growth factor cascades and inflammation. *Trends Neurosci.* 30, 464–472. doi: 10.1016/j.tins.2007.06.011

Cotter, D. R., Pariante, C. M., and Everall, I. P. (2001). Glial cell abnormalities in major psychiatric disorders: the evidence and implications. *Brain Res. Bull.* 55, 585–595. doi: 10.1016/S0361-9230(01)00527-5

de Groot, J., van Milligen, F. J., Moonen-Leusen, B. W., Thomas, G., and Koolhaas, J. M. (1999). A single social defeat transiently suppresses the anti-viral immune response in mice. *J. Neuroimmunol.* 95, 143–151. doi: 10.1016/S0165-5728(99)00005-3

de Jong, J. G., van der Vegt, B. J., Buwalda, B., and Koolhaas, J. M. (2005). Social environment determines the long-term effects of social defeat. *Physiol. Behav.* 84, 87–95. doi: 10.1016/j.physbeh.2004.10.013

Devanand, D., Michaels-Marston, K. S., Liu, X., Pelton, G. H., Padilla, M., Marder, K., et al. (2000). Olfactory deficits in patients with mild cognitive impairment predict Alzheimer's disease at follow-up. *Am. J. Psychiatry* 157, 1399–1405. doi: 10.1176/appi.ajp.157.9.1399

Diamond, M. C. (2001). Response of the brain to enrichment. *An. Acad. Bras. Ciênc.* 73, 211–220. doi: 10.1590/S0001-37652001000200006

Dilger, R. N., and Johnson, R. W. (2008). Aging, microglial cell priming, and the discordant central inflammatory response to signals from the peripheral immune system. *J. Leukoc. Biol.* 84, 932–939. doi: 10.1189/jlb.0208108

Dinse, H. R. (2004). Sound case for enrichment. Focus on "environmental enrichment improves response strength, threshold, selectivity, and latency of auditory cortex neurons". *J. Neurophysiol.* 92, 36–37. doi: 10.1152/jn.00213.2004

Dodel, R., Du, Y., Depboylu, C., Hampel, H., Frölich, L., Haag, A., et al. (2004). Intravenous immunoglobulins containing antibodies against β-amyloid for the treatment of Alzheimer's disease. *J. Neurol. Neurosurg. Psychiatry* 75, 1472–1474. doi: 10.1136/jnnp.2003.033399

Dong, C., Davis, R. J., and Flavell, R. A. (2002). MAP kinases in the immune response. *Annu. Rev. Immunol.* 20, 55–72. doi: 10.1146/annurev.immunol.20.091301.131133

Dong, Y., and Benveniste, E. N. (2001). Immune function of astrocytes. *Glia* 36, 180–190. doi: 10.1002/glia.1107

Draganski, B., Gaser, C., Busch, V., Schuierer, G., Bogdahn, U., and May, A. (2004). Neuroplasticity: changes in grey matter induced by training. *Nature* 427, 311–312. doi: 10.1038/427311a

Draganski, B., Gaser, C., Kempermann, G., Kuhn, H. G., Winkler, J., Büchel, C., et al. (2006). Temporal and spatial dynamics of brain structure changes during

extensive learning. *J. Neurosci.* 26, 6314–6317. doi: 10.1523/JNEUROSCI.4628-05.2006

Drollette, E. S., Scudder, M. R., Raine, L. B., Moore, R. D., Saliba, B. J., Pontifex, M. B., et al. (2013). Acute exercise facilitates brain function and cognition in children who need it most: an ERP study of individual differences in inhibitory control capacity. *Dev. Cogn. Neurosci.* 7C, 53–64. doi: 10.1016/j.dcn.2013.11.001

Du Clos, T. W. (2000). Function of C-reactive protein. *Ann. Med.* 32, 274–278. doi: 10.3109/07853890009011772

Duman, C. H., Schlesinger, L., Russell, D. S., and Duman, R. S. (2008). Voluntary exercise produces antidepressant and anxiolytic behavioral effects in mice. *Brain Res.* 1199, 148–158. doi: 10.1016/j.brainres.2007.12.047

Ehninger, D., and Kempermann, G. (2003). Regional effects of wheel running and environmental enrichment on cell genesis and microglia proliferation in the adult murine neocortex. *Cereb. Cortex* 13, 845–851. doi: 10.1093/cercor/13.8.845

Ekdahl, C. T., Kokaia, Z., and Lindvall, O. (2009). Brain inflammation and adult neurogenesis: the dual role of microglia. *Neuroscience* 158, 1021–1029. doi: 10.1016/j.neuroscience.2008.06.052

Engelhardt, B. (2006). Molecular mechanisms involved in T cell migration across the blood–brain barrier. *J. Neural Transm.* 113, 477–485. doi: 10.1007/s00702-005-0409-y

Engineer, N. D., Percaccio, C. R., Pandya, P. K., Moucha, R., Rathbun, D. L., and Kilgard, M. P. (2004). Environmental enrichment improves response strength, threshold, selectivity, and latency of auditory cortex neurons. *J. Neurophysiol.* 92, 73–82. doi: 10.1152/jn.00059.2004

Erickson, K. I., Voss, M. W., Prakash, R. S., Basak, C., Szabo, A., Chaddock, L., et al. (2011). Exercise training increases size of hippocampus and improves memory. *Proc. Natl. Acad. Sci.* 108, 3017–3022. doi: 10.1073/pnas.1015950108

Eyre, H., and Baune, B. T. (2012). Neuroimmunological effects of physical exercise in depression. *Brain Behav. Immun.* 26, 251–266. doi: 10.1016/j.bbi.2011.09.015

Eyre, H., Papps, E., and Baune, B. T. (2013). Treating depression and depression-like behavior with physical activity: an immune perspective. *Front. Psychiatry* 4:3. doi: 10.3389/fpsyt.2013.00003

Faherty, C. J., Raviie Shepherd, K., Herasimtschuk, A., and Smeyne, R. J. (2005). Environmental enrichment in adulthood eliminates neuronal death in experimental Parkinsonism. *Mol. Brain Res.* 134, 170–179. doi: 10.1016/j.molbrainres.2004.08.008

Fan, Y., Liu, Z., Weinstein, P. R., Fike, J. R., and Liu, J. (2007). Environmental enrichment enhances neurogenesis and improves functional outcome after cranial irradiation. *Eur. J. Neurosci.* 25, 38–46. doi: 10.1111/j.1460-9568.2006.05269.x

Fano, E., Sánchez-Martín, J. R., Arregi, A., Castro, B., Alonso, A., Brain, P., et al. (2001). Social stress paradigms in male mice: variations in behavior, stress and immunology. *Physiol. Behav.* 73, 165–173. doi: 10.1016/S0031-9384(01)00445-0

Fedorova, I., and Salem, N. Jr. (2006). Omega-3 fatty acids and rodent behavior. *Prostaglandins Leukot. Essent. Fatty Acids* 75, 271–289. doi: 10.1016/j.plefa.2006.07.006

Felten, D. (2008). Direct innervation of lymphoid organs: substrate for neurotransmitter signaling of cells of the immune system. *Neuropsychobiology* 28, 110–112. doi: 10.1159/000119011

Fernandez-Teruel, A., Escorihuela, R., Castellano, B., Gonzalez, B., and Tobena, A. (1997). Neonatal handling and environmental enrichment effects on emotionality, novelty/reward seeking, and age-related cognitive and hippocampal impairments: focus on the Roman rat lines. *Behav. Genet.* 27, 513–526. doi: 10.1023/A:1021400830503

Ferrini, F., and De Koninck, Y. (2013). Microglia control neuronal network excitability via BDNF signalling. *Neural Plast.* 2013:429815. doi: 10.1155/2013/429815

Fleshner, M., Laudenslager, M. L., Simons, L., and Maier, S. (1989). Reduced serum antibodies associated with social defeat in rats. *Physiol. Behav.* 45, 1183–1187. doi: 10.1016/0031-9384(89)90107-8

Ford, A., Flicker, L., Alfonso, H., Thomas, J., Clarnette, R., Martins, R., et al. (2010). Vitamins B12, B6, and folic acid for cognition in older men. *Neurology* 75, 1540–1547. doi: 10.1212/WNL.0b013e3181f962c4

Francis, D. D., Diorio, J., Plotsky, P. M., and Meaney, M. J. (2002). Environmental enrichment reverses the effects of maternal separation on stress reactivity. *J. Neurosci.* 22, 7840–7843.

Fratiglioni, L., Paillard-Borg, S., and Winblad, B. (2004). An active and socially integrated lifestyle in late life might protect against dementia. *Lancet Neurol.* 3, 343–353. doi: 10.1016/S1474-4422(04)00767-7

Frick, K. M., and Fernandez, S. M. (2003). Enrichment enhances spatial memory and increases synaptophysin levels in aged female mice. *Neurobiol. Aging* 24, 615–626. doi: 10.1016/S0197-4580(02)00138-0

Frick, K. M., Stearns, N. A., Pan, J.-Y., and Berger-Sweeney, J. (2003). Effects of environmental enrichment on spatial memory and neurochemistry in middle-aged mice. *Learn. Mem.* 10, 187–198. doi: 10.1101/lm.50703

Fuglestad, A. J., Rao, R., Georgieff, M. K., and Code, M. M. (2008). "The role of nutrition in cognitive development," in *Handbook in Developmental Cognitive Neuroscience*, 2nd Edn, eds C. A. Nelson and M. Luciana (Cambridge, MA: MIT Press), 623–642.

Garden, G. A., and Möller, T. (2006). Microglia biology in health and disease. *J. Neuroimmune Pharmacol.* 1, 127–137. doi: 10.1007/s11481-006-9015-5

Gates, N., Fiatarone Singh, M. A., Sachdev, P. S., and Valenzuela, M. (2013). The effect of exercise training on cognitive function in older adults with mild cognitive impairment: a meta-analysis of randomized controlled trials. *Am. J. Geriatr. Psychiatry* 21, 1086–1097. doi: 10.1016/j.jagp.2013.02.018

Gilman, S. E., Kawachi, I., Fitzmaurice, G. M., and Buka, S. L. (2002). Socioeconomic status in childhood and the lifetime risk of major depression. *Int. J. Epidemiol.* 31, 359–367. doi: 10.1093/ije/31.2.359

Gleeson, M., Bishop, N. C., Stensel, D. J., Lindley, M. R., Mastana, S. S., and Nimmo, M. A. (2011). The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. *Nat. Rev. Immunol.* 11, 607–615. doi: 10.1038/nri3041

Gleeson, M., McFarlin, B., and Flynn, M. (2006). Exercise and Toll-like receptors. *Exerc. Immunol. Rev.* 12, 34–53.

Glezer, I., Simard, A., and Rivest, S. (2007). Neuroprotective role of the innate immune system by microglia. *Neuroscience* 147, 867–883. doi: 10.1016/j.neuroscience.2007.02.055

Gobbo, O. L., and O'Mara, S. M. (2004). Impact of enriched-environment housing on brain-derived neurotrophic factor and on cognitive performance after a transient global ischemia. *Behav. Brain Res.* 152, 231–241. doi: 10.1016/j.bbr.2003.10.017

Gottlieb, S. S., Khatta, M., Friedmann, E., Einbinder, L., Katzen, S., Baker, B., et al. (2004). The influence of age, gender, and race on the prevalence of depression in heart failure patients. *J. Am. Coll. Cardiol.* 43, 1542–1549. doi: 10.1016/j.jacc.2003.10.064

Gould, E., Tanapat, P., Rydel, T., and Hastings, N. (2000). Regulation of hippocampal neurogenesis in adulthood. *Biol. Psychiatry* 48, 715–720. doi: 10.1016/S0006-3223(00)01021-0

Griffin, E. W., Bechara, R. G., Birch, A. M., and Kelly, Á. M. (2009). Exercise enhances hippocampal-dependent learning in the rat: evidence for a BDNF-related mechanism. *Hippocampus* 19, 973–980. doi: 10.1002/hipo.20631

Gutman, D. A., and Nemeroff, C. B. (2002). Neurobiology of early life stress: rodent studies. *Semin. Clin. Neuropsychiatry* 7, 89–95. doi: 10.1053/scnp.2002.31781

Haller, J., and Halasz, J. (2000). Anxiolytic effects of repeated victory in male Wistar rats. *Aggressive Behavior* 26, 257–261. doi: 10.1002/(SICI)1098-2337(2000)26:3<257::AID-AB5>3.0.CO;2-7

Hamann, S. B., Ely, T. D., Grafton, S. T., and Kilts, C. D. (1999). Amygdala activity related to enhanced memory for pleasant and aversive stimuli. *Nat. Neurosci.* 2, 289–293. doi: 10.1038/6404

Hamm, R. J., Temple, M. D., O'Dell, D. M., Pike, B. R., and Lyeth, B. G. (1996). Exposure to environmental complexity promotes recovery of cognitive function after traumatic brain injury. *J. Neurotrauma* 13, 41–47. doi: 10.1089/neu.1996.13.41

Hanisch, U. K. (2002). Microglia as a source and target of cytokines. *Glia* 40, 140–155. doi: 10.1002/glia.10161

Hannan, A. J. (2014). Environmental enrichment and brain repair: harnessing the therapeutic effects of cognitive stimulation and physical activity to enhance experience-dependent plasticity. *Neuropathol. Appl. Neurobiol.* 40, 13–25. doi: 10.1111/nan.12102

Hansen, L., and Berthelsen, H. (2000). The effect of environmental enrichment on the behaviour of caged rabbits (*Oryctolagus cuniculus*). *Appl. Anim. Behav. Sci.* 68, 163–178. doi: 10.1016/S0168-1591(00)00093-9

Hanser, S. B., and Thompson, L. W. (1994). Effects of a music therapy strategy on depressed older adults. *J. Gerontol.* 49, P265–P269. doi: 10.1093/geronj/49.6.P265

Harati, H., Majchrzak, M., Cosquer, B., Galani, R., Kelche, C., Cassel, J. C., et al. (2011). Attention and memory in aged rats: impact of lifelong environmental enrichment. *Neurobiol. Aging* 32, 718–736. doi: 10.1016/j.neurobiolaging.2009.03.012

Hardy, C.-A., Quay, J., Livnat, S., and Ader, R. (1990). Altered T-lymphocyte response following aggressive encounters in mice. *Physiol. Behav.* 47, 1245–1251. doi: 10.1016/0031-9384(90)90378-H

Harris, L. D., Custer, L. B., Soranaka, E. T., Burge, J. R., and Ruble, G. R. (2001). Evaluation of objects and food for environmental enrichment of NZW rabbits. *J. Am. Assoc. Lab. Anim. Sci.* 40, 27–30.

Hars, M., Herrmann, F. R., Gold, G., Rizzoli, R., and Trombetti, A. (2014). Effect of music-based multitask training on cognition and mood in older adults. *Age Ageing* 43, 196–200. doi: 10.1093/ageing/aft163

Hawkley, L. C., and Cacioppo, J. T. (2004). Stress and the aging immune system. *Brain Behav. Immun.* 18, 114–119. doi: 10.1016/j.bbi.2003.09.005

Hedden, T., and Gabrieli, J. D. (2004). Insights into the ageing mind: a view from cognitive neuroscience. *Nat. Rev. Neurosci.* 5, 87–96. doi: 10.1038/nrn1323

Herz, R. S. (2009). Aromatherapy facts and fictions: a scientific analysis of olfactory effects on mood, physiology and behavior. *Int. J. Neurosci.* 119, 263–290. doi: 10.1080/00207450802333953

Hickey, W., Hsu, B., and Kimura, H. (1991). T-lymphocyte entry into the central nervous system. *J. Neurosci. Res.* 28, 254–260. doi: 10.1002/jnr.490280213

Hillman, C. H., Erickson, K. I., and Kramer, A. F. (2008). Be smart, exercise your heart: exercise effects on brain and cognition. *Nat. Rev. Neurosci.* 9, 58–65. doi: 10.1038/nrn2298

Horch, H. W. (2004). Local effects of BDNF on dendritic growth. *Rev. Neurosci.* 15, 117–130. doi: 10.1515/REVNEURO.2004.15.2.117

Horch, H. W., and Katz, L. C. (2002). BDNF release from single cells elicits local dendritic growth in nearby neurons. *Nat. Neurosci.* 5, 1177–1184. doi: 10.1038/nn927

Hotting, K., and Roder, B. (2013). Beneficial effects of physical exercise on neuroplasticity and cognition. *Neurosci. Biobehav. Rev.* 37(Pt B), 2243–2257. doi: 10.1016/j.neubiorev.2013.04.005

Ickes, B. R., Pham, T. M., Sanders, L. A., Albeck, D. S., Mohammed, A. H., and Granholm, A.-C. (2000). Long-term environmental enrichment leads to regional increases in neurotrophin levels in rat brain. *Exp. Neurol.* 164, 45–52. doi: 10.1006/exnr.2000.7415

Iwasaki, A., and Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 5, 987–995. doi: 10.1038/ni1112

Jakkamsetti, V., Chang, K. Q., and Kilgard, M. P. (2012). Reorganization in processing of spectral and temporal input in the rat posterior auditory field induced by environmental enrichment. *J. Neurophysiol.* 107, 1457–1475. doi: 10.1152/jn.01057.2010

Janeway, C. A., Travers, P., Walport, M., and Shlomchik, M. (2001). "The complement system and innate immunity," in *Immuno Biology: The Immune System in Health and Disease*, 5th Edn (New York: Garland), 43–64.

Jankowsky, J. L., Melnikova, T., Fadale, D. J., Xu, G. M., Slunt, H. H., Gonzales, V., et al. (2005). Environmental enrichment mitigates cognitive deficits in a mouse model of Alzheimer's disease. *J. Neurosci.* 25, 5217–5224. doi: 10.1523/JNEUROSCI.5080-04.2005

Joseph, J., Cole, G., Head, E., and Ingram, D. (2009). Nutrition, brain aging, and neurodegeneration. *J. Neurosci.* 29, 12795–12801. doi: 10.1523/JNEUROSCI.3520-09.2009

Jurgens, H. A., and Johnson, R. W. (2012). Environmental enrichment attenuates hippocampal neuroinflammation and improves cognitive function during influenza infection. *Brain Behav. Immun.* 26, 1006–1016. doi: 10.1016/j.bbi.2012.05.015

Kaufman, J. C., Harris, T. J., Higgins, J., and Maisel, A. S. (1994). Exercise-induced enhancement of immune function in the rat. *Circulation* 90, 525–532. doi: 10.1161/01.CIR.90.1.525

Kay, G., Tarcic, N., Poltyrev, T., and Weinstock, M. (1998). Prenatal stress depresses immune function in rats. *Physiol. Behav.* 63, 397–402. doi: 10.1016/S0031-9384(97)00456-3

Keeney, A., and Hogg, S. (1999). Behavioural consequences of repeated social defeat in the mouse: preliminary evaluation of a potential animal model of depression. *Behav. Pharmacol.* 10, 753–764. doi: 10.1097/00008877-199912000-00007

Keller, C., Keller, P., Giralt, M., Hidalgo, J., and Pedersen, B. K. (2004). Exercise normalises overexpression of TNF-α in knockout mice. *Biochem. Biophys. Res. Commun.* 321, 179–182. doi: 10.1016/j.bbrc.2004.06.129

Kelly, J., Wrynn, A., and Leonard, B. (1997). The olfactory bulbectomized rat as a model of depression: an update. *Pharmacol. Ther.* 74, 299–316. doi: 10.1016/S0163-7258(97)00004-1

Kemper, K. J., and Danhauer, S. C. (2005). Music as therapy. *South. Med. J.* 98, 282–288. doi: 10.1097/01.SMJ.0000154773.11986.39

Kempermann, G., Gast, D., and Gage, F. H. (2002). Neuroplasticity in old age: sustained fivefold induction of hippocampal neurogenesis by long-term environmental enrichment. *Ann. Neurol.* 52, 135–143. doi: 10.1002/ana.10262

Kennedy, D. O., and Haskell, C. F. (2011). Vitamins and cognition. *Drugs* 71, 1957–1971. doi: 10.2165/11594130-000000000-00000

Kim, J. S. (1996). Cytokines and adhesion molecules in stroke and related diseases. *J. Neurol. Sci.* 137, 69–78. doi: 10.1016/0022-510X(95)00338-3

Knight, R. T. (1996). Contribution of human hippocampal region to novelty detection. *Nature* 383, 256–259. doi: 10.1038/383256a0

Kobayashi, S., Ohashi, Y., and Ando, S. (2002). Effects of enriched environments with different durations and starting times on learning capacity during aging in rats assessed by a refined procedure of the Hebb–Williams maze task. *J. Neurosci. Res.* 70, 340–346. doi: 10.1002/jnr.10442

Koelsch, S. (2010). Towards a neural basis of music-evoked emotions. *Trends Cogn. Sci.* 14, 131–137. doi: 10.1016/j.tics.2010.01.002

Kohut, M., McCann, D., Russell, D., Konopka, D., Cunnick, J., Franke, W., et al. (2006). Aerobic exercise, but not flexibility/resistance exercise, reduces serum IL-18, CRP, and IL-6 independent of β-blockers, BMI, and psychosocial factors in older adults. *Brain Behav. Immun.* 20, 201–209. doi: 10.1016/j.bbi.2005.12.002

Koletzko, B. (2003). Exercise and C-reactive protein. *HEALTHY WEIGHT* 17, 18.

Kozorovitskiy, Y., Gross, C. G., Kopil, C., Battaglia, L., McBreen, M., Stranahan, A. M., et al. (2005). Experience induces structural and biochemical changes in the adult primate brain. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17478–17482. doi: 10.1073/pnas.0508817102

Kreutzberg, G. W. (1996). Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* 19, 312–318. doi: 10.1016/0166-2236(96)10049-7

Kuo, H.-K., Yen, C.-J., Chang, C.-H., Kuo, C.-K., Chen, J.-H., and Sorond, F. (2005). Relation of C-reactive protein to stroke, cognitive disorders, and depression in the general population: systematic review and meta-analysis. *Lancet Neurol.* 4, 371–380. doi: 10.1016/S1474-4422(05)70099-5

Laviola, G., Hannan, A. J., Macri, S., Solinas, M., and Jaber, M. (2008). Effects of enriched environment on animal models of neurodegenerative diseases and psychiatric disorders. *Neurobiol. Dis.* 31, 159–168. doi: 10.1016/j.nbd.2008.05.001

Laviola, G., Rea, M., Morley-Fletcher, S., Di Carlo, S., Bacosi, A., De Simone, R., et al. (2004). Beneficial effects of enriched environment on adolescent rats from stressed pregnancies. *Eur. J. Neurosci.* 20, 1655–1664. doi: 10.1111/j.1460-9568.2004.03597.x

Lazarov, O., Robinson, J., Tang, Y.-P., Hairston, I. S., Korade-Mirnics, Z., Lee, V. M.-Y., et al. (2005). Environmental enrichment reduces Aβ levels and amyloid deposition in transgenic mice. *Cell* 120, 701–713. doi: 10.1016/j.cell.2005.01.015

Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., et al. (1994). A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 372, 739–746. doi: 10.1038/372739a0

Lees, C., and Hopkins, J. (2013). Effect of aerobic exercise on cognition, academic achievement, and psychosocial function in children: a systematic review of randomized control trials. *Prev. Chronic Dis.* 10, E174. doi: 10.5888/pcd10.130010.

Leggio, M. G., Mandolesi, L., Federico, F., Spirito, F., Ricci, B., Gelfo, F., et al. (2005). Environmental enrichment promotes improved spatial abilities and enhanced dendritic growth in the rat. *Behav. Brain Res.* 163, 78–90. doi: 10.1016/j.bbr.2005.04.009

Liberati, A., Altman, D. G., Tetzlaff, J., Mulrow, C., Gøtzsche, P. C., Ioannidis, J. P., et al. (2009). The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. *Ann. Intern. Med.* 151, W-65–W-94. doi: 10.7326/0003-4819-151-4-200908180-00136

Licastro, F., and Chiappelli, M. (2003). Brain immune responses cognitive decline and dementia: relationship with phenotype expression and genetic background. *Mech. Ageing Dev.* 124, 539–548. doi: 10.1016/S0047-6374(03)00034-4

Licastro, F., Pedrini, S., Caputo, L., Annoni, G., Davis, L. J., Ferri, C., et al. (2000). Increased plasma levels of interleukin-1, interleukin-6 and α-1-antichymotrypsin in patients with Alzheimer's disease: peripheral inflammation or signals from the brain? *J. Neuroimmunol.* 103, 97–102. doi: 10.1016/S0165-5728(99)00226-X

Lin, S.-T., Yang, P., Lai, C.-Y., Su, Y.-Y., Yeh, Y.-C., Huang, M.-F., et al. (2011). Mental health implications of music: insight from neuroscientific and clinical studies. *Harv. Rev. Psychiatry* 19, 34–46. doi: 10.3109/10673229.2011.549769

Liu, B., and Hong, J.-S. (2003). Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. *J. Pharmacol. Exp. Ther.* 304, 1–7. doi: 10.1124/jpet.102.035048

Lorant, V., Deliège, D., Eaton, W., Robert, A., Philippot, P., and Ansseau, M. (2003). Socioeconomic inequalities in depression: a meta-analysis. *Am. J. Epidemiol.* 157, 98–112. doi: 10.1093/aje/kwf182

Lupien, S. J., McEwen, B. S., Gunnar, M. R., and Heim, C. (2009). Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat. Rev. Neurosci.* 10, 434–445. doi: 10.1038/nrn2639

Ma, X.-C., Jiang, D., Jiang, W.-H., Wang, F., Jia, M., Wu, J., et al. (2011). Social isolation-induced aggression potentiates anxiety and depressive-like behavior in male mice subjected to unpredictable chronic mild stress. *PLoS ONE* 6:e20955. doi: 10.1371/journal.pone.0020955

Maier, S. F., and Watkins, L. R. (1998). Cytokines for psychologists: implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. *Psychol. Rev.* 105, 83. doi: 10.1037/0033-295X.105.1.83

Malatynska, E., and Knapp, R. J. (2005). Dominant–submissive behavior as models of mania and depression. *Neurosci. Biobehav. Rev.* 29, 715–737. doi: 10.1016/j.neubiorev.2005.03.014

Marais, L., Stein, D. J., and Daniels, W. M. (2009). Exercise increases BDNF levels in the striatum and decreases depressive-like behavior in chronically stressed rats. *Metab. Brain Dis.* 24, 587–597. doi: 10.1007/s11011-009-9157-2

Marashi, V., Barnekow, A., Ossendorf, E., and Sachser, N. (2003). Effects of different forms of environmental enrichment on behavioral, endocrinological, and immunological parameters in male mice. *Horm. Behav.* 43, 281–292. doi: 10.1016/S0018-506X(03)00002-3

Marashi, V., Barnekow, A., and Sachser, N. (2004). Effects of environmental enrichment on males of a docile inbred strain of mice. *Physiol. Behav.* 82, 765–776. doi: 10.1016/j.physbeh.2004.05.009

Maratos, A. S., Gold, C., Wang, X., and Crawford, M. J. (2008). Music therapy for depression. *Cochrane Database Syst. Rev.* 2008:CD004517. doi: 10.1002/14651858.CD004517.pub2

Mars, M., Govender, S., Weston, A., Naicker, V., and Chuturgoon, A. (1998). High intensity exercise: a cause of lymphocyte apoptosis? *Biochem. Biophys. Res. Commun.* 249, 366–370. doi: 10.1006/bbrc.1998.9156

Mathur, N., and Pedersen, B. K. (2008). Exercise as a mean to control low-grade systemic inflammation. *Mediators Inflamm.* 2008:109502. doi: 10.1155/2008/109502

McAfoose, J., and Baune, B. T. (2009). Evidence for a cytokine model of cognitive function. *Neurosci. Biobehav. Rev.* 33, 355–366. doi: 10.1016/j.neubiorev.2008.10.005

McAllister, A. K., Lo, D. C., and Katz, L. C. (1995). Neurotrophins regulate dendritic growth in developing visual cortex. *Neuron* 15, 791–803. doi: 10.1016/0896-6273(95)90171-X

McCarthy, D., and Dale, M. (1988). The leucocytosis of exercise. *Sports Med.* 6, 333–363. doi: 10.2165/00007256-198806060-00002

McCraty, R., Barrios-Choplin, B., Atkinson, M., and Tomasino, D. (1998). The effects of different types of music on mood, tension, and mental clarity. *Altern. Ther. Health Med.* 4, 75.

McGeer, E. G., Klegeris, A., and McGeer, P. L. (2005). Inflammation, the complement system and the diseases of aging. *Neurobiol. Aging* 26, 94–97. doi: 10.1016/j.neurobiolaging.2005.08.008

McGeer, E. G., and McGeer, P. L. (2003). Inflammatory processes in Alzheimer's disease. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 27, 741–749. doi: 10.1016/S0278-5846(03)00124-6

McGeer, P. L., Kawamata, T., Walker, D. G., Akiyama, H., Tooyama, I., and McGeer, E. G. (1993). Microglia in degenerative neurological disease. *Glia* 7, 84–92. doi: 10.1002/glia.440070114

McGeer, P., McGeer, E. G., and Yasojima, K. (2000). "Alzheimer disease and neuroinflammation," in *Advances in Dementia Research*, eds K. Jellinger, R. Schmidt, and M. Windisch (Vienna: Springer), 53–57.

McGeer, P. L., and McGeer, E. G. (1995). The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. *Brain Res. Rev.* 21, 195–218. doi: 10.1016/0165-0173(95)00011-9

McGeer, P. L., and McGeer, E. G. (2004). Inflammation and neurodegeneration in Parkinson's disease. *Parkinsonism Relat. Disord.* 10, S3–S7. doi: 10.1016/j.parkreldis.2004.01.005

McGurk, S. R., Twamley, E. W., Sitzer, D. I., McHugo, G. J., and Mueser, K. T. (2007). A meta-analysis of cognitive remediation in schizophrenia. *Am. J. Psychiatry* 164, 1791. doi: 10.1176/appi.ajp.2007.07060906

Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* 1, 135–145. doi: 10.1038/35100529

Mélik-Parsadaniantz, S., and Rostène, W. (2008). Chemokines and neuromodulation. *J. Neuroimmunol.* 198, 62–68. doi: 10.1016/j.jneuroim.2008.04.022

Mesholam, R. I., Moberg, P. J., Mahr, R. N., and Doty, R. L. (1998). Olfaction in neurodegenerative disease: a meta-analysis of olfactory functioning in Alzheimer's and Parkinson's diseases. *Arch. Neurol.* 55, 84. doi: 10.1001/archneur.55.1.84

Miller, K. J., Dye, R. V., Kim, J., Jennings, J. L., O'Toole, E., Wong, J., et al. (2013). Effect of a computerized brain exercise program on cognitive performance in older adults. *Am. J. Geriatr. Psychiatry* 21, 655–663. doi: 10.1016/j.jagp.2013.01.077

Mirescu, C., Peters, J. D., and Gould, E. (2004). Early life experience alters response of adult neurogenesis to stress. *Nat. Neurosci.* 7, 841–846. doi: 10.1038/nn1290

Möckel, M., Röcker, L., Störk, T., Vollert, J., Danne, O., Eichstädt, H., et al. (1994). Immediate physiological responses of healthy volunteers to different types of music: cardiovascular, hormonal and mental changes. *Eur. J. Appl. Physiol. Occup. Physiol.* 68, 451–459. doi: 10.1007/BF00599512

Moher, D., Liberati, A., Tetzlaff, J., and Altman, D. G. (2009). Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Ann. Intern. Med.* 151, 264–269. doi: 10.7326/0003-4819-151-4-200908180-00135

Montgomery, D. (1994). Astrocytes: form, functions, and roles in disease. *Vet. Pathol.* 31, 145–167. doi: 10.1177/030098589403100201

Mora, F., Segovia, G., and del Arco, A. (2007). Aging, plasticity and environmental enrichment: structural changes and neurotransmitter dynamics in several areas of the brain. *Brain Res. Rev.* 55, 78–88. doi: 10.1016/j.brainresrev.2007.03.011

Mössner, R., and Lesch, K.-P. (1998). Role of serotonin in the immune system and in neuroimmune interactions. *Brain Behav. Immun.* 12, 249–271. doi: 10.1006/brbi.1998.0532

Mrak, R. E., and Griffin, W. S. T. (2005). Glia and their cytokines in progression of neurodegeneration. *Neurobiol. Aging* 26, 349–354. doi: 10.1016/j.neurobiolaging.2004.05.010

Nagele, R. G., Wegiel, J., Venkataraman, V., Imaki, H., Wang, K.-C., and Wegiel, J. (2004). Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. *Neurobiol. Aging* 25, 663–674. doi: 10.1016/j.neurobiolaging.2004.01.007

Najbauer, J., and Leon, M. (1995). Olfactory experience modulates apoptosis in the developing olfactory bulb. *Brain Res.* 674, 245–251. doi: 10.1016/0006-8993(94)01448-Q

Nakamura, H., Kobayashi, S., Ohashi, Y., and Ando, S. (1999). Age-changes of brain synapses and synaptic plasticity in response to an enriched environment. *J. Neurosci. Res.* 56, 307–315. doi: 10.1002/(SICI)1097-4547(19990501)56:3<307::AID-JNR10>3.0.CO;2-3

Nanda, B., Balde, J., and Manjunatha, S. (2013). The acute effects of a single bout of moderate-intensity aerobic exercise on cognitive functions in healthy adult males. *J. Clin. Diagn. Res.* 7, 1883–1885. doi: 10.7860/JCDR/2013/5855.3341

Navarro, A., Gómez, C., Sánchez-Pino, M.-J., González, H., Bández, M. J., Boveris, A. D., et al. (2005). Vitamin E at high doses improves survival, neurological performance, and brain mitochondrial function in aging male mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 289, R1392–R1399. doi: 10.1152/ajpregu.00834.2004

Nehlsen-Cannarella, S. L. (1998). Cellular responses to moderate and heavy exercise. *Can. J. Physiol. Pharmacol.* 76, 485–489. doi: 10.1139/y98-050

Neiman, D., and Pedersen, B. (1999). Exercise and immune function: recent developments. *Sports Med.* 27, 73–80. doi: 10.2165/00007256-199927020-00001

Nichol, K., Deeny, S. P., Seif, J., Camaclang, K., and Cotman, C. W. (2009). Exercise improves cognition and hippocampal plasticity in APOE ϵ4 mice. *Alzheimers Dement.* 5, 287–294. doi: 10.1016/j.jalz.2009.02.006

Nielsen, H. B., Secher, N. H., Christensen, N. J., and Pedersen, B. K. (1996). Lymphocytes and NK cell activity during repeated bouts of maximal exercise. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 271, R222–R227.

Nilsson, M., Perfilieva, E., Johansson, U., Orwar, O., and Eriksson, P. S. (1999). Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. *J. Neurobiol.* 39, 569–578. doi: 10.1002/(SICI)1097-4695(19990615)39:4<569::AID-NEU10>3.0.CO;2-F

Nimmerjahn, F., and Ravetch, J. V. (2008). Anti-inflammatory actions of intravenous immunoglobulin. *Annu. Rev. Immunol.* 26, 513–533. doi: 10.1146/annurev.immunol.26.021607.090232

Núñez, M. J., Mañá, P., Liñares, D., Riveiro, M. P., Balboa, J., Suárez-Quintanilla, J., et al. (2002). Music, immunity and cancer. *Life Sci.* 71, 1047–1057. doi: 10.1016/S0024-3205(02)01796-4

Okuda, H., Tatsumi, K., Makinodan, M., Yamauchi, T., Kishimoto, T., and Wanaka, A. (2009). Environmental enrichment stimulates progenitor cell proliferation in the amygdala. *J. Neurosci. Res.* 87, 3546–3553. doi: 10.1002/jnr.22160

Olsson, I. A. S., and Dahlborn, K. (2002). Improving housing conditions for laboratory mice: a review of 'environmental enrichment'. *Lab. Anim.* 36, 243–270. doi: 10.1258/002367702320162379

Opal, S. M., and DePalo, V. A. (2000). Anti-inflammatory cytokines. *Chest* 117, 1162–1172. doi: 10.1378/chest.117.4.1162

Ortega, E., Collazos, M., Maynar, M., Barriga, C., and De la Fuente, M. (1993). Stimulation of the phagocytic function of neutrophils in sedentary men after acute moderate exercise. *Eur. J. Appl. Physiol. Occup. Physiol.* 66, 60–64. doi: 10.1007/BF00863401

Ortega, E., Giraldo, E., Hinchado, M. D., Martín, L., García, J. J., and De la Fuente, M. (2007). Neuroimmunomodulation during exercise: role of catecholamines as 'stress mediator' and/or 'danger signal' for the innate immune response. *Neuroimmunomodulation* 14, 206–212. doi: 10.1159/000110648

Ostrowski, K., Rohde, T., Asp, S., Schjerling, P., and Pedersen, B. K. (2001). Chemokines are elevated in plasma after strenuous exercise in humans. *Eur. J. Appl. Physiol.* 84, 244–245. doi: 10.1007/s004210170012

Ownby, R. L. (2010). Neuroinflammation and cognitive aging. *Curr. Psychiatry Rep.* 12, 39–45. doi: 10.1007/s11920-009-0082-1

Padilla, N. D., Bleeker, W. K., Lubbers, Y., Rigter, G. M., Van Mierlo, G. J., Daha, M. R., et al. (2003). Rat C-reactive protein activates the autologous complement system. *Immunology* 109, 564–571. doi: 10.1046/j.1365-2567.2003.01681.x

Papaioannou, A., Gerozissis, K., Prokopiou, A., Bolaris, S., and Stylianopoulou, F. (2002). Sex differences in the effects of neonatal handling on the animal's response to stress and the vulnerability for depressive behaviour. *Behav. Brain Res.* 129, 131–139. doi: 10.1016/S0166-4328(01)00334-5

Passineau, M. J., Green, E. J., and Dietrich, W. D. (2001). Therapeutic effects of environmental enrichment on cognitive function and tissue integrity following severe traumatic brain injury in rats. *Exp. Neurol.* 168, 373–384. doi: 10.1006/exnr.2000.7623

Pedersen, B. K., and Hoffman-Goetz, L. (2000). Exercise and the immune system: regulation, integration, and adaptation. *Physiol. Rev.* 80, 1055–1081.

Petersen, A. M. W., and Pedersen, B. K. (2005). The anti-inflammatory effect of exercise. *J. Appl. Physiol.* 98, 1154–1162. doi: 10.1152/japplphysiol.00164.2004

Petersen, A. M. W., and Pedersen, B. K. (2006). The role of IL-6 in mediating the anti-inflammatory effects of exercise. *J. Physiol. Pharmacol.* 57, 43–51.

Pham, T., Ickes, B., Albeck, D., Söderström, S., Granholm, A., and Mohammed, A. (1999a). Changes in brain nerve growth factor levels and nerve growth factor receptors in rats exposed to environmental enrichment for one year. *Neuroscience* 94, 279–286. doi: 10.1016/S0306-4522(99)00316-4

Pham, T. M., Söderström, S., Winblad, B., and Mohammed, A. H. (1999b). Effects of environmental enrichment on cognitive function and hippocampal NGF in the non-handled rats. *Behav. Brain Res.* 103, 63–70. doi: 10.1016/S0166-4328(99)00019-4

Pham, T. M., Winblad, B., Granholm, A.-C., and Mohammed, A. H. (2002). Environmental influences on brain neurotrophins in rats. *Pharmacol. Biochem. Behav.* 73, 167–175. doi: 10.1016/S0091-3057(02)00783-9

Piccinelli, M., and Wilkinson, G. (2000). Gender differences in depression critical review. *Br. J. Psychiatry* 177, 486–492. doi: 10.1192/bjp.177.6.486

Piche, M., Robert, S., Miceli, D., and Bronchti, G. (2004). Environmental enrichment enhances auditory takeover of the occipital cortex in anophthalmic mice. *Eur. J. Neurosci.* 20, 3463–3472. doi: 10.1111/j.1460-9568.2004.03823.x

Poli, A., Kmiecik, J., Domingues, O., Hentges, F., Bléry, M., Chekenya, M., et al. (2013). NK cells in central nervous system disorders. *J. Immunol.* 190, 5355–5362. doi: 10.4049/jimmunol.1203401

Proost, P., Wuyts, A., and Van Damme, J. (1996). The role of chemokines in inflammation. *Int. J. Clin. Lab. Res.* 26, 211–223. doi: 10.1007/BF02602952

Rabin, M. D. (1988). Experience facilitates olfactory quality discrimination. *Percept. Psychophys.* 44, 532–540. doi: 10.3758/BF03207487

Ragneskog, H., Bråne, G., Karlsson, I., and Kihlgren, M. (1996). Influence of dinner music on food intake and symptoms common in dementia. *Scand. J. Caring Sci.* 10, 11. doi: 10.1111/j.1471-6712.1996.tb00304.x

Rasin, M. R., Darmopil, S., Petanjek, Z., Tomic-Mahecic, T., Mohammed, A. H., and Bogdanovic, N. (2011). Effect of environmental enrichment on morphology of deep layer III and layer V pyramidal cells of occipital cortex in oldest-old rat – a quantitative Golgi cox study. *Coll. Antropol.* 35(Suppl. 1), 253–258.

Richardson, A. J. (2003). The importance of omega-3 fatty acids for behaviour, cognition and mood. *Food Nutr. Res.* 47, 92–98. doi: 10.3402/fnr.v47i2.1476

Rickard, N. S., Toukhsati, S. R., and Field, S. E. (2005). The effect of music on cognitive performance: insight from neurobiological and animal studies. *Behav. Cogn. Neurosci. Rev.* 4, 235–261. doi: 10.1177/1534582305285869

Rinaldi, M., Graffi, G., Gallone, S., and Massa, E. R. (2014). Metabolic syndrome and cognitive performance in the elderly. *J. Biol. Res.-Boll. Soc. Ital. Biol. Sper.* 87, 21–23. doi: 10.4081/jbr.2014.2132

Roberts, J. E. (1995). Visible light induced changes in the immune response through an eye-brain mechanism (photoneuroimmunology). *J. Photochem. Photobiol. B Biol.* 29, 3–15. doi: 10.1016/1011-1344(95)90241-4

Roberts, J. E. (2000). Light and immunomodulation. *Ann. N. Y. Acad. Sci.* 917, 435–445. doi: 10.1111/j.1749-6632.2000.tb05408.x

Rochefort, C., Gheusi, G., Vincent, J.-D., and Lledo, P.-M. (2002). Enriched odor exposure increases the number of newborn neurons in the adult olfactory bulb and improves odor memory. *J. Neurosci.* 22, 2679–2689.

Ron-Harel, N., Cardon, M., and Schwartz, M. (2011). Brain homeostasis is maintained by "danger" signals stimulating a supportive immune response within the brain's borders. *Brain Behav. Immun.* 25, 1036–1043. doi: 10.1016/j.bbi.2010.12.011

Rosselli-Austin, L., and Williams, J. (1990). Enriched neonatal odor exposure leads to increased numbers of olfactory bulb mitral and granule cells. *Dev. Brain Res.* 51, 135–137. doi: 10.1016/0165-3806(90)90267-3

Rossi, C., Angelucci, A., Costantin, L., Braschi, C., Mazzantini, M., Babbini, F., et al. (2006). Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment. *Eur. J. Neurosci.* 24, 1850–1856. doi: 10.1111/j.1460-9568.2006.05059.x

Rothwell, N. J., Luheshi, G., and Toulmond, S. (1996). Cytokines and their receptors in the central nervous system: physiology, pharmacology, and pathology. *Pharmacol. Ther.* 69, 85–95. doi: 10.1016/0163-7258(95)02033-0

Ruis, M., Te Brake, J., Buwalda, B., De Boer, S., Meerlo, P., Korte, S., et al. (1999). Housing familiar male wildtype rats together reduces the long-term adverse behavioural and physiological effects of social defeat. *Psychoneuroendocrinology* 24, 285–300. doi: 10.1016/S0306-4530(98)00050-X

Sadato, N., Pascual-Leone, A., Grafman, J., Ibañez, V., Deiber, M.-P., Dold, G., et al. (1996). Activation of the primary visual cortex by Braille reading in blind subjects. *Nature* 380, 526–528. doi: 10.1038/380526a0

Sampedro-Piquero, P., Zancada-Menendez, C., Begega, A., Rubio, S., and Arias, J. L. (2013). Effects of environmental enrichment on anxiety responses, spatial memory and cytochrome *c* oxidase activity in adult rats. *Brain Res. Bull.* 98, 1–9. doi: 10.1016/j.brainresbull.2013.06.006

Särkämö, T., Tervaniemi, M., Laitinen, S., Forsblom, A., Soinila, S., Mikkonen, M., et al. (2008). Music listening enhances cognitive recovery and mood after middle cerebral artery stroke. *Brain* 131, 866–876. doi: 10.1093/brain/awn013

Sawada, M., Kondo, N., Suzumura, A., and Marunouchi, T. (1989). Production of tumor necrosis factor-alpha by microglia and astrocytes in culture. *Brain Res.* 491, 394–397. doi: 10.1016/0006-8993(89)90078-4

Schaal, B. (1988). Olfaction in infants and children: developmental and functional perspectives. *Chem. Senses* 13, 145–190. doi: 10.1093/chemse/13.2.145

Schaller, M., Miller, G. E., Gervais, W. M., Yager, S., and Chen, E. (2010). Mere visual perception of other people's disease symptoms facilitates a more aggressive immune response. *Psychol. Sci.* 21, 649–652. doi: 10.1177/0956797610368064

Schwartz, M., Butovsky, O., Brück, W., and Hanisch, U.-K. (2006). Microglial phenotype: is the commitment reversible? *Trends Neurosci.* 29, 68–74. doi: 10.1016/j.tins.2005.12.005

Schwarz, M. J., Chiang, S., Müller, N., and Ackenheil, M. (2001). T-helper-1 and T-helper-2 responses in psychiatric disorders. *Brain Behav. Immun.* 15, 340–370. doi: 10.1006/brbi.2001.0647

Segovia, G., Yagüe, A. G., García-Verdugo, J. M., and Mora, F. (2006). Environmental enrichment promotes neurogenesis and changes the extracellular concentrations of glutamate and GABA in the hippocampus of aged rats. *Brain Res. Bull.* 70, 8–14. doi: 10.1016/j.brainresbull.2005.11.005

Senchina, D. S., and Kohut, M. L. (2007). Immunological outcomes of exercise in older adults. *Clin. Interv. Aging* 2, 3. doi: 10.2147/ciia.2007.2.1.3

Shephard, R. J., and Shek, P. N. (1998). Immune responses to inflammation and trauma: a physical training model. *Can. J. Physiol. Pharmacol.* 76, 469–472. doi: 10.1139/y98-048

Sherratt, K., Thornton, A., and Hatton, C. (2004). Music interventions for people with dementia: a review of the literature. *Aging Ment. Health* 8, 3–12. doi: 10.1080/13607860310001613275

Sherwin, C. (2004). Mirrors as potential environmental enrichment for individually housed laboratory mice. *Appl. Anim. Behav. Sci.* 87, 95–103. doi: 10.1016/j.applanim.2003.12.014

Sherwin, C., and Glen, E. (2003). Cage colour preferences and effects of home cage colour on anxiety in laboratory mice. *Anim. Behav.* 66, 1085–1092. doi: 10.1006/anbe.2003.2286

Shi, L., Fatemi, S. H., Sidwell, R. W., and Patterson, P. H. (2003). Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. *J. Neurosci.* 23, 297–302.

Shinkai, S., Konishi, M., and Shephard, R. J. (1998). Aging and immune response to exercise. *Can. J. Physiol. Pharmacol.* 76, 562–572. doi: 10.1139/y98-043

Simon, G. E., Von Korff, M., Saunders, K., Miglioretti, D. L., Crane, P. K., van Belle, G., et al. (2006). Association between obesity and psychiatric disorders in the US adult population. *Arch. Gen. Psychiatry* 63, 824. doi: 10.1001/archpsyc.63.7.824

Smith, J., Telford, R., Mason, I., and Weidemann, M. (1990). Exercise, training and neutrophil microbicidal activity. *Int. J. Sports Med.* 11, 179–187. doi: 10.1055/s-2007-1024788

Soffié, M., Hahn, K., Terao, E., and Eclancher, F. (1999). Behavioural and glial changes in old rats following environmental enrichment. *Behav. Brain Res.* 101, 37–49. doi: 10.1016/S0166-4328(98)00139-9

Spector, A., Thorgrimsen, L., Woods, B., Royan, L., Davies, S., Butterworth, M., et al. (2003). Efficacy of an evidence-based cognitive stimulation therapy programme for people with dementia: randomised controlled trial. *Br. J. Psychiatry* 183, 248–254. doi: 10.1192/bjp.183.3.248

Speisman, R. B., Kumar, A., Rani, A., Foster, T. C., and Ormerod, B. K. (2013). Daily exercise improves memory, stimulates hippocampal neurogenesis and modulates immune and neuroimmune cytokines in aging rats. *Brain Behav. Immun.* 28, 25–43. doi: 10.1016/j.bbi.2012.09.013

Sredni-Kenigsbuch, D. (2002). TH1/TH2 cytokines in the central nervous system. *Int. J. Neurosci.* 112, 665–703. doi: 10.1080/00207450290025725

Staecker, H., Kopke, R., Malgrange, B., Lefebvre, P., and Van De Water, T. R. (1996). NT-3 and/or BDNF therapy prevents loss of auditory neurons following loss of hair cells. *Neuroreport* 7, 889–894. doi: 10.1097/00001756-199603220-00011

Stefanski, V. (2001). Social stress in laboratory rats: behavior, immune function, and tumor metastasis. *Physiol. Behav.* 73, 385–391. doi: 10.1016/S0031-9384(01)00495-4

Stefanski, V., and Engler, H. (1998). Effects of acute and chronic social stress on blood cellular immunity in rats. *Physiol. Behav.* 64, 733–741. doi: 10.1016/S0031-9384(98)00127-9

Stefanski, V., and Engler, H. (1999). Social stress, dominance and blood cellular immunity. *J. Neuroimmunol.* 94, 144–152. doi: 10.1016/S0165-5728(98)00242-2

Strait, D. L., and Kraus, N. (2014). Biological impact of auditory expertise across the life span: musicians as a model of auditory learning. *Hear. Res.* 308, 109–121. doi: 10.1016/j.heares.2013.08.004

Streit, W. J. (2004). Microglia and Alzheimer's disease pathogenesis. *J. Neurosci. Res.* 77, 1–8. doi: 10.1002/jnr.20093

Streit, W. J. (2005). Microglia and neuroprotection: implications for Alzheimer's disease. *Brain Res. Rev.* 48, 234–239. doi: 10.1016/j.brainresrev.2004.12.013

Strous, R. D., and Shoenfeld, Y. (2006). To smell the immune system: olfaction, autoimmunity and brain involvement. *Autoimmun. Rev.* 6, 54–60. doi: 10.1016/j.autrev.2006.07.002

Tansey, M. G., and Goldberg, M. S. (2010). Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention. *Neurobiol. Dis.* 37, 510–518. doi: 10.1016/j.nbd.2009.11.004

Tarkowski, E., Liljeroth, A. M., Minthon, L., Tarkowski, A., Wallin, A., and Blennow, K. (2003). Cerebral pattern of pro- and anti-inflammatory cytokines in dementias. *Brain Res. Bull.* 61, 255–260. doi: 10.1016/S0361-9230(03)00088-1

Tees, R. C. (1999). The influences of sex, rearing environment, and neonatal choline dietary supplementation on spatial and nonspatial learning and memory in adult rats. *Dev. Psychobiol.* 35, 328–342. doi: 10.1002/(SICI)1098-2302(199912)35:4<328::AID-DEV7>3.0.CO;2-4

Tha, K. K., Okuma, Y., Miyazaki, H., Murayama, T., Uehara, T., Hatakeyama, R., et al. (2000). Changes in expressions of proinflammatory cytokines IL-1β, TNF-α and IL-6 in the brain of senescence accelerated mouse (SAM) P8. *Brain Res.* 885, 25–31. doi: 10.1016/S0006-8993(00)02883-3

Tomporowski, P. D. (2003). Effects of acute bouts of exercise on cognition. *Acta Psychol.* 112, 297–324. doi: 10.1016/S0001-6918(02)00134-8

Tooyama, I., Kimura, H., Akiyama, H., and McGeer, P. (1990). Reactive microglia express class I and class II major histocompatibility complex antigens in Alzheimer's disease. *Brain Res.* 523, 273–280. doi: 10.1016/0006-8993(90)91496-4

Torasdotter, M., Metsis, M., Henriksson, B. G., Winblad, B., and Mohammed, A. H. (1998). Environmental enrichment results in higher levels of nerve growth factor mRNA in the rat visual cortex and hippocampus. *Behav. Brain Res.* 93, 83–90. doi: 10.1016/S0166-4328(97)00142-3

Trollor, J. N., Smith, E., Agars, E., Kuan, S. A., Baune, B. T., Campbell, L., et al. (2012). The association between systemic inflammation and cognitive performance in the elderly: the Sydney Memory and Ageing Study. *Age (Dordr.)* 34, 1295–1308. doi: 10.1007/s11357-011-9301-x

Trøseid, M., Lappegård, K. T., Claudi, T., Damås, J. K., Mørkrid, L., Brendberg, R., et al. (2004). Exercise reduces plasma levels of the chemokines MCP-1 and IL-8 in subjects with the metabolic syndrome. *Eur. Heart J.* 25, 349–355. doi: 10.1016/j.ehj.2003.12.006

Tuan, T.-C., Hsu, T.-G., Fong, M.-C., Hsu, C.-F., Tsai, K. K., Lee, C.-Y., et al. (2008). Deleterious effects of short-term, high-intensity exercise on immune function: evidence from leucocyte mitochondrial alterations and apoptosis. *Br. J. Sports Med.* 42, 11–15. doi: 10.1136/bjsm.2006.029314

Tung, J., Barreiro, L. B., Johnson, Z. P., Hansen, K. D., Michopoulos, V., Toufexis, D., et al. (2012). Social environment is associated with gene regulatory variation in the rhesus macaque immune system. *Proc. Natl. Acad. Sci. U.S.A.* 109, 6490–6495. doi: 10.1073/pnas.1202734109

Turetsky, B. I., Moberg, P. J., Yousem, D. M., Doty, R. L., Arnold, S. E., and Gur, R. E. (2000). Reduced olfactory bulb volume in patients with schizophrenia. *Am. J. Psychiatry* 157, 828–830. doi: 10.1176/appi.ajp.157.5.828

Tvede, N., Pedersen, B., Hansen, F., Bendix, T., Christensen, L., Galbo, H., et al. (1989). Effect of physical exercise on blood mononuclear cell subpopulations and in vitro proliferative responses. *Scand. J. Immunol.* 29, 383–389. doi: 10.1111/j.1365-3083.1989.tb01137.x

Uauy, R., and Dangour, A. D. (2006). Nutrition in brain development and aging: role of essential fatty acids. *Nutr. Rev.* 64, S24–S33. doi: 10.1301/nr.2006.may.S24-S33

Van de Weerd, H., Van Loo, P., Van Zutphen, L., Koolhaus, J., and Baumans, V. (1998a). Preferences for nest boxes as environmental enrichment for laboratory mice. *Anim. Welf.* 7, 11–26.

Van de Weerd, H., Van Loo, P., Van Zutphen, L., Koolhaas, J., and Baumans, V. (1998b). Strength of preference for nesting material as environmental enrichment for laboratory mice. *Appl. Anim. Behav. Sci.* 55, 369–382. doi: 10.1016/S0168-1591(97)00043-9

van de Weerd, H. A., and Day, J. E. (2009). A review of environmental enrichment for pigs housed in intensive housing systems. *Appl. Anim. Behav. Sci.* 116, 1–20. doi: 10.1016/j.applanim.2008.08.001

Van der Borght, K., Havekes, R., Bos, T., Eggen, B. J., and Van der Zee, E. A. (2007). Exercise improves memory acquisition and retrieval in the Y-maze task: relationship with hippocampal neurogenesis. *Behav. Neurosci.* 121, 324. doi: 10.1037/0735-7044.121.2.324

Vandewalle, G., Collignon, O., Hull, J. T., Daneault, V., Albouy, G., Lepore, F., et al. (2013). Blue light stimulates cognitive brain activity in visually blind individuals. *J. Cogn. Neurosci.* 25, 2072–2085. doi: 10.1162/jocn_a_00450

Van Loo, P., Kruitwagen, C., Koolhaas, J., Van de Weerd, H., Van Zutphen, L., and Baumans, V. (2002). Influence of cage enrichment on aggressive behaviour and physiological parameters in male mice. *Appl. Anim. Behav. Sci.* 76, 65–81. doi: 10.1016/S0168-1591(01)00200-3

Van Loo, P., Van de Weerd, H., Van Zutphen, L., and Baumans, V. (2004). Preference for social contact versus environmental enrichment in male laboratory mice. *Lab. Anim.* 38, 178–188. doi: 10.1258/002367704322968867

Van Praag, H., Shubert, T., Zhao, C., and Gage, F. H. (2005). Exercise enhances learning and hippocampal neurogenesis in aged mice. *J. Neurosci.* 25, 8680–8685. doi: 10.1523/JNEUROSCI.1731-05.2005

Vega, S. R., Knicker, A., Hollmann, W., Bloch, W., and Struder, H. (2010). Effect of resistance exercise on serum levels of growth factors in humans. *Horm. Metab. Res.* 42, 982–986. doi: 10.1055/s-0030-1267950

Venable, N., Fernandez, V., Diaz, E., and Pinto-Hamuy, T. (1989). Effects of preweaning environmental enrichment on basilar dendrites of pyramidal neurons in occipital cortex: a Golgi study. *Brain Res. Dev. Brain Res.* 49, 140–144. doi: 10.1016/0165-3806(89)90068-0

Veyrac, A., Sacquet, J., Nguyen, V., Marien, M., Jourdan, F., and Didier, A. (2008). Novelty determines the effects of olfactory enrichment on memory and neurogenesis through noradrenergic mechanisms. *Neuropsychopharmacology* 34, 786–795. doi: 10.1038/npp.2008.191

Vivinetto, A. L., Suárez, M. M., and Rivarola, M. A. (2013). Neurobiological effects of neonatal maternal separation and post-weaning environmental enrichment. *Behav. Brain Res.* 240, 110–118. doi: 10.1016/j.bbr.2012.11.014

von Büdingen, H.-C., Kuo, T. C., Sirota, M., van Belle, C. J., Apeltsin, L., Glanville, J., et al. (2012). B cell exchange across the blood–brain barrier in multiple sclerosis. *J. Clin. Investig.* 122, 4533. doi: 10.1172/JCI63842

Wainwright, P. E. (2002). Dietary essential fatty acids and brain function: a developmental perspective on mechanisms. *Proc. Nutr. Soc.* 61, 61–69. doi: 10.1079/PNS2001130

Wainwright, P. E., Huang, Y.-S., Bulman-Fleming, B., Levesque, S., and McCutcheon, D. (1994). The effects of dietary fatty acid composition combined with environmental enrichment on brain and behavior in mice. *Behav. Brain Res.* 60, 125–136. doi: 10.1016/0166-4328(94)90139-2

Wasantwisut, E. (1996). Nutrition and development: other micronutrients' effect on growth and cognition. *Southeast Asian J. Trop. Med. Public Health* 28, 78–82.

Welberg, L., Thrivikraman, K. V., and Plotsky, P. M. (2006). Combined pre- and postnatal environmental enrichment programs the HPA axis differentially in male and female rats. *Psychoneuroendocrinology* 31, 553–564. doi: 10.1016/j.psyneuen.2005.11.011

Wells, D. L. (2009). Sensory stimulation as environmental enrichment for captive animals: a review. *Appl. Anim. Behav. Sci.* 118, 1–11. doi: 10.1016/j.applanim.2009.01.002

Williams, B. M., Luo, Y., Ward, C., Redd, K., Gibson, R., Kuczaj, S. A., et al. (2001). Environmental enrichment: effects on spatial memory and hippocampal CREB immunoreactivity. *Physiol. Behav.* 73, 649–658. doi: 10.1016/S0031-9384(01)00543-1

Williamson, L. L., Chao, A., and Bilbo, S. D. (2012). Environmental enrichment alters glial antigen expression and neuroimmune function in the adult rat hippocampus. *Brain Behav. Immun.* 26, 500–510. doi: 10.1016/j.bbi.2012.01.003

Willis, S. L., Tennstedt, S. L., Marsiske, M., Ball, K., Elias, J., Koepke, K. M., et al. (2006). Long-term effects of cognitive training on everyday functional outcomes in older adults. *JAMA* 296, 2805–2814. doi: 10.1001/jama.296.23.2805

Wilund, K. (2007). Is the anti-inflammatory effect of regular exercise responsible for reduced cardiovascular disease? *Clin. Sci.* 112, 543–555. doi: 10.1042/CS20060368

Winner, B., Kohl, Z., and Gage, F. H. (2011). Neurodegenerative disease and adult neurogenesis. *Eur. J. Neurosci.* 33, 1139–1151. doi: 10.1111/j.1460-9568.2011.07613.x

Wu, A., Ying, Z., and Gomez-Pinilla, F. (2004). Dietary omega-3 fatty acids normalize BDNF levels, reduce oxidative damage, and counteract learning disability after traumatic brain injury in rats. *J. Neurotrauma* 21, 1457–1467. doi: 10.1089/neu.2004.21.1457

Wykes, T., Huddy, V., Cellard, C., McGurk, S. R., and Czobor, P. (2011). A meta-analysis of cognitive remediation for schizophrenia: methodology and effect sizes. *Am. J. Psychiatry* 168, 472–485. doi: 10.1176/appi.ajp.2010.10060855

Xie, Z., Morgan, T. E., Rozovsky, I., and Finch, C. E. (2003). Aging and glial responses to lipopolysaccharide in vitro: greater induction of IL-1 and IL-6, but smaller induction of neurotoxicity. *Exp. Neurol.* 182, 135–141. doi: 10.1016/S0014-4886(03)00057-8

Xu, J., Yu, L., Cai, R., Zhang, J., and Sun, X. (2009). Early auditory enrichment with music enhances auditory discrimination learning and alters NR2B protein expression in rat auditory cortex. *Behav. Brain Res.* 196, 49–54. doi: 10.1016/j.bbr.2008.07.018

Yasojima, K., Schwab, C., McGeer, E. G., and McGeer, P. L. (1999). Up-regulated production and activation of the complement system in Alzheimer's disease brain. *Am. J. Pathol.* 154, 927–936. doi: 10.1016/S0002-9440(10)65340-0

Yirmiya, R., and Goshen, I. (2011). Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain Behav. Immun.* 25, 181–213. doi: 10.1016/j.bbi.2010.10.015

Zald, D. H., and Pardo, J. V. (1997). Emotion, olfaction, and the human amygdala: amygdala activation during aversive olfactory stimulation. *Proc. Natl. Acad. Sci. U.S.A.* 94, 4119–4124. doi: 10.1073/pnas.94.8.4119

Zhang, H., Cai, R., Zhang, J., Pan, Y., and Sun, X. (2009). Environmental enrichment enhances directional selectivity of primary auditory cortical neurons in rats. *Neurosci. Lett.* 463, 162–165. doi: 10.1016/j.neulet.2009.07.054

Zhang, L. I., Bao, S., and Merzenich, M. M. (2001). Persistent and specific influences of early acoustic environments on primary auditory cortex. *Nat. Neurosci.* 4, 1123–1130. doi: 10.1038/nn745

Zheng, H., Liu, Y., Li, W., Yang, B., Chen, D., Wang, X., et al. (2006). Beneficial effects of exercise and its molecular mechanisms on depression in rats. *Behav. Brain Res.* 168, 47–55. doi: 10.1016/j.bbr.2005.10.007

Ziv, Y., Ron, N., Butovsky, O., Landa, G., Sudai, E., Greenberg, N., et al. (2006). Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nat. Neurosci.* 9, 268–275. doi: 10.1038/nn1629

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 31 January 2014; accepted: 14 March 2014; published online: 03 April 2014.*
*Citation: Singhal G, Jaehne EJ, Corrigan F and Baune BT (2014) Cellular and molecular mechanisms of immunomodulation in the brain through environmental enrichment. Front. Cell. Neurosci.* ***8****:97. doi: 10.3389/fncel.2014.00097*
*This article was submitted to the journal Frontiers in Cellular Neuroscience.*
*Copyright © 2014 Singhal, Jaehne, Corrigan and Baune. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*

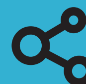
## ADVANTAGES OF PUBLISHING IN FRONTIERS

**FAST PUBLICATION**

Average 90 days from submission to publication

**COLLABORATIVE PEER-REVIEW**

Designed to be rigorous – yet also collaborative, fair and constructive



**RESEARCH NETWORK**

Our network increases readership for your article

**OPEN ACCESS**

Articles are free to read, for greatest visibility

**TRANSPARENT**

Editors and reviewers acknowledged by name on published articles

**GLOBAL SPREAD**

Six million monthly page views worldwide

**COPYRIGHT TO AUTHORS**

No limit to article distribution and re-use

**IMPACT METRICS**

Advanced metrics track your article's impact

**SUPPORT**

By our Swiss-based editorial team

frontiers

EPFL Innovation Park · Building I · 1015 Lausanne · Switzerland
T +41 21 510 17 00 · info@frontiersin.org · frontiersin.org