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# Advances in Neuroimmunology

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Edited by  
Donna Gruol

Printed Edition of the Special Issue Published in *Brain Sciences*

# Advances in Neuroimmunology

Special Issue Editor  
Donna Gruol

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*Special Issue Editor*  
Donna Gruol  
The Scripps Research Institute  
USA

*Editorial Office*  
MDPI AG  
St. Alban-Anlage 66  
Basel, Switzerland

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**Damir Nizamutdinov and Lee A. Shapiro**

Overview of Traumatic Brain Injury: An Immunological Context

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## About the Special Issue Editor

**Donna Gruol** is an Associate Professor in the Department of Neuroscience at the Scripps Research Institute. She has studied the role of neuroimmune factors in normal brain physiology and disease states for over fifteen years, and has made significant contributions to an understanding of the actions of neuroimmune factors on neuronal excitability and synaptic function. Her current research focuses on the role of neuroimmune factors in the effect of alcohol on brain structure and function.



# Preface to “Advances in Neuroimmunology”

It is now widely accepted that an innate immune system exists within the brain and plays an important role in both physiological and pathological processes [1,2]. This neuroimmune system is comprised of brain cells that produce and secrete chemicals that are historically considered signaling factors of the peripheral immune system, such as cytokines and chemokines. Cells of the brain, primarily glia cells (e.g., astrocytes and microglia) but also neurons under some conditions, produce a large number of immune factors. In addition, endothelial cells of the brain and peripheral immune cells that enter the brain can contribute to the immune environment of the brain [3].

In general, pathological conditions are associated with elevated levels of neuroimmune factors in the brain, whereas low levels of neuroimmune factors are found in the normal brain. For example, elevated levels of neuroimmune factors in the brain have been reported for a number of conditions including brain injury, infection, neurodegenerative and psychiatric disorders, and drug abuse [4–6]. Considerable effort has been devoted to identifying the neuroimmune factors that play a role in these conditions, but much work is yet to be done, especially with respect to the biological actions of individual neuroimmune factors and their role in specific brain disorders.

Neuroimmune factors, like their counterpart in the periphery, produce their biological actions through interactions with cognate membrane receptor systems that translate the chemical signal through the intervention of intracellular signaling pathways. These signaling systems are complex and many have yet to be fully elucidated. Of importance is that during pathological conditions, typically multiple signaling factors are simultaneously present in the cellular environment and may activate different signaling pathways on the same cell. These intracellular pathways may interact, a complexity that is a challenge to an understanding of mechanisms responsible for the biological actions associated with a particular brain condition and the development of specific therapeutic strategies.

In this Special issue, recent advances in an understanding of the neuroimmune system of the brain and the actions of neuroimmune factors are presented for ten areas under study; most areas are associated with pathological conditions. Together these studies are illustrative of the breadth and status of the field, the experimental approaches being employed, and areas for future research.

The review by Gruol [7], summarizes studies on the effects of three neuroimmune factors, the proinflammatory cytokine IL-6, the chemokine CCL2, and the chemokine CXCL10, on an essential aspect of brain function, synaptic transmission. The goal of these studies is to understand the actions of specific neuroimmune factors on this process. The majority of the studies discussed employ transgenic mice that express elevated levels of a neuroimmune factor (IL-6, CCL2 or CXCL10) in the brain through increased expression by astrocytes.

Transgenic mice that express elevated levels of IL-6 in the brain through increased astrocyte expression are also used in studies reported in the original article by Erta et al [8]. Transgenic mice null for astrocyte IL-6 expression are also used. The goal of these studies is to identify the role of astrocyte production of IL-6 in the symptomatology of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis in humans.

The review by Mukandala et al [9] summarizes studies that investigate the role of neuroimmune factors in acute and chronic hypoxia, and the consequences of neuroinflammation induced by hypoxia on hippocampal synaptic function. Hypoxia and neuroinflammation are two conditions that play a central role in ischemia. Complex signaling pathways involving the proinflammatory cytokine TNF- $\alpha$  and other factors are described along with their proposed roles in hypoxia and altered synaptic function associated with hypoxia.

Mori et al. [10] review the current state of knowledge on the expression and actions of two cytokines, IL-13 and IL-4, in the brain. Production of these cytokines by neurons and glia of the brain has been reported, but information is still limited. Both IL-13 and IL-4 can signal through a receptor complex comprised of IL-13 and IL-4 receptor subunits, although IL-4 also interacts with a separate IL-4 receptor. Evidence of a role for one of both of these cytokines in hypoxia, EAE and Parkinson’s disease is presented, along with evidence for modulatory actions on dopaminergic neurons.



Parkinson's disease is also a topic of the review by Grimmig et al. [11]. This review focuses on the role of neuroimmunology and neuron-glia interactions in the pathophysiology of Parkinson's disease in the context of aging. Pathological mechanisms are described along with potential therapeutic agents and strategies. Fractalkine, a protein constitutively expressed by neurons in the brain, and the antioxidant astaxanthin, a xanthophyll carotenoid that occurs naturally, are discussed as potential therapeutic agents.

Three original articles in this Special issue focus on the role of neuroimmune factors in the actions of drugs of abuse on the brain. Recent studies have revealed that several abused drugs, including alcohol and morphine, induce glial cells of the brain, primarily astrocytes and microglia, to secrete neuroimmune factors [2,12,13]. Microglial activation and elevated secretion of neuroimmune factors are thought to contribute to neuronal damage and cognitive dysfunction associated excessive drug use and other pathological conditions [14].

The original article by Marshall et al. [15] reports results from studies on the effects of a binge pattern of alcohol exposure on microglial activation and expression of neuroimmune factors in the brain of rats. Differences in the consequences of single versus repetitive alcohol exposure on microglial activation are addressed. In the original article by Knapp et al. [16], studies are reported that examine the expression of neuroimmune mRNAs in the brain after treatment of rats to an experimental paradigm involving chronic alcohol exposure followed by alcohol withdrawal. Results from the alcohol exposure/withdrawn animals are compared to neuroimmune mRNA expression produced in rats by stress, which is a risk factor for alcohol relapse.

Chang et al. [17] report effects of the bacterial endotoxin lipopolysaccharide (LPS) on expression of genes for proteins localized in multi-protein complexes called inflammasomes, which are important producers of neuroimmune factors and regulators of the inflammatory response. A number of different inflammasomes have been identified [18]. The studies focus on LPS-induced expression of genes for proteins housed in the inflammasomes in the context of morphine tolerance, which results from prolonged exposure to morphine. LPS is used in these studies to model invasion by a pathogen, which causes an inflammatory response. Morphine is known to affect the inflammatory response elicited by pathogens. A variety of inflammasome-related genes (e.g., for neuroimmune factors and downstream signaling partners) are examined in brains of morphine naïve rats and rats chronically exposed to morphine in these studies.

The review article by Liu et al. [19] focuses on another brain glial cell, the oligodendrocyte, and injury that occurs to this brain cell during HIV-1 infection. Oligodendrocytes are responsible for axonal myelination, which is essential for normal neuronal and synaptic processes that mediate brain function. Oligodendrocytes also contribute to the immunology of the brain by producing a wide range of neuroimmune mediators [20]. Process and mediators involved in oligodendrocyte and myelin damage as a consequence of HIV-1 are discussed in this article.

Nizamutdinov and Shapiro [21] provide a comprehensive review of the traumatic brain injury (TBI), and the role of neuroimmunity and peripheral immunity in the complex pathology of this condition. Traumatic brain injury is a broad area that encompasses many types of brain injury. A number of TBI experimental models are discussed along with mechanisms of neuropathology and the involvement of neuroimmunity. Neuroimmune factors have been reported to play a critical role in TBI outcomes.

**Donna Gruol**

*Special Issue Editor*

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Review

# Impact of Increased Astrocyte Expression of IL-6, CCL2 or CXCL10 in Transgenic Mice on Hippocampal Synaptic Function

Donna Gruol

Molecular and Cellular Neuroscience Department, The Scripps Research Institute, La Jolla, CA 92037, USA; gruol@scripps.edu; Tel.: +1-858-784-7060; Fax: +1-858-784-7393

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**Abstract:** An important aspect of CNS disease and injury is the elevated expression of neuroimmune factors. These factors are thought to contribute to processes ranging from recovery and repair to pathology. The complexity of the CNS and the multitude of neuroimmune factors that are expressed in the CNS during disease and injury is a challenge to an understanding of the consequences of the elevated expression relative to CNS function. One approach to address this issue is the use of transgenic mice that express elevated levels of a specific neuroimmune factor in the CNS by a cell type that normally produces it. This approach can provide basic information about the actions of specific neuroimmune factors and can contribute to an understanding of more complex conditions when multiple neuroimmune factors are expressed. This review summarizes studies using transgenic mice that express elevated levels of IL-6, CCL2 or CXCL10 through increased astrocyte expression. The studies focus on the effects of these neuroimmune factors on synaptic function at the Schaffer collateral to CA1 pyramidal neuron synapse of the hippocampus, a brain region that plays a key role in cognitive function.

**Keywords:** pyramidal neurons; Schaffer collaterals; LTP; neuroimmune; alcohol; field potential recordings; cytokine; chemokine

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## 1. Introduction

Several lines of evidence have confirmed the existence of a neuroimmune system in the CNS, and a role for neuroimmune communication in CNS homeostasis, function, and pathology. Glial cells, and in particular astrocytes and microglia, are the main cellular components of the CNS neuroimmune system. Glial cells initiate neuroimmune communication primarily through the production of small protein signaling factors with distinct structure and function. These neuroimmune factors include members of the cytokine superfamily such as proinflammatory cytokines and chemokines. Typically, proinflammatory cytokines and chemokines are present at low levels in the normal CNS, while elevated levels are associated with CNS disease and injury. For example, elevated levels of proinflammatory cytokines and/or chemokines in the CNS are typical hallmarks of CNS inflammatory and neurodegenerative diseases such as HIV infection [1], Alzheimer's disease [2], epilepsy [3], multiple sclerosis [4], alcoholism and fetal alcohol spectrum disorders [5–7], and psychiatric disorders (e.g., autism spectrum disorders, schizophrenia, depression) [8–10]. The elevated levels are thought to contribute to pathological processes occurring in these conditions, although protective actions could also play a role. Elevated levels of these neuroimmune factors also occur in normal aging, and may play a role in cognitive decline that can occur with normal aging [11,12].

CNS glial cells are capable of producing a variety of proinflammatory cytokines and chemokines, but the specific biological actions and roles of these neuroimmune factors have yet to be fully elucidated, and are likely to depend on the cell source and physiological or pathological context. During conditions associated with CNS disease and injury, multiple neuroimmune factors are commonly, and often chronically produced. The complexity of this situation makes it difficult to identify the actions of specific neuroimmune factors and the cell source, especially if pharmacological, biological, or other types of tools are lacking. A number of approaches have been used to circumvent this problem. This article focuses on one approach, the use of transgenic mice that endogenously produce elevated levels of a specific neuroimmune factor in the CNS by a cell type that normally produces it, and within the anatomical integrity and physiological pathways of the CNS. The transgenic mice of interest in this review express elevated levels of the proinflammatory cytokine Interleukin-6 (IL-6), the chemokine CCL2 (CC chemokine ligand 2, previously known as monocyte chemoattractant protein-1 or MCP-1), or the chemokine CXCL10 (previously known as interferon-gamma inducible protein 10 or IP10) through increased astrocyte expression. The review summarizes studies on the consequences of the increased astrocyte expression on a basic mechanism of CNS function, synaptic function, and in particular, hippocampal synaptic function. The hippocampus plays a critical role in learning and memory, and alterations in hippocampal synaptic function can significantly affect cognition [13]. Studies in experimental models have shown that altered hippocampal synaptic function is associated with CNS conditions known to involve elevated expression of neuroimmune factors (e.g., [14–26]). The transgenic mice have also been a useful model for a number of other types of studies related to CNS conditions during disease and injury, a topic that is not addressed in this review (e.g., [27–34]).

## **2. Astrocytes Are a Primary Source of Neuroimmune Factors in the CNS**

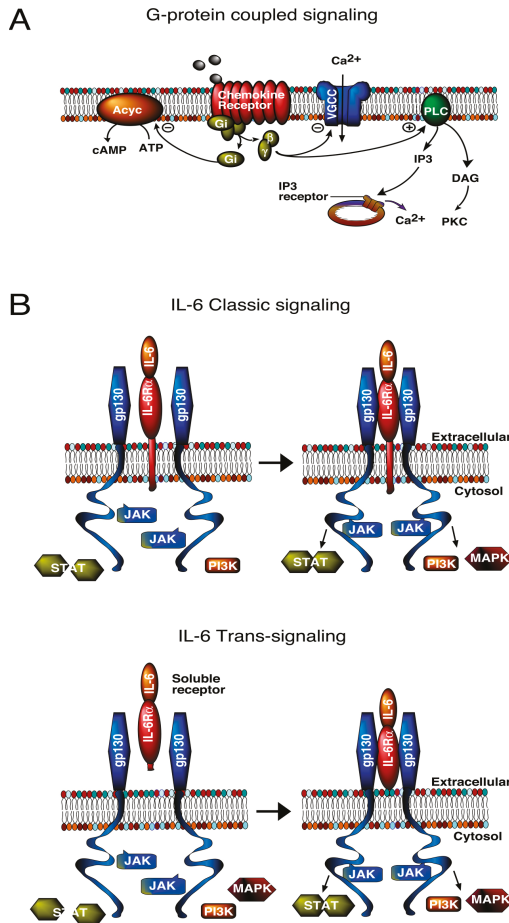
Astrocytes are the most abundant cell type in the CNS and a key component of the neuroimmune system of the CNS [35]. Astrocytes play a variety of roles in the CNS, as regulators/mediators of normal physiology and responders to adverse conditions, such as those occurring during injury and infection, when astrocytes contribute to repair and recovery processes [36,37]. A large number of cytokines and chemokines are produced by astrocytes, including IL-6, CCL2, and CXCL10, but relatively little is known about the specific roles and biological actions of these factors under physiological or pathophysiological conditions when astrocytes are the initial cell source of these factors. Astrocytes are in close association with neurons and synapses, making them ideally positioned to influence neuronal circuit activity, which is essential for normal CNS function and is often compromised in CNS disorders [38,39]. In this review, studies on the consequence of elevated astrocyte expression IL-6, CCL2, or CXCL10 on synaptic function at the Schaffer collateral to CA1 pyramidal neuron synapse of the hippocampus are summarized. The Schaffer collateral to CA1 pyramidal neuron synapse is one of the most highly studied synapse in the CNS [40]. Output from the CA1 region provides important input to other brain regions and plays a key role in learning, memory, and other cognitive functions.

## **3. Signal Transduction Pathways**

IL-6, CCL2 and CXCL10 initiate biological actions through the activation of specific membrane receptors, IL-6R, CCR2, and CXCR3, respectively. However, downstream signal transduction pathways differ. CCR2 and CXCR3 are G-protein coupled receptors (GPCRs), whereas IL-6R is linked to a tyrosine kinase signal transduction pathway (Figure 1). Moreover, IL-6R associated signal transduction can occur through two pathways, a classic pathway and trans-signaling [41] (Figure 1).

The classic IL-6 pathway involves membrane bound IL-6R, which interacts with another membrane bound protein, gp130, the signaling subunit of IL-6R and other cytokine receptors. Trans-signaling involves IL-6R that has been released from cells into the extracellular fluid and is referred to as soluble IL-6R. Soluble IL-6R can bind to IL-6 in the extracellular fluid and the ligand/receptor complex can then bind to membrane bound gp130. Because gp130 is ubiquitously expressed in CNS cells, trans-signaling can occur in cells that do not express membrane bound IL-6R, and

consequently trans-signaling greatly expands the target area of IL-6 actions. Trans-signaling appears to be the primary pathway involved in the pathological actions of IL-6 in the CNS [42].



**Figure 1.** Diagrams showing signal transduction pathways used by chemokines and the proinflammatory cytokine IL-6. A plus sign within a circle indicates activation of the target molecule and a minus sign within a circle indicates inhibition of the target molecule. (A) Agonist binding to the G-protein coupled receptors (GPCR) initiates dissociation of the G-protein heterotrimer coupled to the receptor into G $\alpha$  and G $\beta\gamma$  subunits. The G $\alpha$  and G $\beta\gamma$  subunits then activate or inhibit downstream effectors. These effectors include ion channels, such as voltage-gated calcium channels (VGCC), and signal transduction molecules including phospholipase C (PLC) and adenylate cyclase (Acyc). Activation of PLC leads to the production of other signaling molecules including diacylglycerol (DAG) and inositol trisphosphate (IP3), and downstream activation of protein kinase C (PKC) and inositol trisphosphate receptors (IP3R), which regulate the release of calcium from intracellular stores; (B) IL-6 can signal through either a membrane bound (classic signaling) or a soluble (trans-signaling) IL-6R. The IL-6/IL-6R complex interacts with gp130 to activate the JAK/STAT signaling pathway. In addition, the IL-6/IL-6R/gp130 complex can activate RAS/mitogen-activated protein kinase (p44/42 MAPK, also called ERK1/2; MAPK) and phosphatidylinositol-3 kinase (PI3K) signaling pathways. All three signaling pathways activate additional downstream signaling molecules and effectors.

The differences in signal transduction pathways utilized by IL-6 and chemokines could indicate different biological actions. However, signal transduction pathways downstream of the G-protein and tyrosine kinase step can merge at common pathway partners or targets and lead to similar biological actions. Thus, it is not surprising that all three neuroimmune factors have neuronal or synaptic actions, although the actions are not identical.

Both neurons and glial cells express receptors and signal transduction pathways utilized by IL-6R [41,43], CCR2 [44–46], and CXCR3 [47,48], and are potential downstream cellular targets of the astrocyte produced neuroimmune factors. Because of the close association of astrocytes with neurons and synapses [39], actions of cytokines or chemokines on either cell type could potentially alter neuronal and synaptic function. Downstream molecular targets of GPCR and IL-6R pathways can regulate gene expression, which may be instrumental in directing neuroadaptive changes associated with elevated expression of IL-6, CCL2, and CXCL10 in the CNS of the transgenic mice.

#### **4. IL-6, CCL2, or CXCL10 Transgenic Mice**

All three lines of transgenic mice with increased astrocyte expression of IL-6, CCL2, or CXCL10 were generated by a similar approach, insertion of the transgene (mouse or human) for the neuroimmune factor under transcriptional control of the glial fibrillary acidic protein (GFAP) gene promoter [29,34,49,50]. GFAP is an intermediate filament protein expressed almost exclusively by astrocytes in the adult CNS and commonly used as a marker for astrocytes [50,51]. More than one line was generated for each neuroimmune factor. Heterozygotes from the following lines were used for the studies discussed in this review: IL-6 transgenic line 167 (IL-6 tg), CXCL10 transgenic line CXCL10-10 (CXCL10 tg), CCL2 transgenic line on a SJL background (CCL2-tg SJL mice), and CCL2 transgenic line on a C57Bl/6J background (CCL2-tg), which were developed from the CCL2-tg SJL mice. Non-transgenic littermates of the respective transgenic line were used as controls. In general, elevated expression of other neuroimmune factors was not evident, or at low level in these transgenic lines [29,34,52], enabling investigation of the consequences of elevated expression of the transgene alone or in combination with other experimental manipulations.

##### *4.1. Expression of IL-6, CCL2, or CXCL10 in the Transgenic Mice*

Because transgene expression in the transgenic mice is under control of the GFAP promoter, elevated expression of IL-6, CCL2, or CXCL10 is linked to GFAP expression. GFAP expression in astrocytes is initiated during the developmental period, which occurs primarily during the first 3 weeks of postnatal life in mice. GFAP expression in the mouse hippocampus is evident at 1 day postnatal, increases with age until 6 days postnatal, and then levels off and remains stable through adulthood [53]. Thus, neuronal/synaptic exposure to these neuroimmune factors in the transgenic mice occurs during an important period of structural and synaptic development and could affect developmental patterns. Evidence is limited on this topic, but in general, neuropathology in the hippocampus of the IL-6, CCL2, and CXCL10 heterozygous mice is absent or minimal up to 3–6 months of age, although homozygous mice can show pathology at early ages [29,32,54,55]. Thus, if the elevated expression of IL-6, CCL2, or CXCL10 altered CNS development in the transgenic mice, the effects on development were not pathological or were compensated for by other changes. In this review, discussion of the transgenic mice refers to the heterozygotes.

CNS expression of IL-6, CCL2, or CXCL10 has been quantified in the respective transgenic mice at the mRNA and/or protein levels. Studies of IL-6-tg mice showed that IL-6 mRNA was evident in the CNS at 7 days postnatal, increased with age and reached a peak at 3 months postnatal (adult stage), after which a decline was observed [52]. IL-6 transgene expression was demonstrated in hippocampal astrocytes by expression of the lacZ reporter gene and immunohistochemical detection of  $\beta$ -gal [55]. Constitutive secretion of IL-6 from astrocytes was demonstrated in studies of astrocyte cultures prepared from CNS of the IL-6 tg mice [49]. IL-6 levels were ~150 pg/mL in the supernatant from astrocyte cultures prepared from CNS of IL-6 tg mice, compared with <5 pg/mL for supernatant

from astrocyte cultures prepared from CNS of non-tg mice. Interestingly, ELISA analysis of IL-6 levels in the hippocampus have revealed low levels and no differences between the IL-6 tg and non-tg hippocampus, although higher levels and genotypic differences were noted in the cerebellum [52,56]. The cerebellum is the CNS region with the highest level of IL-6 mRNA expression in the transgenic mice, particularly in the Bergman glial [49]. These results may indicate that IL-6 produced by hippocampal astrocytes *in vivo* is rapidly released and degraded. Others have noted difficulty in measuring IL-6 levels in CNS tissue using commercial ELISA kits, which may mean that there are technical issues to be resolved [57]. In spite of the lack of differences in measurable levels of IL-6 protein, increase expression of IL-6 regulated genes (e.g., GFAP, eb22, Socs3) and elevated levels of STAT3 and the activated form of STAT3 (phosphoSTAT3), the downstream partner of IL-6 signal transduction through which IL-6 acts to increase GFAP [58–60], were observed in the CNS of IL-6 tg mice. These results are consistent with actions of elevated levels of IL-6 in the IL-6 tg CNS.

Protein measurements in the CNS of the two CCL2 transgenic lines showed that the older CCL2-tg SJL mice express higher levels of CCL2 in the hippocampus than in the CCL2-tg mice. CCL2 levels measured by ELISA were ~1.3 ng/mL at 3–4 months of age and ~3.0 ng/mL at 7–9 months of age in hippocampal homogenate from the CCL2-tg SJL mice [61]. In the CCL2-tg mice, CCL2 levels measured by ELISA were ~1.2 ng/mL at 3–5 months of age and ~1.5 ng/mL at 7–9 [58]. CCL2 levels were ~0.2 ng/mL in hippocampal homogenates from the non-tg mice from both the CCL2-tg and CCL2-tg SJL lines. Studies of supernatants from astrocyte cultures prepared from CNS of CCL2-tg SJL mice showed that astrocytes constitutively secrete large amounts of CCL2 (e.g., ~3.5 ng/mL) [34].

Expression of CXCL10 in the CNS of CXCL10-tg mice has been characterized at the mRNA level by *in situ* hybridization [29]. The highest levels of CXCL10 mRNA were observed in the hippocampus, olfactory bulb, periventricular zone, cortical areas, cerebellum, and choroid plexus of the CXCL10-tg CNS (mice 5–6 months of age). Western blot studies confirmed high levels of CXCL10 protein in the hippocampus, and immunohistochemical staining confirmed expression of CXCL10 protein in astrocytes [29]. No CXCL10 mRNA or protein expression was observed in non-tg mice. Levels of CXCL10 protein in the CNS of CXCL10-tg mice have not been measured by ELISA.

Elevated levels of neuroimmune factors are typically associated with pathological conditions, whereas low levels appear to exist under physiological conditions. However, the range of protein levels expressed during physiological and pathophysiological conditions has yet to be fully elucidated for most neuroimmune factors. Although elevated levels IL-6, CCL2 and CXCL10 mRNA and/or protein have been documented in the CNS of the respective transgenic mice, it is unknown if protein levels for the three transgenic lines are functionally comparable. However, mRNA or protein levels were shown to be within the range associated with experimentally induced pathophysiological conditions in the CNS of IL-6 tg [62], CXCL10-tg [29] and CCL2-tg SJL mice [34].

#### 4.2. Neuropathology

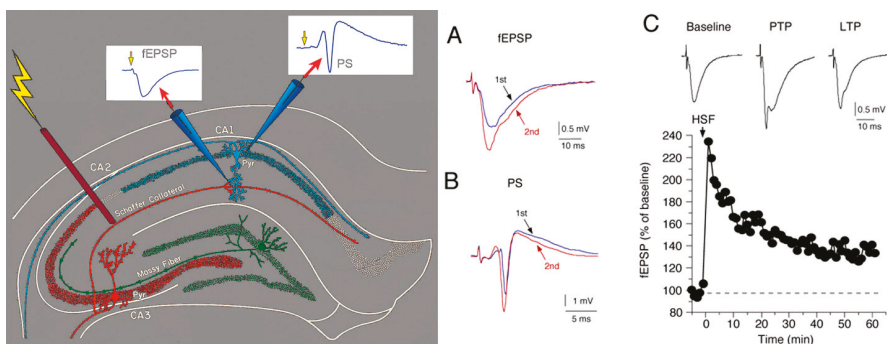
In general, before 3–6 months of age, the heterozygous IL-6, CCL2, and CXCL10 transgenic mice show relatively little neuropathology. In the IL-6 tg mice, the cerebellum shows the highest levels of IL-6 mRNA expression in the CNS of the IL-6 tg mice and greatest neuropathological changes, the most prominent being neovascularization [49,63]. Age-dependent neuropathological changes in the cortex and hippocampus of the IL-6 tg mice were evident in immunohistochemical studies of synaptic and cellular proteins. The neuropathological changes included reduced immunostaining for the presynaptic protein synapsin I indicative of synaptic damage (cortex, 12 months of age), reduced immunostaining for microtubule associated protein-2 (MAP-2) indicative of dendritic damage (cortex at 3 and 12 months of age), reduced immunostaining for parvalbumin, a calcium binding protein expressed by inhibitory interneurons (hippocampus at 3 and 12 months of age), and eventual loss of the interneurons, and reduced immunostaining for calbindin, a calcium binding protein expressed by inhibitory interneurons (cortex, 12 months of age) [49,54].



Histological studies of the CNS of CCL2-tg and CXCL10-tg mice are limited. However, CCL2-tg SJL mice have been reported to be free of neurological impairment before 6 month of age [34]. Routine histological analysis of the CNS of the CXCL10 mice showed no apparent neuropathological changes relative to the CNS of the non-tg mice [29].

### 5. Synaptic Function in the Hippocampus from IL-6, CCL2, and CXCL10 Transgenic Mice

For all three transgenic lines, physiological studies to assess synaptic function have been carried out at the Schaffer collateral to CA1 pyramidal neuron synapse of the hippocampus using a similar protocol that involved extracellular field potential recordings from acutely isolated slices of hippocampus (Figure 2). This approach has been extensively used for physiological studies of hippocampal synaptic function. One potential limitation to this approach is that the normal level of neuroimmune factors could be altered by the slice preparation and recording procedures. However, such effects would presumably also occur in the non-tg slices and thus be controlled for.



**Figure 2.** Measurement of synaptic function using extracellular recordings in hippocampal slices. (**Left Panel**) Simplified diagram showing the placement of stimulating and recording electrodes and recorded responses in a field potential recording of synaptic transmission at the Schaffer collateral to CA1 pyramidal neuron synapse in a hippocampal slice. Synaptic transmission is initiated experimentally by electrical stimulation of Schaffer collaterals, axons of the CA3 pyramidal neurons of the hippocampus. Stimulation of the Schaffer collateral elicits a fEPSP in the dendritic region and, depending on the strength of the stimulation, a PS in the somatic region; (**Right panel**) Repetitive stimulation can result in a change in the magnitude of synaptic responses. (**A**) Repetitive stimulation with a 40 ms interval between the first and second stimulation resulted in an enhancement of the fEPSP (2nd) evoked by the second stimulation relative to the fEPSP (1st) evoked by the first stimulation; (**B**) Repetitive stimulation with a 10 ms interval between the first and second stimulation resulted in an enhancement of the PS (2nd) evoked by the second stimulation relative to the PS (1st) evoked by the first stimulation in this slice; (**C**) High frequency stimulation (HSF) induces a long-term enhancement of the fEPSP. The graph shows the magnitude of the fEPSP enhancement relative to baseline levels before high frequency stimulation was applied (at the arrow). The initial, large enhancement of the fEPSP is referred to as post-tetanic potentiation (PTP). The delayed, stable increase in the magnitude of the fEPSP is referred to as long-term potentiation (LTP). Representative recordings are shown above the graph.

Synaptic transmission to CA1 pyramidal neurons was elicited by electrical stimulation of the Schaffer collaterals. Both baseline synaptic transmission elicited by single stimulations and synaptic plasticity elicited by repetitive stimulation were studied. The response to synaptic transmission was measured in the dendritic region of the CA1 neurons as a field excitatory postsynaptic potential (fEPSP), which reflects the membrane depolarization produced by synaptic transmission in a population of CA1 neurons (Figure 2). In some studies, recordings were also made in the somatic region of the CA1

pyramidal neurons, where population spikes (PS) were recorded (Figure 2). The PS reflects action potentials occurring in the soma/dendritic region that were generated by synaptic depolarizations in a population of CA1 pyramidal neurons. Data from hippocampal slices from the transgenic mice were compared to data from hippocampal slices from the respective non-tg littermate controls. Results are summarized in Table 1. In addition to the studies of IL-6 tg mice discussed in this review, two other studies of synaptic function in the hippocampus have appeared, both in the dentate region [64,65]. In addition, one study on synaptic function in the cerebellum has appeared [66].

**Table 1.** Genotypic differences in synaptic function in the hippocampus.

| Measurement                   | IL-6 tg vs. Non-tg |      | CCL2-tg vs. Non-tg | CCL2-tg SJL vs. Non-tg | CXCL10-tg vs. Non-tg |
|-------------------------------|--------------------|------|--------------------|------------------------|----------------------|
|                               | 1–2                | 3–6  | 2–3                | 7–12                   | 5–6                  |
| Synaptic transmission         |                    |      |                    |                        |                      |
| -fEPSP                        | ↑                  | ↑    | no Δ               | ↓                      | no Δ                 |
| -PS                           | ↑                  | ↑    | ↑                  | ↓                      | no Δ                 |
| P-P synaptic plasticity       |                    |      |                    |                        |                      |
| -fEPSP (PPF)                  | no Δ               | no Δ | no Δ               | ↑                      | no Δ                 |
| -PS (PPR)                     | no Δ               | no Δ | no Δ               | ↑                      | no Δ                 |
| Long-term synaptic plasticity |                    |      |                    |                        |                      |
| -PTP                          | ↓                  | no Δ | no Δ               | ↑                      | no Δ                 |
| -LTP                          | no Δ               | no Δ | no Δ               | no Δ                   | no Δ                 |
| Reference                     | [60]               |      | [67]               | [61]                   | [68]                 |

↓ = decrease, ↑ = increase, no Δ = no difference.

### 5.1. Synaptic Transmission

The hippocampus from the IL-6 tg mice was studied at two ages, young mice 1–2 months of age and adult mice 3–6 months of age. Results were similar for the two age groups and showed that the fEPSP was enhanced in the hippocampus from the IL-6 tg mice compared to the hippocampus from non-tg mice of the same age group [60]. As a consequence of the enhanced fEPSP, the PS was also enhanced in the IL-6 tg hippocampus [60].

There was no difference in the fEPSP magnitude between the hippocampus from the CCL2-tg and non-tg mice at 2–3 months of age, whereas the PS was significantly larger in the hippocampus from the CCL2-tg mice [67]. Thus, the hippocampus from both the IL-6 tg and CCL2-tg mice showed an increase in the PS, indicative of increased excitability. However the increased PS in the hippocampus from the IL-6 tg mice could be explained by a larger fEPSP, but the increased PS in the hippocampus from the CCL2-tg mice could not. This difference indicates that although the functional consequence at the level of the PS was similar for the IL-6 tg and CCL2-tg hippocampus, different underlying mechanisms were involved. The increased excitability in the IL-6 tg mice could underlie the enhanced sensitivity to glutamate receptor agonists-induced seizure activity [69] and enhanced alcohol withdrawal hyperexcitability [70] observed in the IL-6 tg mice compared to the non-tg mice. The CCL2-tg mice did not show the enhanced alcohol withdrawal hyperexcitability observed in the IL-6 tg mice [70]. Effects glutamate receptor agonist on seizure activity has not been tested in the CCL2-tg mice.

In contrast to the CCL2-tg mice where only the PS was altered and an enhancement was observed, in the hippocampus from the CCL2-tg SJL mice at 7–12 months of age, both the fEPSP and PS showed a reduction in magnitude compared to non-tg hippocampus [61]. This difference between CCL2-tg and CCL2-tg SJL hippocampus may be due to the older age or the higher level of CCL2 expression in the CCL2-tg SJL hippocampus. In contrast to the IL-6 tg, CCL2-tg and CCL2-tg SJL hippocampus, there was no significant difference in the magnitude of the fEPSP or PS between the CXCL10 tg and non-tg hippocampus from 5–6 months old mice [68].

## 5.2. Synaptic Plasticity in IL-6 tg, CCL2-tg and CXCL-10 tg Mice

Synaptic plasticity is a change in the magnitude of synaptic responses that results when a synapse is repetitively stimulated. Synaptic plasticity is considered to be an important cellular mechanism of memory and learning [71]. Short-term and/or long-term synaptic plasticity at the Schaffer collateral to CA1 pyramidal neuron synapse has been studied in one or more of the transgenic lines. Results are summarized in Table 1.

### 5.2.1. Short-Term Synaptic Plasticity

In this form of synaptic plasticity, repetitive activation of a synapse at short intervals (<1 s) elicits a transient increase or decrease in the magnitude of the synaptic response. Short-term synaptic plasticity is experimentally determined by applying repetitive stimulation to the Schaffer collaterals using a paired-pulse (P-P) paradigm. The magnitude of the plasticity is indicated by the paired-pulse ratio (PPR, magnitude of the response to the 2nd stimulation divided by magnitude of the response to the 1st stimulation). At the Schaffer collateral to CA1 pyramidal neuron synapse the paired-pulse protocol results in an enhancement of the fEPSP (*i.e.*, a PPR greater than 1, Figure 2A). This enhancement is referred to as paired-pulse facilitation (PPF). PPF reflects greater transmitter release with the 2nd stimulation due to actions of residual  $Ca^{2+}$  on the probability of transmitter release in the presynaptic terminals of the Schaffer collaterals [72–74].

There was no difference in PPF of the fEPSP between the hippocampus from IL-6 tg and non-tg mice at either age studied (1–2 and 3–6 months of age) [60]. The hippocampus from CCL2-tg and non-tg mice studied at 2–3 months of age also showed no difference in PPF of the fEPSP [67]. In the 7–12 months CCL2-tg SJL mice, PPF of the fEPSP was increased at the 40 ms paired-pulse interval but not at longer intervals compared to the hippocampus from non-tg mice, indicating activity-induced presynaptic changes that impact excitatory synaptic transmission in a limited manner [61]. There was no significant difference in the PPF between the CXCL10 tg and non-tg hippocampus from 5–6 months old mice [68].

A second form of short-term plasticity induced by synaptic activation occurs in the somatic region of the CA1 neurons and affects the PS that is generated by the fEPSP. Plasticity of the PS can result in a PPR greater than one (less inhibition; Figure 2B) or less than one (more inhibition) depending on the relative contribution of somatic/dendritic excitability and recurrent inhibition to the somatic region. There was no difference in PPR of the PS between the hippocampus from IL-6 tg and non-tg mice at either age studied (1–2 and 3–5 months of age) [60], or between the hippocampus from CCL2-tg and non-tg mice at 2–3 months of age [67]. PPR of the PS in the hippocampus from 7–12 months old CCL2-tg SJL mice was increased compared to the hippocampus from non-tg mice, indicating decreased inhibitory influences in the soma/dendritic region [61]. There was no significant difference in PPR of the PS between the CXCL10 tg and non-tg hippocampus from 5–6 months old mice [68].

### 5.2.2. Long-Term Synaptic Plasticity

Long-lasting changes in synaptic transmission are also observed at the Schaffer collateral to CA1 pyramidal neuron synapse. These changes can involve an increase in the magnitude of the synaptic response, referred to as long-term potentiation (LTP), or a decrease in the magnitude of the synaptic response, referred to as long-term depression (LTD). LTP is experimentally induced by brief, high frequency stimulation of the Schaffer collaterals, whereas LTD is induced experimentally by prolonged stimulation of the Schaffer collaterals at low frequency. LTP has been studied in hippocampal slices from the IL-6 tg, CCL2-tg, CCL2-tg SJL and CXCL10-tg mice, but studies on LTD have not appeared.

High frequency stimulation (HFS) of the Schaffer collaterals induces an immediate and dramatic increase in the amplitude of the fEPSP, after which the enhancement declines somewhat to a steady, stable level reflecting LTP (Figure 2C). The initial enhancement is a shorter form of synaptic plasticity referred to as post-tetanic potentiation (PTP). PTP results from the impact of HFS on presynaptic

mechanisms involved in transmitter release. LTP, the delayed, persistent, stable increase in the magnitude of the fEPSP is primarily a result of activity-induced changes in post-synaptic mechanisms. Results from studies of long-term synaptic plasticity are shown in Table 1.

In the IL-6 tg line, there was no genotypic difference in LTP between IL-6 and non-tg hippocampus from both young (1–2 months of age) and adult (3–6 months of age) mice. PTP was reduced in the hippocampus from young (1–2 months of age) IL-6 tg mice compared to the hippocampus from non-tg mice, indicating changes in presynaptic function, a genotypic effect that was not observed for IL-6 and non-tg hippocampus from adult mice (3–6 months of age) [60]. In both the CCL2-tg and CXCL10-tg lines, no genotypic effect on PTP or LTP was observed between the hippocampus from transgenic *vs.* non-tg mice [67,68]. PTP was enhanced in the hippocampus from the CCL2-tg SJL mice compared with the hippocampus from the non-tg mice, but there was no genotypic difference in LTP.

### 5.3. Effect of Acute Application of Neuroimmune Factors on Synaptic Function

In addition to studies of the hippocampus from transgenic mice, studies on the effects of acute, exogenous applied IL-6 or CXCL10 on synaptic transmission and plasticity at the Schaffer collateral to CA1 pyramidal synapse have been carried out in hippocampal slices from rat or mice. The effect of acute exposure is of interest because it presumably reflects to some degree the actions of the endogenous cytokine or chemokine during the initial stages of elevated expression in the transgenic mice. Results are summarized in Table 2. There was no significant effect of exogenously applied IL-6 on the fEPSP or PS of rat hippocampal slices. There was also no significant effect of exogenously applied CXCL10 on the fEPSP in hippocampal slices from the CXCL10 tg and non-tg mice [68]. The effect of acute, exogenous applied CCL2 on synaptic transmission was studied in the rat hippocampal slices using whole cell voltage clamp techniques. CCL2 enhanced the excitatory postsynaptic currents elicited by stimulation of the Schaffer collaterals, an effect shown to result from actions of CCL2 on presynaptic mechanisms [75,76].

Although acute, exogenous application of IL-6 or CXCL10 had no effect on baseline synaptic transmission, IL-6 significantly reduced PTP and LTP in rat hippocampal slices [77,78]. Exogenous application of CXCL-10 also significantly reduced both PTP and LTP hippocampal slices from the non-tg mice, but only LTP in hippocampal slices from CXCL10-tg mice [68]. The lack of effect of CXCL-10 on PTP in the CXCL10-tg hippocampus, suggest neuroadaptive changes in the CXCL10-tg mice that prevent the actions of acute CXCL-10.

**Table 2.** Effects of exogenous application of neuroimmune factor on synaptic function in hippocampus.

| Measurement                    | Neuroimmune Factor |              |             |               |              |
|--------------------------------|--------------------|--------------|-------------|---------------|--------------|
|                                | IL-6               |              | CCL2        | CXCL10 non-tg | CXCL10 tg    |
| species                        | rat                | rat          | rat         | mouse         | mouse        |
| Age (months) or weight (gm)    | 2–3 months         | 200–250 gm   | 0.5–1 month | 5–6 months    | 5–6 months   |
| Concentration                  | 1, 5, 50 ng/mL     | 50–2000 U/mL | 2.3 nM      | 10 ng/mL      | 10 ng/mL     |
| Synaptic transmission          |                    |              |             |               |              |
| -fEPSP or EPSC                 | nd                 | no $\Delta$  | $\uparrow$  | no $\Delta$   | nd           |
| -Population spike              | no $\Delta$        | nd           | nd          | nd            | nd           |
| Short-term synaptic plasticity |                    |              |             |               |              |
| -fEPSP (PPF)                   | no $\Delta$        | nd           | nd          | $\uparrow$    | no $\Delta$  |
| -Population spike (PPR)        | nd                 | nd           | nd          | nd            | nd           |
| Long-term synaptic plasticity  |                    |              |             |               |              |
| -PTP                           | $\downarrow$       | $\downarrow$ | nd          | $\downarrow$  | no $\Delta$  |
| -LTP                           | $\downarrow$       | $\downarrow$ | nd          | $\downarrow$  | $\downarrow$ |
| Reference                      | [79]               | [78]         | [75]        | [68]          |              |

$\downarrow$  = decrease,  $\uparrow$  = increase, no  $\Delta$  = no difference, nd = not determined. EPSC = excitatory postsynaptic current.

Taken together, these results show that mechanisms that induce LTP and PTP are sensitive to acute exposure to IL-6 or CXCL10. Thus, the lack of genotypic differences in LTP and PTP between the IL-6 tg and non-tg hippocampus and the CXCL10-tg and non-tg hippocampus may reflect neuroadaptive changes in mechanisms that induce LTP and PTP. These neuroadaptive changes produced an apparent normalization of function. Effects of acute, exogenous application of CCL2 on PTP and LTP have not been reported.

## 6. Protein Levels in Hippocampus

IL-6, CCL2, and CXCL10 signal transduction pathways can lead to downstream effects on gene expression and, consequently, changes in the levels of important cellular and synaptic proteins. Changes in protein levels could also occur through other regulatory mechanisms. Such neuroadaptive changes could impact synaptic function. Western blot studies were carried out to identify potential changes in protein levels in the hippocampus from IL-6 tg and CCL2-tg mice. CXCL10-tg mice have not examined. Relatively few changes in protein levels were observed in the hippocampus from the IL-6 tg and CCL2-tg mice compared to hippocampus from their respective non-tg mice, as shown in Table 3. These results are consistent with the relative lack of neuropathological changes observed in the hippocampus from the IL-6 tg and CCL2-tg mice at the ages studied. However, some differences were observed that could affect synaptic function.

**Table 3.** Genotypic differences on protein levels in hippocampus.

| Measurement           | IL-6 tg vs. Non-tg |      | CCL2-tg vs. Non-tg |      | CCL2-tg SJL vs. Non-tg |      |
|-----------------------|--------------------|------|--------------------|------|------------------------|------|
|                       | 1–2                | 3–5  | 1–3                | 3–5  | 3–4                    | 7–9  |
| Age (months)          |                    |      |                    |      |                        |      |
| Housekeeping proteins |                    |      |                    |      |                        |      |
| -β-actin              | no Δ               | no Δ | no Δ               | no Δ | no Δ                   | no Δ |
| Astrocyte proteins    |                    |      |                    |      |                        |      |
| -GFAP                 | ↑                  | ↑    | no Δ               | no Δ | no Δ                   | ↑    |
| -Glutamine synthetase | no Δ               | no Δ | no Δ               | no Δ | nd                     | nd   |
| Microglial protein    |                    |      |                    |      |                        |      |
| -CD11b                | nd                 | no Δ | no Δ               | nd   | ↑                      | no Δ |
| Neuronal proteins     |                    |      |                    |      |                        |      |
| -Enolase              | no Δ               | no Δ | no Δ               | no Δ | no Δ                   | no Δ |
| -GAD65/67             | no Δ               | ↓    | no Δ               | no Δ | no Δ                   | no Δ |
| Synaptic proteins     |                    |      |                    |      |                        |      |
| -Synapsin 1           | no Δ               | no Δ | no Δ               | ↑    | no Δ                   | no Δ |
| -VGLUT1               | nd                 | no Δ | no Δ               | nd   | nd                     | nd   |
| -GluA1                | no Δ               | no Δ | no Δ               | no Δ | no Δ                   | no Δ |
| -GluN1                | no Δ               | no Δ | ↑                  | ↑    | no Δ                   | no Δ |
| Signal transduction   |                    |      |                    |      |                        |      |
| -STAT3                | ↑                  | ↑    | no Δ               | nd   | nd                     | nd   |
| -p42/44 MAPK          | no Δ               | no Δ | no Δ               | no Δ | nd                     | nd   |
| Reference             | [58,60]            |      | [58,67]            |      | [61]                   |      |

↓ = decrease, ↑ = increase, no Δ = no difference, nd = not determined.

Compared to the hippocampus from non-tg mice, the hippocampus from IL-6 tg mice showed elevated levels of GFAP and STAT3, the signal transduction molecule that is involved in IL-6 regulation of GFAP gene expression [59,60]. The level of phosphorylated (*i.e.*, activated) STAT3 was also elevated in the hippocampus from IL-6 tg mice [59,60]. Another astrocytic protein, glutamate synthetase, which is involved in glutamate cycling, an important aspect of excitatory synaptic transmission [80], was not altered in the hippocampus from the IL-6 tg mice [60], suggesting that the increased levels of GFAP do not reflect a general action of IL-6 on astrocytic protein levels. The increased levels of activated STAT3

in the hippocampus of IL-6 tg mice could affect synaptic function. STAT3 has been shown to be highly expressed in CNS neurons, where it is present in the postsynaptic density, and to regulate synaptic plasticity (LTD) in the hippocampus [1]. In addition to increased levels of GFAP and STAT3, reduced levels of GAD65/67, the synthetic enzyme for the inhibitory transmitter GABA, were observed in the IL-6 tg hippocampus [60], consistent with the immunohistochemical studies indicating a negative effect of the elevated levels of IL-6 on the structure of inhibitory interneurons [49,54].

Compared to the hippocampus from non-tg mice, the hippocampus from CCL2-tg mice showed elevated levels of synapsin 1, a presynaptic protein involved in transmitter release, and GluN1, the essential subunit of NMDA receptors [58,67]. NMDA receptors play a critical role in neuronal development, synaptic plasticity, and neuronal toxicity, and are an important target site for therapeutic intervention in a number of neurological disorders [81,82]. The neuroadaptive changes in synapsin 1 and GluN1 levels were not evident in the CCL2-tg SJL hippocampus, where the only changes were an increase in CD11b and GFAP [61]. Taken together, these results show that neuroadaptive changes occur at the level of synaptic proteins in the IL-6 tg and CCL2-tg hippocampus. The differences in proteins targeted in the IL-6 tg and CCL2-tg hippocampus could contribute to differences in the synaptic properties altered in the two transgenic lines.

## 7. Behavioral Studies

Alterations in synaptic function can result in changes in behavior. Two behavioral tests that evaluate the functioning of the hippocampus are the avoidance learning test and the contextual fear conditioning test. These behavioral tests were used to examine hippocampal function in the IL-6 tg or CCL2-tg lines. Behavior has not been tested in the CXCL10-tg line. The IL-6 tg mice did not show a behavioral deficit compared to non-tg mice in the avoidance learning when tested at 3 months of age. However, by 6 months of age the IL-6 tg mice exhibited a significant deficit in their ability to learn the avoidance response, which declined further by 12 months of age [54]. The CCL2-tg mice were examined in behavioral tests for contextual fear conditioning at 2–3 months of age. There were no significant differences between the CCL2-tg and non-tg mice in these tests [67]. These results suggest the lack of significant hippocampal dysfunction at 3 months of age in both the IL-6 tg and CCL2 tg mice, at least under baseline conditions in these tests.

## 8. Covert Neuroadaptive Changes

Taken together, studies of synaptic function and protein expression in the hippocampus from IL-6 tg and CCL2-tg mice revealed relatively few neuroadaptive changes produced by the respective neuroimmune factor under baseline conditions, although the observed changes could significantly alter CNS function depending on physiological or pathological context. However, studies on the effects of acute alcohol on synaptic function in the hippocampus from IL-6 tg and CCL2-tg mice and their respective non-tg controls revealed covert neuroadaptive changes that resulted in an altered response to alcohol (Table 4). For example, although there was no difference in the magnitude of PTP and LTP in hippocampal slices from IL-6 tg or CCL2-tg mice compared to their respective non-tg controls under baseline conditions, exposure to acute alcohol (60 mM) depressed PTP and LTP in hippocampal slices from non-tg mice from both the IL-6 and CCL-2 lines, while PTP and LTP in hippocampal slices from the IL-6 tg and CCL2-tg hippocampus were resistant to this effect of acute alcohol [67,70]. Thus, the hippocampus from the IL-6 tg and CCL2-tg mice showed a similar resistance to the depressing effects of alcohol on LTP and PTP. Differences in the response to alcohol were also observed between IL-6 tg and CCL2-tg mice in the effects of alcohol on the fEPSP and PS. For example, 60 mM acute alcohol reduced the fEPSP and PS in hippocampal slices from non-tg mice from the IL-6 and CCL2 lines and in hippocampal slices from CCL2-tg mice, whereas in hippocampal slices from IL-6 tg mice the same dose of alcohol increased the fEPSP and PS [67,70]. 60 mM alcohol is a pharmacologically relevant dose that would produce severe intoxication in humans.

**Table 4.** Effects of alcohol on synaptic function in hippocampus.

| Measurement                   | 60 mM Alcohol vs. Baseline |         |        |         |
|-------------------------------|----------------------------|---------|--------|---------|
|                               | Non-tg                     | IL-6 tg | Non-tg | CCL2-tg |
| Synaptic transmission         |                            |         |        |         |
| -fEPSP                        | ↓                          | ↑       | ↓      | ↓       |
| -PS                           | ↓                          | ↑       | ↓      | ↓       |
| P-P synaptic plasticity       |                            |         |        |         |
| -fEPSP (PPF)                  | no Δ                       | no Δ    | no Δ   | no Δ    |
| -PS (PPR)                     | ↑                          | no Δ    | ↑      | no Δ    |
| Long-term synaptic plasticity |                            |         |        |         |
| -PTP                          | ↓                          | no Δ    | ↓      | no Δ    |
| -LTP                          | ↓                          | no Δ    | ↓      | no Δ    |
| Reference                     | [70]                       |         | [67]   |         |

↓ = decrease, ↑ = increase, no Δ = no difference.

A difference in response to alcohol was also observed in behavioral studies of alcohol actions. In one study of alcohol withdrawal hyperexcitability, IL-6 tg and CCL2-tg mice and their non-tg littermates were exposed to an acute, high dose of alcohol (4 gm/kg, i.p.), which initially causes sedation, but during the phase of declining blood alcohol levels, CNS hyperexcitability is produced. The hyperexcitability was measured by handling induced convulsions (HIC) [83,84]. The IL-6 tg mice showed significantly higher HIC scores than their non-tg controls, indicating greater hyperexcitability, whereas CCL2-tg and their non-tg mice showed similar HIC scores [70]. In behavioral tests for contextual fear conditioning, there were no significant differences between the CCL2-tg and non-tg mice under baseline conditions. Acute alcohol (1 gm/kg, i.p.) significantly impaired the non-tg mice but not the CCL2-tg mice in this behavioral test [67]. In contrast, in the rotorod test, which is considered primarily a cerebellar mediated behavior, CCL2-tg and non-tg mice show no difference in recovery from the effects of acute alcohol (2 gm/kg, i.p.) [67]. A similar result was obtained for the effects of acute alcohol (2 gm/kg, i.p.) on IL-6 tg and non-tg mice in the rotarod test (recovery time =  $176.2 \pm 9.3$  min for non-tg and  $171.2 \pm 9.0$  min for IL-6 tg).

Covert changes were also revealed in other studies of the IL-6 tg mice. Systemic exposure (i.p. injection) to a low dose of kainate or NMDA induced prominent seizures and lethality in IL-6-tg mice but not in the non-tg mice, which required a higher dose to produce such effects [69]. Also, basal plasma corticosterone levels were normal in IL-6-tg mice but, after restraint stress, abnormally increased levels were observed in the IL-6 tg mice compared to non-tg mice [85]. Thus, in addition to the detected neuroadaptive changes in baseline functions and behavior, covert neuroadaptive changes are produced by the chronic exposure to IL-6 and CCL2 and can be revealed within certain contexts. Such neuroadaptive changes could play an important role in pathophysiological conditions.

## 9. Conclusions

Although a large literature has demonstrated elevated CNS expression of cytokines and chemokines in CNS disease and injury, a relatively small number of studies have examined the consequences of the elevated expression at the synaptic level. The transgenic approach provides tools for such studies. Transgenic models that target astrocyte production of neuroimmune factors have enabled studies that provide a basic understanding of the synaptic consequence of persistent elevated expression of a specific neuroimmune factor by this CNS cell type. This information can facilitate identification of potential contributions of the neuroimmune factor to a more complex condition when multiple neuroimmune factors are expressed. This information may also be useful for identification of the actions/role of specific neuroimmune factors in CNS physiology. The astrocyte targeted transgenic models complement traditional approaches involving knock out (KO) models. In the KO model, all cell types are affected and, therefore, the KO models provide more global information about

the involvement of a specific neuroimmune factor in CNS development, function or dysfunction. One caveat to these models is that expression in the transgenic model or lack of expression in the KO model occurs over the lifespan of the animal, which could influence CNS development. It is unclear if or how potential development effects would impact studies in adult animals. However, emerging research on the actions of neuroimmune factors on CNS development is starting to provide answers to this question.

Overall, the studies of synaptic function in the hippocampus from the three transgenic lines revealed relatively few alterations. This result is consistent with the relative lack of neuropathology in the hippocampus of the transgenic mice at the ages studied, and raises the possibility that additional factors may be necessary when pathology is observed. Both similarities and differences were observed in the effects of the three neuroimmune factors on synaptic function, suggesting that similarities and differences exist in underlying mechanisms, and are likely to be reflected in the consequences of elevated expression under different pathological contexts.

Although only a limited number of neuroadaptive changes in synaptic function were identified under basal conditions, several experimental manipulations revealed that covert neuroadaptive changes were produced by elevated expression of the neuroimmune factors. These covert neuroadaptive changes may have been responsible for the apparent normalization of function under baseline conditions such that genotypic differences were not observed. The identification of covert actions illustrates the importance of physiological or pathological context in the consequence of cytokine or chemokine actions in the CNS. Both the identified and covert neuroadaptive changes resulting from increased astrocyte production of the neuroimmune factors could contribute to cognitive impairment in a pathological context.

The mechanisms and molecular targets underlying the neuroadaptive changes produced by IL-6, CCL2, and CXCL10 have yet to be elucidated. Studies to address these issues are an important future direction, and are essential for a more complete understanding of the actions and roles of IL-6, CCL2 and CXCL10 in CNS physiology and pathology. The level of expression, duration of exposure, presence of other neuroimmune factors, and biological context are all likely to be important variables, and their biological impact will also need to be resolved in future studies. Taken together, such information could reveal new targets for therapeutic intervention for a range of pathophysiological conditions that are associated with increased expression of IL-6, CCL2 and/or CXCL10 in the CNS.

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Article

# Astrocytic IL-6 Influences the Clinical Symptoms of EAE in Mice

Maria Erta <sup>1,2</sup>, Mercedes Giralte <sup>1,2</sup>, Silvia Jiménez <sup>1</sup>, Amalia Molinero <sup>1,2</sup>, Gemma Comes <sup>1,2</sup> and Juan Hidalgo <sup>1,2,\*</sup>

<sup>1</sup> Department of Cellular Biology, Physiology and Immunology, Animal Physiology Unit, Faculty of Biosciences, Universitat Autònoma de Barcelona, Bellaterra 08193, Spain; mariaerta@gmail.com (M.E.); merce.giralte@uab.es (M.G.); siljiba@gmail.com (S.J.); amalia.molinero@uab.es (A.M.); gemma.comes@uab.es (G.C.)

<sup>2</sup> Institute of Neurosciences, Universitat Autònoma de Barcelona, Bellaterra 08193, Spain

\* Correspondence: Juan.Hidalgo@uab.es; Tel.: +34-935-812-037; Fax: +34-935-812-390

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**Abstract:** Interleukin-6 (IL-6) is a multifunctional cytokine that not only plays major roles in the immune system, but also serves as a coordinator between the nervous and endocrine systems. IL-6 is produced in multiple cell types in the CNS, and in turn, many cells respond to it. It is therefore important to ascertain which cell type is the key responder to IL-6 during both physiological and pathological conditions. In order to test the role of astrocytic IL-6 in neuroinflammation, we studied an extensively-used animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), in mice with an IL-6 deficiency in astrocytes (Ast-IL-6 KO). Results indicate that lack of astrocytic IL-6 did not cause major changes in EAE symptomatology. However, a delay in the onset of clinical signs was observed in Ast-IL-6 KO females, with fewer inflammatory infiltrates and decreased demyelination and some alterations in gliosis and vasogenesis, compared to floxed mice. These results suggest that astrocyte-secreted IL-6 has some roles in EAE pathogenesis, at least in females.

**Keywords:** interleukin-6; astrocyte; EAE

## 1. Introduction

Interleukin 6 (IL-6) is a multifunctional four-helix bundle cytokine, originally identified as a B-cell differentiation factor in 1985 [1], which has been linked to numerous biological functions. It is now known to be one of the main cytokines controlling the immune system, and in addition, it acts as a coordinator between the nervous and endocrine systems. IL-6 plays an essential role in the central nervous system (CNS) in many physiological, inflammatory and disease conditions, being able to exert dual actions (for a review, see [2]). Although many cells in the CNS produce IL-6 [3], astrocytes are the main producer [2,4].

Multiple sclerosis (MS) is one of the most common inflammatory disorders of the CNS and a leading cause of disability in young adults. It is estimated that 2–2.5 million people are currently living with this demyelinating disease worldwide [5]. Its pathological hallmarks consist of local demyelination, inflammation and variable axonal destruction. Experimental autoimmune encephalomyelitis (EAE) [6] is a well-known animal model to study this inflammatory condition. Cytokines are key mediators in the pathogenesis of inflammatory lesions in CNS, presenting both helpful and harmful effects [7]. IL-6 plays a crucial role in MS as demonstrated by its presence in acute and chronic active plaques of MS patients [8]. In addition, it has been shown that IL-6-deficient mice are resistant to EAE [9–13] presumably because of a lack of differentiation of naive T cells into MOG-specific T helper cells producing IL-17 (Th17) and the subsequent reduction of their infiltration into the CNS [14], although the exact mechanism is not fully understood.

A thorough understanding of the role of the cellular context in this and other diseases is necessary to clarify the putative roles of IL-6 in the CNS [15,16]. We recently characterized the role of astrocytic IL-6 in normal (basal) conditions by using transgenic mice with astrocyte-specific IL-6 deficiency, showing effects on behavior and body weight in a sex-dependent manner [17,18]. The role of astrocytic IL-6 during pathological situations remained to be assessed, and here, we report the initial studies with EAE.

## 2. Materials and Methods

### 2.1. Animals

The generation of astrocyte-IL-6 KO (Ast-IL6 KO) and floxed littermate mice, which served as controls, was as described previously [17]. A number of studies have shown that GFAP is primarily expressed in astrocytes of the CNS, with minimal expression in peripheral regions [19]. All mice were kept under constant temperature with free access to food (Harlan global diet 2918) and water. Ethical approval for the use and experimentation of all mice in this study was obtained from the Animal and Human Experimentation Ethics Committee of the Universitat Autònoma de Barcelona (n<sup>o</sup> 4017, approved 3 September 2015).

### 2.2. EAE Induction and Clinical Evaluation

For the induction of EAE, two-month-old male and female mice were used. EAE was induced by active immunization with MOG<sub>35–55</sub> peptide. On Day 0, all mice, under isoflurane anesthesia, were injected subcutaneously into the hind flanks with an emulsion of 100 µL MOG<sub>35–55</sub> (3 mg/mL) and 100 µL Complete Freund's Adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 4 mg/mL *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI, USA). In addition, animals received an intraperitoneal injection of 500 ng pertussis toxin (Sigma-Aldrich, St. Louis, MO, USA), which was repeated two days later.

After immunization, mice were examined daily, weighed and scored for EAE. The EAE clinical score was assessed for each animal according to the following criteria: 0 = no signs of disease, 0.5 = partial loss of tail tonus, 1 = loss of tail tonus, 2 = moderate hind limb paraparesis, 2.5 = severe hind limb paraparesis, 3 = partial hind limb paralysis, 3.5 = hind limb paralysis, 4 = hind limb paralysis plus partial front leg paralysis, 4.5 = moribund/total paralysis and 5 = death. Finally, for each animal, we determined the time to disease onset (clinical score  $\geq 1$ ), time to peak disease, peak-score, cumulative score (sum of all scores from disease onset to Day 20) and grade of remission (difference between peak score and outcome).

Three independent EAE experiments were carried out (Table 1). Experiment 1 was carried out for 0–22 days post-immunization (dpi); Experiment 2 was carried out for 0–20 dpi; and Experiment 3 was carried out for 0–46 dpi. For each experiment, littermates were used. Female mice from 20–22 dpi were grouped before comparison to 46 dpi mice. Male Ast-IL-6 KO mice did not differ from male floxed mice at 20–22 dpi and, thus, were not examined at 46 dpi. In all cases, all surviving mice were euthanized at the indicated days post-immunization by decapitation. Spinal cords were immediately removed and fixed for 48 h in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry (IHC) and histochemistry (HC) analyses. Spinal cords from additional healthy female mice were processed in parallel.

**Table 1.** Number of mice per genotype, sex and experiment.

| Genotype    | Experiment 1 (0–22 dpi) |         | Experiment 2 (0–20 dpi) |         | Experiment 3 (0–46 dpi) |
|-------------|-------------------------|---------|-------------------------|---------|-------------------------|
|             | Males                   | Females | Males                   | Females | Females                 |
| Floxed      | 7                       | 3       | 11                      | 17      | 8                       |
| Ast-IL-6 KO | 3                       | 7       | 8                       | 8       | 8                       |

dpi, days post-immunization; Ast, astrocyte.

### 2.3. IHC and HC Analysis

Embedded paraffin tissues were cut into 8  $\mu\text{m}$ -wide sections in a microtome (Leica, Germany) and mounted in Superfrost slides (Thermo Scientific, Waltham, MA, USA).

Microglia were identified by lectin HC (tomato lectin from *Lycopersicon esculentum*, Sigma-Aldrich 1:500 in Tris-buffered saline (TBS) with 0.5% triton X-100 (TBS-t)). Astrocytes were identified by GFAP IHC (rabbit anti-Glial Fibrillary Acidic Protein from DakoCytomation Denmark, 1:1200 in blocking buffer). Lymphocytes were identified by CD3 IHC (rabbit anti-human CD3, Dako A0452, 1:100 in blocking buffer). Spinal cord sections were also stained with hematoxylin-eosin and with Luxol Fast Blue solution (LFB) (0.1%, overnight at 37 °C) and counterstained with Cresyl violet (0.1%, 1 min), to assess the number of cellular infiltrates and demyelination in grey matter, respectively.

Sections for IHC and HC were preincubated for 1 h with the blocking buffer (0.5% BSA in TBS-t) and then incubated with the primary antibodies (GFAP, CD3) or tomato lectin overnight at 4 °C, followed by 1 h at room temperature (RT) (GFAP, CD3) or at 37 °C (lectin). For CD3 IHC, a previous antigen retrieval step was performed with protease type XIV (Sigma P5147). After washing in TBS, sections were incubated with either horseradish peroxidase-coupled streptavidin (Vector Labs, Burlingame, CA, USA, 1:600, 1 h) for HC or biotinylated secondary antibody (Vector Labs, Burlingame, CA, USA, 1:300, 1 h at RT) followed by washes and horseradish peroxidase-coupled streptavidin (Vector Labs, Burlingame, CA, USA, 1:600, 1 h) for IHC. The immunoreactivity was visualized by using 0.033%  $\text{H}_2\text{O}_2$  in 0.5 mg/mL 3,3-diaminobenzidine-tetrahydrochloride (DAB) in Tris buffer (TB) for 4–30 min at room temperature. Reaction was stopped with TB, washed, dehydrated and mounted in DPX (distyrene-plasticiser-xylene) (Sigma, St Louis, MO, USA). Images at 100 $\times$  (GFAP) or 200 $\times$  (lectin, CD3) were taken in a bright field Nikon Eclipse 90i microscope (Nikon Instruments Europe BV, Amsterdam, The Netherlands) and acquired with a Nikon digital camera DXm1200F (Nikon Instruments Europe BV, Amsterdam, The Netherlands) and Nikon Act-1 version 2.70 software (Nikon Instruments Europe BV, Amsterdam, The Netherlands) from different brain areas and spinal cord. Finally, to quantify staining areas, intensity and the number of cells, images were analyzed using ImageJ software (NIH, Bethesda, MD, USA). Histological analyses were performed on at least two sections per mouse. Control sections for non-specific binding analysis (where primary antibody or tomato lectin was not used) were included routinely.

For GFAP IHC, the percentage of stained area (at 100 $\times$ ) was measured in different regions of the spinal cord from EAE-induced mice. Six to twelve images per animal were examined. White and grey matter areas of spinal cords were also analyzed (12 images per animal and area). A threshold was set for each area to better define cells from tissue background. Because tomato lectin HC stains both microglia and vessels, the quantification of staining using ImageJ was supplemented with manual counts of microglial cells showing a basal (ramified), reactive or fully-activated (round) morphology and of the number of vessels; these were assessed in the same regions as for GFAP IHC analysis, at 200 $\times$ . In addition, total numbers of microglia/macrophage infiltration areas in spinal cord were recorded. For CD3 IHC, the percentage of stained area was measured in the spinal cord (10 images per area and animal). In LFB/Cresyl violet staining, total numbers of cellular infiltrates in grey matter of spinal cord were counted, and afterwards, a color deconvolution plugin from ImageJ software was used in order to separate colors from LFB and Cresyl violet to be able to quantify the demyelination by analyzing the percentage of LFB-stained area in spinal cord. The threshold set gave 100% of area of the spinal cord covered by LFB staining for healthy female mice. To standardize, the same threshold was used for female and male mice. Demyelination and a decrease of the area covered by LFB caused by EAE were readily detected in both male and female mice.

### 2.4. Statistical Analysis

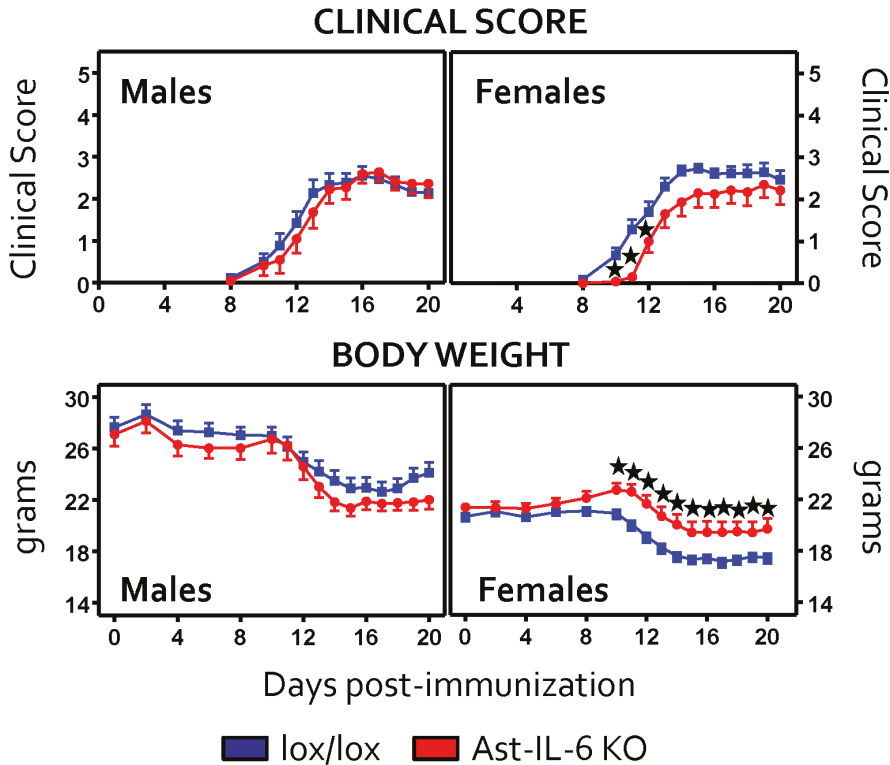
Values in text and figures are shown as the mean  $\pm$  standard error of the mean (SEM). Statistical analyses were performed using SPSS statistical software (version 17.0 for Windows, SPSS Inc., Chicago, IL, USA).  $p \leq 0.05$  was considered significant in all analyses.

For clinical evaluations and body weight changes, we used the general linear model (GzLM) for repeated measures, the generalized estimated equations test (GEE), with genotype (floxed *vs.* Ast-IL-6 KO mice) and sex as the main factors. The *post-hoc* Student *t*-test or the Mann–Whitney *U*-test was used to identify significant differences between Ast-IL-6 KO and floxed animals at specific time periods. For the remaining variables analyzed, two-way ANOVA (with genotype and sex as the main factors) or GzLM was used for each sex separately followed by *post-hoc* tests when appropriate.

### 3. Results

#### 3.1. Lack of Astrocytic IL-6 Alters the Clinical Course of EAE in Ast-IL-6 KO Mice and Ameliorates EAE Symptomatology in a Sex-Dependent Manner

Both genotypes showed the prototypical ascending paralysis course with body weight loss (Figure 1). The clinical score and the body weight changes observed following MOG<sub>35–55</sub> immunization up to 20 dpi in the Ast-IL6 KO and floxed mice are shown in Figure 1. Ast-IL6 KO females showed a significantly reduced clinical score and increased body weight with respect to female floxed mice, a genotypic difference that was not observed in the male mice. Average incidence of the disease ranged from 90% to 100%, and there were no significant differences in mortality rate (Table 2).



**Figure 1.** Clinical course of EAE, in both floxed ( $n = 18$  males, 28 females) and Ast-IL-6 KO ( $n = 11$  males, 23 females) mice up to 20 dpi. Results are the mean  $\pm$  SEM of data pooled from Experiments 1–3 (Days 0–20 dpi; Table 1).  $\star$   $p$  at least  $<0.05$  versus floxed mice at specific days following a *post-hoc* analysis.



Table 2. EAE disease.

| EAE          | Females        |              | Males          |              |
|--------------|----------------|--------------|----------------|--------------|
|              | Ast-IL-6 KO    | Floxed       | Ast-IL-6 KO    | Floxed       |
| Incidence    | 21/23          | 28/28        | 11/11          | 17/18        |
| Mortality    | 3/23           | 0/28         | 0/11           | 0/18         |
| Day of onset | 13.38 ± 0.59 * | 11.57 ± 0.29 | 12.27 ± 0.52 * | 11.65 ± 0.49 |

Results shown are pooled data from Experiments 1–3 separated by sex and genotype. For each animal, time to disease onset was defined by a clinical score  $\geq 1$ . For statistical analysis, a two-way ANOVA for genotype (floxed *vs.* Ast-IL-6 KO mice) and sex as the main factors was performed. \*  $p < 0.05$  *vs.* floxed mice.

When the day of disease onset was analyzed using sex and genotype as the main factors, a significant genotypic difference was observed for both sexes (Table 2). In contrast, the peak score, cumulative score and grade of remission at 20 dpi were not different between genotypes for either sex (Table 3). These results suggest that while the disease is delayed and the severity is somewhat lower at the early stages in female mice, it is not affected by astrocytic IL-6 deficiency later on.

Table 3. EAE clinical course.

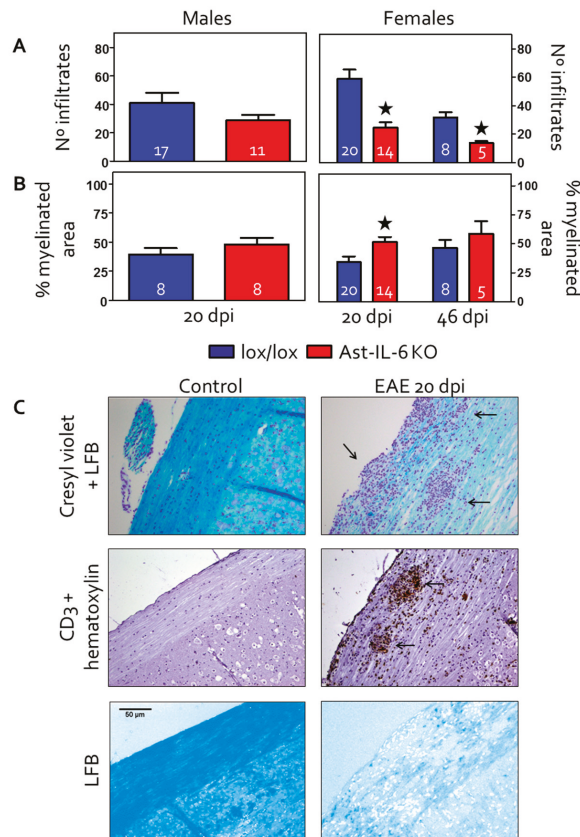
| Clinical Course    | Females (0–20 dpi)   |                 | Females (46 dpi)    |                | Males (0–20 dpi)     |                 |
|--------------------|----------------------|-----------------|---------------------|----------------|----------------------|-----------------|
|                    | Ast-IL-6 KO (n = 15) | Floxed (n = 20) | Ast-IL-6 KO (n = 8) | Floxed (n = 8) | Ast-IL-6 KO (n = 11) | Floxed (n = 18) |
| Time of peak score | 14.30 ± 0.53         | 14.80 ± 0.49    | 22.5 ± 2.46 *       | 15.5 ± 1.92    | 15.18 ± 0.80         | 13.76 ± 0.6     |
| Peak score         | 2.80 ± 0.23          | 3.45 ± 0.16     | 3.68 ± 0.50         | 3.18 ± 0.23    | 3.18 ± 0.18          | 3.14 ± 0.12     |
| Cumulative score   | 19.40 ± 2.55         | 24.16 ± 1.32    | 88.87 ± 21.84       | 72.18 ± 10.35  | 20.32 ± 1.64         | 22.61 ± 1.34    |
| Grade of Remission | 0.64 ± 0.13          | 0.91 ± 0.11     | 1.00 ± 0.34         | 1.43 ± 0.27    | 0.82 ± 0.26          | 20.32 ± 1.64    |

Results shown are pooled data from Experiments 1–3 separated by sex and genotype. For simplicity, data from 20–22 dpi were grouped and are referred to as 20 dpi. For each animal, we determined time to peak disease, peak score, cumulative score (sum of all scores from disease onset to Days 20 and 22 combined or 46), and the grade of remission (difference between peak score and outcome). Results are the mean  $\pm$  SEM. For statistical analysis at 0–20 dpi, a two-way ANOVA for genotype (floxed *vs.* Ast-IL-6 KO mice) and sex as the main factors was performed. For the females at 46 dpi, a one-way ANOVA for genotype (floxed *vs.* Ast-IL-6 KO mice) was performed. \*  $p < 0.05$  *vs.* floxed mice.

### 3.2. Reduced Cellular Infiltrates and Demyelination in the Spinal Cord of Ast-IL-6 KO Female Mice

The number of inflammatory infiltrates in the longitudinal lumbar-cervical spinal cord of control and EAE-induced animals was assessed in females and males at 20–22 dpi (for simplicity, data from 20–22 dpi were grouped and are referred to as 20 dpi). Females were also assessed at 46 dpi (Figure 2A,C). The total number of infiltrates in white matter was counted in a Cresyl violet/Luxol Fast Blue staining and in a CD3 IHC counterstained with hematoxylin; a mean value of both stainings was then calculated for each animal and used for statistical analysis. As shown in Figure 2A, there is a significant decrease in the number of infiltrates in Ast-IL6 KO females at both 20–22 and 46 dpi compared to floxed female mice. Ast-IL-6 KO males did not show a significant difference compared to floxed males (Figure 2A).

Demyelination in spinal cord white matter was assessed measuring the percentage of Luxol Fast Blue-stained area (Figure 2B,C). Healthy animals (0 dpi) had 100% area covered by LFB staining. Following EAE, Ast-IL-6 KO females showed a significantly lower Luxol Fast Blue-stained area, indicating greater demyelination, compared to floxed female mice at 20 dpi, but not at 46 dpi (Figure 2B). Ast-IL-6 KO males did not show a significant difference in Luxol Fast Blue-stained area compared to floxed males (Figure 2B).



**Figure 2.** Assessment of the total number of cellular infiltrates (A) and demyelination (B) in the longitudinal lumbar-cervical spinal cord white matter from EAE-induced animals at 20 dpi (for both females and males Ast-IL6 KO and floxed mice) and 46 dpi (females). Number of mice per group as indicated.  $\star p < 0.05$  between Ast-IL6 KO and floxed mice. (C) Representative sections from female mice showing infiltrates (arrows) in spinal cord stained with Cresyl violet (top) and CD3 (middle) in non-immunized (control) and immunized (20 dpi) floxed mice. The bottom panel shows the demyelination of the spinal cord as revealed by Luxol Fast Blue (LFB) staining following color deconvolution. All images at magnification 100 $\times$ .

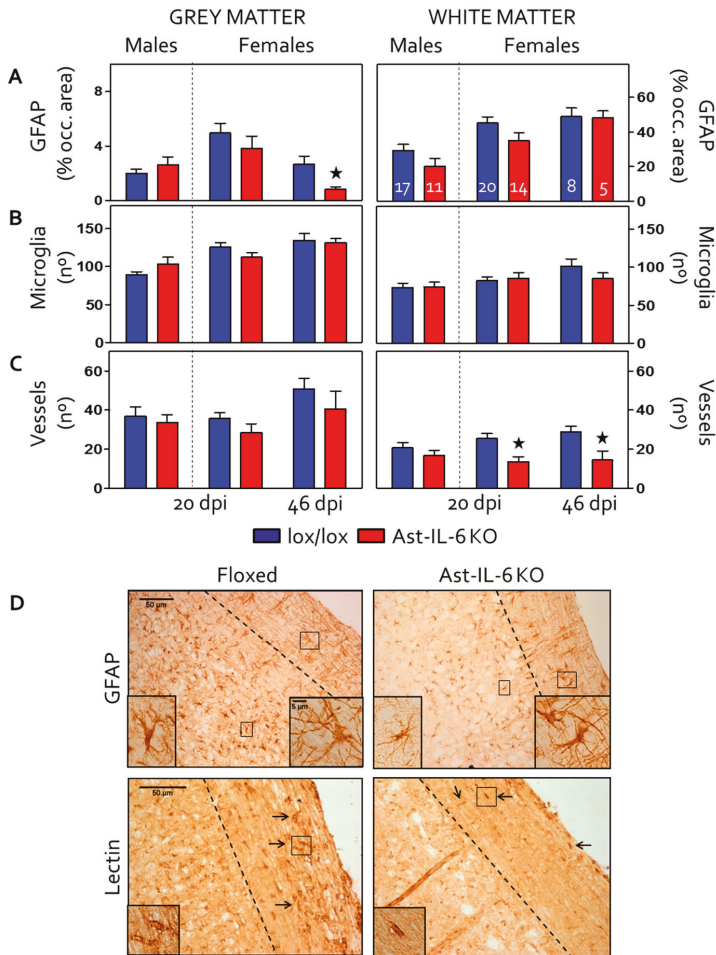
### 3.3. Reduced Gliosis and Vasogenesis in Spinal Cord and Brain of EAE-Induced Ast-IL-6 KO Females

Gliosis was evaluated by GFAP (astrocytes) and lectin (microglia) staining in the spinal cord of the EAE-induced mice (Figure 3A,B,D). The area occupied by GFAP immunostaining was measured in both the grey and white matter (Figure 3A). A significant decrease in GFAP immunostaining in grey matter was observed in Ast-IL-6 KO female mice compared to floxed female mice at 46 dpi, accompanied by a less reactive morphology of astrocytes (Figure 3D, top, left inset). No genotypic difference in GFAP immunostaining was observed for females or males in grey matter at 20 dpi or in the white matter for females and males at the times studied.

Regarding microgliosis in spinal cord (Figure 3D, bottom), quantification of the occupied area did not show significant differences between genotypes (data not shown). As quantification of lectin staining is not able to separate microglia from blood vessels, the number of microglia was counted. No significant genotypic difference in the number of microglia was observed for either females or

males in both the grey and white matter of the spinal cord at the days studied (Figure 3B). These results indicated that astrocytic IL-6 deficiency did not impact microgliosis in either the grey or white matter of the spinal cord.

Finally, vasogenesis was analyzed by counting lectin-stained vessels in the spinal cord of EAE-induced mice (Figure 3D, bottom, arrows and insets). In female mice, the number of vessels in the white matter was significantly reduced in Ast-IL-6 KO mice compared to floxed mice at both 20–22 and 46 dpi (Figure 3C). No genotypic difference was observed for the number of vessels in the white matter for males or for the number of vessels in the grey matter for females and males at the times studied.



**Figure 3.** (A–C) Results from GFAP and lectin stainings in the spinal cord (grey and white matter) of EAE-induced animals at 20 dpi (for both females and males Ast-IL6 KO and floxed mice) and 46 dpi (females). Number of mice per group as indicated. GFAP overall immunostaining (A) and the number of lectin-positive microglia (B) and vessels (C) are shown. \*  $p < 0.05$  vs. floxed mice. (D) Representative GFAP at 100 $\times$  (top) and lectin at 150 $\times$  (bottom) stainings of 46 and 20 dpi females, respectively. Arrows indicate vessels. The discontinuous line separates grey matter (left) from white matter (right). All inserts are at 400 $\times$ ; at the top they, show astrocytic morphology in both grey and white matter, and at the bottom, they show a vessel.

#### **4. Discussion**

IL-6 is implicated in the pathogenesis of autoimmune disorders, such as MS in humans [20,21]. A critical role of IL-6 in the animal model of MS, EAE, has been demonstrated by a number of studies: systemic IL-6 KO mice are resistant to EAE [9–13], and neutralization of IL-6 with antibodies leads to a reduced disease [22], by not well-defined mechanisms. Moreover, studies have demonstrated that the transgenic expression of IL-6 in the CNS by viral systems reduces EAE [23] and that the systemic administration of IL-6 also reduces the clinical symptoms in a viral model of EAE [24]. Thus, IL-6 can potentiate, but also inhibit EAE, reflecting the complexity of its actions. Key questions remain, however, including the identity of the key cell types that produce and respond to IL-6 and whether the critical actions of IL-6 are peripheral or central.

Because the production of IL-6 during the course of EAE arises from diverse cellular sources both in the periphery and in the CNS, the specific contribution of each source of IL-6 to the development of the disease needs to be established. We have previously demonstrated a role of CNS IL-6 in regulating EAE, because mice expressing IL-6 only by astrocytes (GFAP-IL6-IL-6 KO mice) were capable of developing the atypical EAE known to occur in GFAP-IL6 mice [13]. Furthermore, in adoptive transfer experiments, EAE is less severe in IL-6KO mice than in wild-type mice, which suggests that IL-6 locally mediates the disease in the CNS [10]. Thus, although EAE has always been considered a disease mostly induced peripherally, it seems that CNS IL-6 may also play an important role. Because astrocytic IL-6 plays a major role in neuroinflammation [25,26] and because astrocytes are the most abundant cell in the CNS, astrocytic IL-6 seemed to be an excellent candidate to examine as the key regulator of EAE in the CNS.

We have previously shown that compared to floxed mice, Ast-IL-6 KO mice exhibit a number of altered behaviors under normal (basal) conditions, including changes in activity, anxiety and learning; a prosurvival role of astrocytic IL-6 is also apparent [17,18]. Here, we present results from studies involving a neuropathological condition, MOG<sub>35–55</sub>-induced EAE. In contrast to results in systemic IL-6 KO mice, astrocytic-specific IL-6 deficiency is unable to prevent typical signs of EAE induction and has no prominent neuropathologic effects. However, astrocyte IL-6 KO mice did show significant delays of the onset of the EAE, at least in female mice, ameliorating the clinical signs in the early stages of EAE.

Autoreactive T-cells can result in inflammatory demyelination of the CNS, and knowing that the frequency of Tregs in MS patients is unchanged from controls [27] (although their function is impaired) could be a clue to the decreased demyelination seen only in Ast-IL-6 KO females, the only group that presented a decrease in T lymphocyte infiltration. Thus, a lower EAE score at early stages could be due to an initial impaired T cell infiltration of the CNS. This possibility is consistent with our results showing that Ast-IL-6 KO female (but not male) mice had a decreased number of infiltrates in the spinal cord and lower scores for clinical signs. However, it is important to note that we only carried out Cresyl violet and CD3 immunostaining for T cells, so we cannot rule out that a change in T cell subpopulations is responsible for the different clinical signs observed in Ast-IL-6 KO female mice. IL-6 has a major role in Th17 cell differentiation from naive CD4<sup>+</sup> T-cells (reviewed in [28]), particularly in the EAE model [14,29]. EAE-resistant IL-6 KO mice show a deficiency in Th17 cell infiltrates in the CNS [30]. When responsiveness to IL-6 is experimentally eliminated in T helper cells, mice show resistance to EAE induction, as the IL-21 pathway is intact, but not active in the absence of IL-6 signaling [31]. Th17 cells produce IL-17 (among other cytokines), which enhances IL-6 production by astrocytes, which, in turn, induces the differentiation of Th17 cells in a positive feed-back loop between IL-17 and IL-6 via activation of NF- $\kappa$ B and STAT-3 [32,33]. This loop may be compromised in our Ast-IL-6 KO animals, where astrocyte production of IL-6 has been eliminated. However, since Ast-IL-6 KO mice only show a delay in EAE, but are not resistant to EAE, we can speculate that this astrocytic loop is not necessary for the development of the disease; and/or that neuronal, endothelial and microglial IL-6 could instead allow this positive feedback between IL-17 and IL-6. In order to test these hypotheses, a detailed study of the exact lymphocytic population present in the infiltrates would be important, particularly with further backcrossing to C57/Bl6 mice.

Somewhat surprisingly, considering the importance of astrocytic IL-6 in neuroinflammation [2,4,25,26], we did not observe dramatic effects of astrocytic IL-6 deficiency on either astrocytes or microglia at the times studied. This result makes it unlikely that changes in glial cell reactivity are underlying the differences in clinical signs between Ast-IL-6 KO mice and floxed mice. Nevertheless, overall staining and cell morphologies are limited approaches, and more detailed studies are needed to assess other roles of glial cells (other than IL-6 production by astrocytes) in clinical signs of EAE. Finally, regarding the number of vessels, we observed a decrease in Ast-IL-6 KO mice, which is in agreement with results in mice overexpressing IL-6, which show extensive revascularization, both in basal conditions [25,26] and after an injury [34], indicative of a role of IL-6 in vasogenesis. Other studies also support the idea that IL-6 promotes vasogenesis [35]. Probably, this is secondary to the number and/or type of inflammatory cells present in the spinal cord, since no differences were observed in male mice.

The extent of the reduction of IL-6 in astrocytes of the Ast-IL-6 KO mice *in vivo* has yet to be determined. However, studies of cultured astrocytes from the Ast-IL-6 KO mice demonstrated that the astrocytes are deficient in IL-6 production. In these studies, analysis of culture supernatant after 24 h of stimulation (10 ng/mL of LPS and 10 ng/mL of INF- $\gamma$ ) showed that astrocytes from floxed mice produced approximately 13 ng/mL of IL-6, whereas astrocytes from Ast-IL-6 KO mice only produced 2 ng/mL IL-6 [17,18]. Regardless of the extent of the astrocytic IL-6 deficiency in the Ast-IL-6 KO mice, a delay in onset to clinical symptoms was evident in females, including less demyelination at 20–22 dpi in accordance with the lower clinical scores. However, this was a transient effect, and sometime after 20–22 dpi, the EAE was similar in both genotypes (as indicated by the lack of significant differences in demyelination at 46 dpi), indicating the astrocytic IL-6 no longer played a role.

## 5. Conclusions

In conclusion, we have shown that lack of astrocytic IL-6 is not sufficient to prevent EAE disease, but it is able to delay the disease and to ameliorate clinical scoring and the inflammatory milieu in female mice. These results support the idea that the local CNS production of IL-6 is important in this disease. Several interesting questions remain to be addressed. For example, due to the delayed onset of clinical signs of disease in Ast-IL-6 KO females, it will be important to analyze in detail the priming and inflammatory infiltrates in the CNS of female and male Ast-IL-6 KO mice and their floxed controls. Furthermore, studies of males at later dpi may reveal genotypic differences at later stages. Moreover, a comparison of the immunized Ast-IL-6 KO and floxed mice with a CFA immunized control group could reveal specific EAE effects in Ast-IL-6 KO mice. Future studies will address these and other relevant issues relative to the role of astrocytic IL-6 in the EAE.

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**Author Contributions:** M.E., M.G., S.J., A.M. and G.C. were involved in several aspects of the experiments carried out. M.E. led most of the experimental work. J.H. conceived of the experiments. M.E. and J.H. wrote the paper.

**Conflicts of Interest:** The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; nor in the decision to publish the results.

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Review

# The Effects of Hypoxia and Inflammation on Synaptic Signaling in the NS

Gatambwa Mukandala, Ronan Tynan, Sinead Lanigan and John J. O'Connor \*

UCD School of Biomolecular and Biomedical Science, UCD Conway Institute of Biomolecular and Biomedical Research, Belfield, Dublin 4, Ireland; Gatambwa.mukandala@ucdconnect.ie (G.M.); ronan.tynan@ucdconnect.ie (R.T.); sinead.lanigan@ucdconnect.ie (S.L.)

\* Correspondence: john.oconnor@ucd.ie; Tel.: +353-1-716-6765

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**Abstract:** Normal brain function is highly dependent on oxygen and nutrient supply and when the demand for oxygen exceeds its supply, hypoxia is induced. Acute episodes of hypoxia may cause a depression in synaptic activity in many brain regions, whilst prolonged exposure to hypoxia leads to neuronal cell loss and death. Acute inadequate oxygen supply may cause anaerobic metabolism and increased respiration in an attempt to increase oxygen intake whilst chronic hypoxia may give rise to angiogenesis and erythropoiesis in order to promote oxygen delivery to peripheral tissues. The effects of hypoxia on neuronal tissue are exacerbated by the release of many inflammatory agents from glia and neuronal cells. Cytokines, such as TNF- $\alpha$ , and IL-1 $\beta$  are known to be released during the early stages of hypoxia, causing either local or systemic inflammation, which can result in cell death. Another growing body of evidence suggests that inflammation can result in neuroprotection, such as preconditioning to cerebral ischemia, causing ischemic tolerance. In the following review we discuss the effects of acute and chronic hypoxia and the release of pro-inflammatory cytokines on synaptic transmission and plasticity in the central nervous system. Specifically we discuss the effects of the pro-inflammatory agent TNF- $\alpha$  during a hypoxic event.

**Keywords:** hypoxia; TNF- $\alpha$ ; adenosine; HIF-1 $\alpha$ ; hippocampus; long-term potentiation; prolyl hydroxylase inhibitor

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## 1. Introduction

In the central nervous system, hypoxia occurs when there is an inadequate supply of oxygen to neuronal tissue. During acute hypoxia multiple oxygen sensors are deployed allowing neurons to adapt to the response. These responses to hypoxia include synaptic signaling decreases usually as a result of anaerobic metabolism changes whilst chronic hypoxia may give rise to more severe perturbations of synaptic transmission and the activation of transcription factors that regulate oxygen homeostasis [1]. Different neurons adapt to a decreased oxygen supply to the brain in many ways, reflecting the diverse role of neuronal functions and also the extent of the hypoxia experienced. It is now known that an hypoxic event in brain tissue can cause ATP to drop by as much as 90% in less than 5 min. Additionally, oxygen-sensitive ion channels including Na<sup>+</sup> and K<sup>+</sup> are activated bringing about changes in excitation and inhibition of neuronal and glial cells [2]. Depolarisation of cells may also take place causing the uptake of Na<sup>+</sup> and Cl<sup>-</sup> into cells followed by passive influx of water, resulting in swelling and oedema [2]. Hypoxic insults may also activate voltage-gated Ca<sup>2+</sup> and K<sup>+</sup> ion channels and glutamate transporters, eventually causing excess glutamate to spill into the synaptic regions causing excitotoxicity. On the other hand, many of the long-term hypoxic responses are mediated by hypoxia inducible factors (HIF), such as HIF-1 $\alpha$  [3,4]. HIF-1 $\alpha$  is a universally expressed transcriptional



mediator of the hypoxic response that is degraded in an oxygen-dependent manner. Under normoxic conditions, HIF-1 $\alpha$  has a half-life of approximately 8 min due to hydroxylation by prolyl hydroxyl domains (PHDs) [5]. These PHDs exist in three different isoforms, PHD1, PHD2, and PHD3 and all require oxygen, iron, ascorbate and 2-oxoglutarate, a product of the oxygen dependent Krebs cycle, to hydroxylate HIF-1 $\alpha$ . Under hypoxic conditions the Krebs cycle is inhibited leading to a reduction in 2-oxoglutarate, preventing the binding of PHDs to the targeting proline domains [4,6]. During hypoxia, the HIF-1 $\alpha$  protein stabilizes allowing it to recruit transcriptional co-activators, which are blocked during normal conditions via factor inhibiting HIF (FIH) [7]. This complex then permits for the transcription of hypoxia-related proteins through binding of the hypoxic responsive element (HRE). HRE binding induces the expression of genes, such as erythropoietin, vascular endothelial growth factor and insulin growth factor. These all play a neuroprotective role in response to the hypoxic insult.

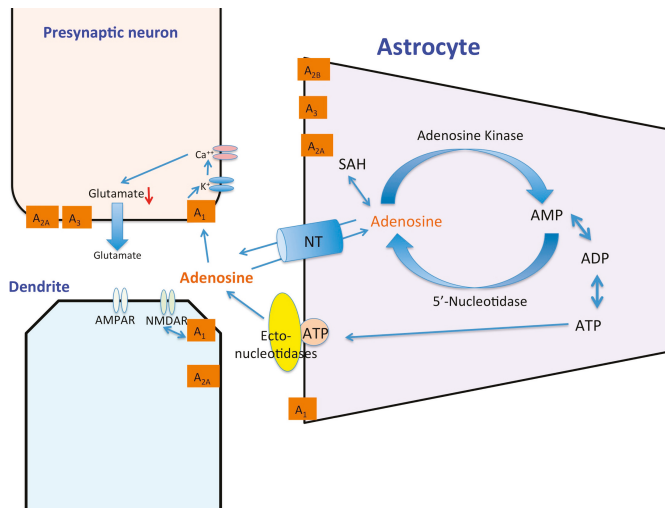
These acute and chronic responses to hypoxia are clearly manifested during ischemic events in the brain. An example of one such event with a hypoxic component is stroke, which is caused by a reduction in blood flow as a result of an obstruction or rupture of blood vessels within the brain and may cause both acute and chronic episodes of hypoxia. This leads to complex pathological changes taking place, which may lead to tissue necrosis through increased inflammation and oxygen deprivation [8]. During an ischemic stroke the eventual restriction of oxygen in the brain due to an obstruction leads to a cascade of events including hypoxia, increased expression of pro-inflammatory cytokines like tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin-1beta (IL-1 $\beta$ ), as well as increased release of the excitatory neurotransmitter glutamate [9]. In this review we will discuss how hypoxia and the release of pro-inflammatory cytokines can effect synaptic transmission and plasticity in the central nervous system (CNS).

## 2. Hypoxia and Synaptic Signaling

Synaptic transmission in the CNS requires approximately 30% to 50% of cerebral oxygen. Therefore many of the changes in the CNS related to acute hypoxia stem from modifications of synaptic excitation and depression. The responses to hypoxia, which occur within seconds, most likely do not involve a role for HIF-1 $\alpha$  stabilization. Additionally, upon re-oxygenation after a short period, synaptic transmission can recover to 100% in many brain regions [10]. This decrease in synaptic signaling during acute hypoxia is thought to protect some neurons during ischemic events. Adenosine is one of many neurotransmitters, which plays a vital role in the neuroprotective response to hypoxia [11]. Adenosine A<sub>1</sub> receptors (A<sub>1</sub>Rs), in particular, play a part in altering neurotransmitter release [12] and have wide expression levels throughout the CNS [13]. This inhibitory neuromodulation by A<sub>1</sub>Rs is coupled to inhibitory G<sub>i</sub> or G<sub>o</sub> containing G-proteins [14]. Activation of the receptor stimulates adenylyl cyclase, activates inwardly rectifying K<sup>+</sup> channels, thus inhibiting Ca<sup>2+</sup> channels and activation of phospholipase C. This inhibits the release of a number of neurotransmitters including glutamate, dopamine, serotonin and acetylcholine thus making it the primary neuroprotective receptor. Adenosine forms through the enzymatic catabolism of adenosine triphosphate (ATP) into adenosine monophosphate (AMP), which then is broken down by ecto'5 nucleotidases into adenosine (see Figure 1). Adenosine kinase is mainly responsible for the removal of adenosine via phosphorylation to AMP [15]. Under hypoxic conditions when there is a build-up of adenosine in the extracellular space, hypoxia induced factors such as HIF-1 $\alpha$  also cause an increase in the ecto'5 nucleotidases CD73, allowing for a breakdown of extracellular ATP into adenosine [16,17].

It is now known that during hypoxia, HIF-1 $\alpha$  inhibits the equilibrative nucleoside transporters ent-1/2 located on the membranes of neurons and glia preventing adenosine reuptake into the neuronal cell [18]. Extracellular adenosine binds to A<sub>1</sub>Rs located on both the postsynaptic and presynaptic membranes. Postsynaptic A<sub>1</sub>R activation inhibits the activation of glutamatergic N-methyl-D-aspartate receptors (NMDARs) and adenosine binding to A<sub>1</sub>Rs located presynaptically [14]. Inhibition of neurotransmitter release can be suppressed by the addition of an A<sub>1</sub>R selective inhibitor, such as 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), suggesting that adenosine binding is necessary for the

reduction of post synaptic potentials [19]. It has also been shown that the A<sub>1</sub>R binding of adenosine inhibits NMDA receptor activation [20]. Creation of knockout mice with the deletion of presynaptic A<sub>1</sub>Rs, uncovered the neuroprotective role that adenosine receptor binding plays in the hypoxic response [21]. Synaptic depression of the excitatory post-synaptic potential (EPSP) was attenuated allowing activation of glutamatergic NMDA receptors and increasing the likelihood for excitotoxicity. More importantly decreased extracellular levels of adenosine have been shown to lead to a loss of hypoxia-induced neuroprotection after repeated exposure to hypoxia [22].



**Figure 1.** The effects of hypoxia on adenosine release in the CNS. Hypoxia causes a breakdown of extracellular ATP and AMP along with activation of membrane-bound transporters such as ectonucleotidases, leading to a build-up of extracellular adenosine. Adenosine binds presynaptically to A<sub>1</sub>Rs attenuating voltage dependent calcium channel (VDCC) function and thus neurotransmitter release and also binds postsynaptically to A<sub>1</sub>Rs receptors inactivating glutamatergic NMDARs. Adenosine is released from astrocytes in response to chronic hypoxia.

The depression of synaptic transmission in longer term hypoxia goes beyond a neuroprotective role. For example during longer duration hypoxia, nicotinamide adenine dinucleotide phosphate-oxidase oxidase production of reactive oxygen species (ROS) such as superoxides by microglial complement receptor 3 can activate protein phosphatase 2A (PP2A), which causes the internalization of postsynaptic  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) through serine-threonine dephosphorylation [23]. This is similar to the discovery that the oxygen sensing *C. elegans* protein *egl-9*, which regulates HIF in an oxygen-dependent manner can also regulate *C. elegans* glutamate receptor-1 (GLR-1) trafficking through the generation of isoform-specific transgenes which interact with the GLR-1 promoter [24,25]. In normoxic conditions, *egl-9* binds to Lin-10 preventing its phosphorylation, this complex then allows for the movement of glutamate receptors to the synapse. Under hypoxic conditions, Lin-10 is phosphorylated, thus preventing the formation of the EGL9/Lin-10 complex leading to a lack of synaptic GluR1 receptors [26].

One particular form of hypoxia, chronic intermittent hypoxia (CIH) may have specific detrimental effects on CNS function. CIH can lead to the over-activation of NMDARs, leading to an overload of intracellular Ca<sup>2+</sup> and a dephosphorylation of extracellular signal-regulated kinases (ERK) [27]. The CA1 region of the hippocampus is thought to be selectively vulnerable to CIH damage due to the high density of glutamate receptors located on its pyramidal neurons [28]. CIH also leads

to a reduction in the levels of the transcription factor cAMP response element-binding protein (CREB) in its phosphorylated form [29]. This reduction in activated CREB leads to a lowering of CREB transcriptional targets, such as brain-derived neurotrophic factor (BDNF), causing cognitive dysfunction [30]. The CIH-induced cognitive dysfunction was shown to be repaired through exogenous application of BDNF to the hypoxic cell [30]. Perinatal hypoxic events may also lead to increases in excitability in hippocampal regions. These events usually occur after asphyxia events just after birth and can lead to long term synaptic changes. Changes in excitability in some local brain regions such as the CA1 region have also been noted [31]. The pursuant neonatal seizures may be related to the phosphorylation of the AMPA GLUA1 receptors on serine 183 and serine 845. This may enhance AMPA receptor excitatory post synaptic currents (EPSCs) which allows for a decrease in the percentage of silent synapses and an increase in AMPA receptor function [32]. This loss of silent synapses is thought to be the mechanism, which attenuates synaptic plasticity in adult life [33]. In critical cases of hypoxia-re-oxygenation the brain loses the ability to form new memories. This anterograde amnesia is decoupled from the hippocampus and its primarily caused by adenosine up-regulation of caspase 1 and then IL-1 $\beta$  in the amygdala [34]. These effects were shown to last up to five hours after re-oxygenation with caspase inhibitors, such as YVAD-CMK, able to shorten the recovery time [34]. The links of hypoxia to cognitive disorders, as well as ability to cause neuronal apoptosis through hyper-excitability, displays the importance of understanding hypoxia and preventing its long-term effects.

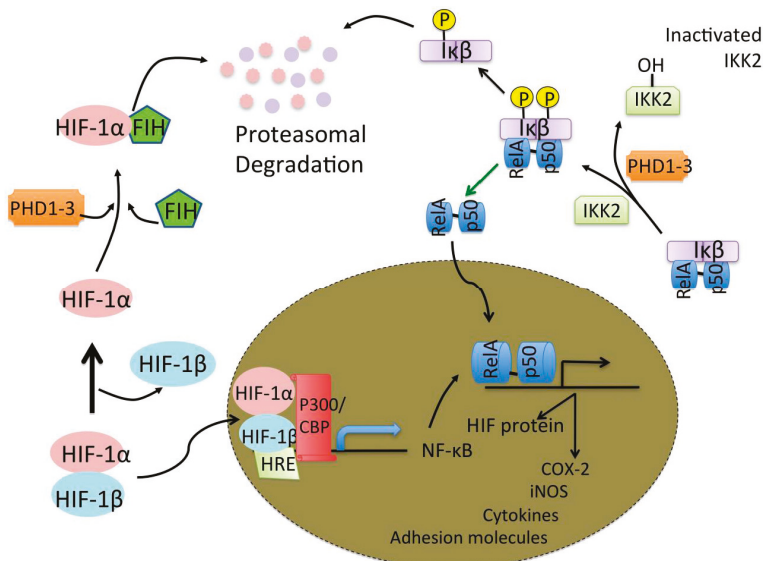
### 3. Hypoxia and Synaptic Plasticity

As previously mentioned, hippocampal neuron exposure to hypoxia may lead to cognitive deficits due to synaptic plasticity impairments [35]. Many studies have investigated the relationship between oxygen deprivation and synaptic plasticity. Early studies indicated that brief periods of hypoxia could disrupt long-term potentiation (LTP) in the CA1 hippocampus and that this effect could be reproduced with brief application of adenosine prior to the induction of LTP [36–38]. It was later discovered that a brief anoxic episode, as opposed to hypoxia, applied to brain slices, could generate a new type of LTP although still voltage-, NMDA- [39], protein kinase C (PKC)- and NO-dependent [40–42]. It is proposed that it is the re-oxygenation and not initial de-oxygenation of neurons and the subsequent high concentration of glutamate that in fact causes the excessive activation of NMDARs and subsequent large influx of Ca<sup>2+</sup> [43]. It has also been shown that chemically-induced hypoxia with the use of PHD inhibitors, and thus hypoxia mimetics, whilst having no effect on synaptic signaling at low concentrations *per se*, could inhibit LTP in the hippocampus [44,45]. Application of the iron chelator deferoxamine mesylate (DFO) or dimethylxaloglycine (DMOG), both non-specific pharmacological inhibitors of PHD, and thus increasers of HIF-1 $\alpha$  expression [46] could impair LTP in the CA1 hippocampus [44,45,47]. Interestingly the application of DMOG to the dentate gyrus region of hippocampal slices did not impair LTP [29]. It is believed that these effects of PHD inhibitors are not HIF-dependent. There is also increasing evidence for a role for CIH in synaptic plasticity and specifically LTP. Initial reports in early 2000 demonstrated that CIH treated animals demonstrated impaired LTP in isolated rat hippocampal slices [48,49]. More recently two reports have put forward evidence for a role for BDNF in this impairment [30,50]. They found that application of BDNF reversed the IH-induced impairment of LTP. In our own laboratories we have implicated a role for PHDs in this inhibition of LTP by intermittent hypoxia [29].

### 4. Hypoxia and Neuroinflammation in the CNS

During an ischemic stroke and resulting hypoxia, inflammatory cytokines are released by microglia, neurons and astrocytes with glutamate largely released by neurons. The up-regulation of pro-inflammatory cytokines through the activation of microglia and astrocytes in the brain contribute a great deal to ischemic brain damage [51]. During hypoxia, HIF-1 $\alpha$  binds to HRE like binding sites allowing for the up-regulation of cytokines, such as IL- $\beta$ , IL-6, IL-8, and TNF- $\alpha$ . Mutations in either the HIF-1 $\alpha$  gene or its binding site at the promoter inhibit this cytokine up-regulation [46].

Up-regulation of IL-1 $\beta$  is related to hypoxic hyperexcitability due to the fact that IL-1 $\beta$  can activate tyrosine kinases, which then phosphorylate the NMDAR subunits, NR2A and NR2. This increase in NMDAR potentiation leads to excessive flow of Ca<sup>2+</sup> leading to hyperexcitability and neuronal injury [52]. Hypoxia also leads to activation of nuclear factor  $\kappa$ B (NF $\kappa$ B) signaling pathways whereby HIF-1 $\alpha$  has a molecular interaction with the inflammatory mediator NF $\kappa$ B. HRE binding, as seen in Figure 2, allows for the expression of NF $\kappa$ B, which then activates the transcription of inflammatory genes and HIF proteins [53]. NF $\kappa$ B expression is increased when hypoxia is followed by a period of re-oxygenation [54]. Reactive oxygen species (ROS) have been shown to both activate and inactivate NF $\kappa$ B, which could explain the importance of the re-oxygenation period. ROS can trigger both apoptotic and necrotic cell death depending on the severity of the oxidative stress [55–57]. Another form of hypoxia, CIH, such as seen in sleep apnea can lead to neuronal cell death and one of the mechanisms involved may be inflammation. Neural inflammation caused by CIH can be region specific with the expression of microglial toll-like receptor-4 (TLR4) increased differentially across areas of the CNS [58]. Hypoxia-re-oxygenation increases microglial levels of inducible nitric oxide synthase (iNOS) leading to neuronal cell loss through apoptosis and memory impairment [59]. Many other insults such as bacterial, viral, cytokines and neurodegenerative insults induce iNOS in microglia [60]. This increase in iNOS raises the levels of NO allowing for the inhibition of neuronal respiration causing glutamate release [61]. Rho-associated protein kinase (ROCK) is thought to play a vital role in this pathway as the introduction of the ROCK inhibitor, fasudil, attenuates the neuronal apoptosis [62]. Thus inflammatory pathways and microglial activation are key components to the hypoxic response whereby their activation allows for formation of ROS as well as having the ability to modulate glutamatergic receptors. The important role they play in causing neuronal cell damage as well their potential to be neuroprotective through hypoxic preconditioning makes the inflammatory response a vital therapeutic target in hypoxia. Only recently has it been reported that patients with obstructive sleep apnea (involving episodes of IH) were 1.37 times more likely to have Parkinson's disease than patients without the disease [63].



**Figure 2.** Hypoxia and NF $\kappa$ B activation. During hypoxic HIF-1 $\alpha$  binding to the HRE induces the expression of NF $\kappa$ B (left). NF $\kappa$ B p50 p65 dimer is able to freely activate the transcription of inflammatory and HIF proteins (right).

## 5. TNF- $\alpha$ and Hypoxia

TNF- $\alpha$ , a pro-inflammatory cytokine produced primarily by monocytes and macrophages in the periphery and microglia and neurons in the CNS, is involved in the promotion of the inflammatory response and cognitive dysfunction [64,65]. TNF- $\alpha$  is initially produced as a 212-amino acid-long type II transmembrane that is stable as a homotrimer. The cleavage of the membrane-integrated form by TNF- $\alpha$  converting enzyme produces a soluble homotrimer, which binds to either of two receptors, TNF receptor type 1 (TNFR1) or TNF receptor type 2 (TNFR2). TNFR1 is constitutively expressed throughout most tissues and is thought to be the main TNF signaling receptor. The activation of TNF-R1 leads to either apoptotic cell death or the activation of either the caspase-8 pathway or c-Jun NH2-terminal kinase (JNK) pathways, or neuroprotection through the binding of I $\kappa$ B kinase (IKK) complex and the subsequent activation of the NF $\kappa$ B pathway [66]. The signaling network in TNF-R1 is interesting due to the extensive crosstalk between the NF $\kappa$ B, and JNK signaling pathways. The cells susceptibility to apoptosis increases in the absence of NF $\kappa$ B. The activation of TNFR2 leads to the activation of the NF $\kappa$ B pathway, phosphatidylinositol-3 kinase (PI3K) and subsequent transcription of neuroprotective mediators like calbindin and manganese superoxide dismutase [67,68]. Specifically in microglia activation of TNFR2 anti-inflammatory pathways may be induced [69]. A putative role for TNF- $\alpha$  has been shown in rats infused with lipopolysaccharide (LPS may promote the secretion of pro-inflammatory cytokines including TNF- $\alpha$  and IL-1 $\beta$ ) into the fourth ventricle to induce chronic neuroinflammation [70]. TNF- $\alpha$  synthesis inhibition was found to restore the neuronal function as well as reverse cognitive deficits induced by the chronic neuroinflammation [70].

It is becoming apparent that TNF- $\alpha$  is one of the most important inflammatory cytokines to be studied in relation to neuronal damage caused by the absence of oxygen due to the fact that it actively participates in the immune-mediated inflammation of stroke and other neurodegenerative diseases with an hypoxia component [71]. The release of TNF- $\alpha$  is a result of the pathogenesis of disorders such as stroke [72], Alzheimer's disease [73], Parkinson's disease [74] and severe infections such as meningitis [75], yet its role during hypoxia is not fully understood. In severe ischemia TNF- $\alpha$  levels appear to be elevated in affected brain tissue after 24 h [76]. One such critical role in neuroinflammation has been illustrated whereby TNF- $\alpha$  can damage dopaminergic neurons and thus anti-TNF agents may ameliorate Parkinson's disease [74]. Despite many research papers in this field few laboratories have investigated the effects of acute hypoxia and inflammatory mediators on synaptic transmission [77,78]. Recently our laboratory reported that recovery of synaptic transmission in CA1 neurons was impaired post-hypoxia in the presence of TNF- $\alpha$  [77]. It also been shown that HIF-1 $\alpha$  has a binding site for the Fas Associated Death Domain promoter, which is an adapter molecule in TNFR1 mediated cell death. Therefore it has a direct role in TNF- $\alpha$  mediated apoptosis which may help explain the poor recovery of EPSPs following a hypoxic insult [79].

A growing body of evidence indicates that TNF- $\alpha$  may play a role in the regulation of tolerance to chronic hypoxia such as occurs in ischemia yet it has a deleterious effect in ischemic brain injury after stroke [80]. It seems that administration of a high dose of lipopolysaccharide (LPS) may induce a robust inflammatory response that can result in lethal septic shock whereas administration of a low dose of LPS may induce a protective state of tolerance to subsequent exposure to LPS at doses that might cause serious injury [81,82]. In fact LPS preconditioning is known to exert neuroprotection from cerebral ischemia [83,84]. In cerebellar granule neurons the neuroprotective effects of LPS preconditioning were said to be independent of endogenous IL-1 $\beta$  but dependent on endogenous TNF- $\alpha$  and also IL-6 [85]. Our laboratories have recently provided evidence that TNF- $\alpha$  has a preconditioning effect following a glutamate toxic insult 24 h later in the CA1 region of rat organotypic slices [65]. We suggested that the preconditioning effects may be as a result of changing resting Ca<sup>2+</sup> levels and Ca<sup>2+</sup> influx in the presence of TNF- $\alpha$ .

## 6. TNF- $\alpha$ and Synaptic Plasticity

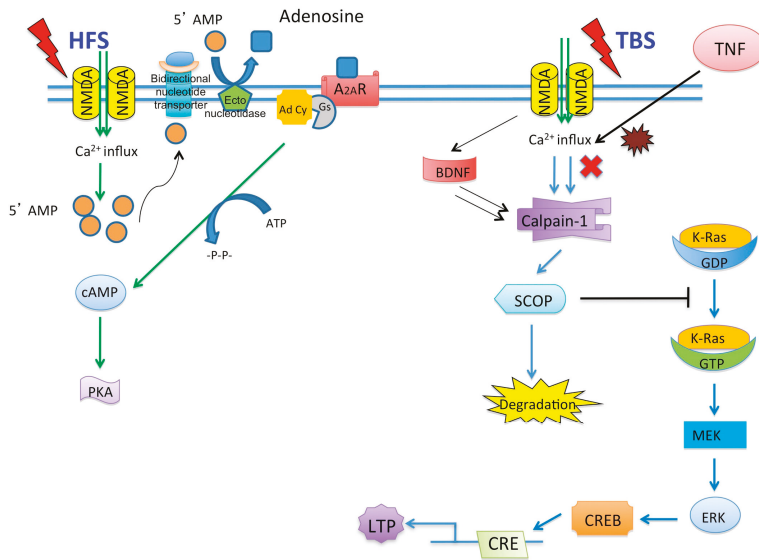
A growing body of evidence has highlighted the role of TNF- $\alpha$  in glutamatergic synaptic plasticity and scaling. It has been shown that TNF- $\alpha$  has an inhibitory effect on LTP in both the CA1 and dentate gyrus [76,86–89]. Studies initially carried out by Tancredi *et al.* (1992) [90] showed an inhibitory effect of TNF- $\alpha$  on LTP induction in the CA1 region, which was concentration-dependent. However, they demonstrated that short-term application of TNF- $\alpha$  (>50 min) did not affect LTP. These findings and others highlight the various parameters involved in the regulatory role that this cytokine plays in synaptic plasticity. The inhibitory actions of TNF- $\alpha$  on LTP have been shown to be mediated through the signaling pathways, P38 MAP kinase and JNK [91]. Butler *et al.* (2004) [88] reported that the inhibition of LTP by TNF- $\alpha$  was in fact a biphasic response. SB203580, a P38 MAPK inhibitor, blocked the early inhibition of LTP by TNF- $\alpha$  but did not reverse its late inhibition (3 h following induction), possibly due to the requirement for new protein synthesis. Using antagonists for metabotropic glutamate receptor 5 (mGluR5) and ryanodine, a potential role for metabotropic glutamate receptors and ryanodine sensitive intracellular Ca<sup>2+</sup> stores in TNF- $\alpha$  mediated inhibition of LTP have also been proposed [87].

Other studies have provided evidence that exogenous application of TNF- $\alpha$  whilst not inhibiting LTP in the CA1 region of the hippocampus may alter homeostatic plasticity (synaptic scaling) rather than synaptic plasticity [92]. These studies have shown that glia released TNF- $\alpha$  is required for synaptic scaling through AMPAR trafficking to the membrane [92–94]. Others have reported that the increase in AMPAR expression on the cell surface is mediated through the P13 kinase pathway and the AMPARs trafficked were lacking the GLR-2 subunit. Since LTP is dependent on synaptic glutamate it is also interesting to note that TNF- $\alpha$  has been shown to increase glutamate release from astrocytes [95], block glutamate transporters [96], and also may have a modulatory effect on the expression of GLT-1 and GLT-2. These effects combined may result in increased glutamate concentrations in the synaptic cleft [97,98]. TNFR1, but not TNFR2, may play an important role in AMPAR localization on the membrane of cortical neurons. Deletion of TNFR1 resulted in a decrease of AMPAR clustering on the synaptic membrane, which was not rescued by exogenous application of TNF- $\alpha$  [99]. These observations indicate a potential therapeutic approach for TNF- $\alpha$  via TNFR1 in mediating AMPAR excitotoxicity. Glutamatergic gliotransmission is an important stimulatory input to excitatory synapses and it has been shown that TNF- $\alpha$  is a modulator of this process in the dentate gyrus [100]. Many of the discrepancies observed with regard to the effects of TNF- $\alpha$  on LTP may be region specific or indeed depend on the induction protocol used to induce LTP. There are many factors regulating the magnitude of LTP induced by different parameters such as high frequency stimulation and theta burst stimulation [101] (Figure 3). Recently, we have shown that the stimulation parameters used to induce LTP may have an influence on TNF- $\alpha$ 's ability to inhibit LTP [102]. TNF- $\alpha$  has no inhibitory effect on LTP when induced with prolonged high frequency stimulation (HFS) whereas full inhibition was observed when LTP was induced by theta burst stimulation (TBS). Figure 3 illustrates a potential mechanism that might explain this discrepancy whereby TBS may trigger alternative signaling cascades to HFS that can be modulated by TNF- $\alpha$ .

## 7. TNF- $\alpha$ , Hypoxia and Synaptic Plasticity

Hippocampal slices exposed to acute hypoxia may recover when oxygen is re-introduced. Recently it has been shown that in the presence of TNF- $\alpha$  there is an impairment in the recovery of synaptic transmission in the CA1 region post-hypoxia [77]. Conversely, hypoxia has also been shown to increase intercellular Ca<sup>2+</sup> levels and activate calmodulin-dependent protein kinase II (CaMKII) through a TNF- $\alpha$  independent mechanism [103]. However CaMKII is also capable of activating the PI3K-PKC $\lambda$ -AMPA signaling pathway. TNF- $\alpha$  has been found to play roles in cell adhesion up-regulation, disruption of the blood brain barrier and is a key component for the participation of glial cells in the physiological control of synaptic transmission and plasticity through the release of glutamate, a process known as glutamatergic gliotransmission [100,104]. TNF- $\alpha$  has been shown to

increase the release of glutamate from astrocytes, maintain glutamate levels through the blocking of glutamate transporters [96] and modulate the expression of Glut-1 and Glut-2. All these effects by TNF- $\alpha$  result in the increase in the concentration of glutamate in the synaptic cleft, which may have an influence on the magnitude of LTP post-hypoxia. Using a robust LTP-inducing stimulus protocol we have been able to demonstrate a significant enhancing effect of TNF- $\alpha$  on LTP post hypoxia but only in the dentate gyrus of the hippocampus [102]. In the presence of DMOG (a non-specific PHD inhibitor) this enhancement of LTP was still evident perhaps indicating a novel HIF/PHD-independent effect of TNF- $\alpha$  [102].



**Figure 3.** Putative signaling pathways activated after HFS- and TBS-induced LTP. HFS-induced LTP may be dependent on the breakdown of 5' AMP into adenosine. Adenosine activates the A<sub>2A</sub>R receptor leading to cAMP and PKA activation. TBS-induced LTP involves the influx of Ca<sup>2+</sup> and subsequent activation of calpain-1. The activation of calpain-1 leads to a calpain-1-mediated suprachiasmatic nucleus circadian oscillator protein degradation and ERK activation. Exogenous TNF- $\alpha$  inhibits LTP induced by TBS only. During hypoxia, TNF- $\alpha$  may have potentiating effect on HFS-induced LTP but not TBS.

## 8. Conclusions

Hypoxia is one of the key components, which can arise from neuropathological conditions such as stroke, Parkinson's or Alzheimer's disease. Hypoxic events can cause the release of pro-inflammatory cytokines from neurons and glial cells, such as TNF- $\alpha$ , which can lead to further neurotoxicity or indeed neuroprotection in the brain. However, the effects of TNF- $\alpha$  on neurons during de- and re-oxygenation of neurons is largely unknown. Many studies have now shown that pro-inflammatory cytokines, such as TNF- $\alpha$ , play a key role in the regulation of synaptic transmission and plasticity in the absence and presence of acute hypoxia, especially within the hippocampus. The mechanisms by which elevated levels of TNF- $\alpha$  have an enhancing or detrimental effect on synaptic signaling and synaptic plasticity in the presence or after a hypoxic event remains to be elucidated.

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## Abbreviations

|                   |  |
|-------------------|--|
| A <sub>1</sub> Rs | Adenosine A <sub>1</sub> receptors                             |
| AMP               | adenosine monophosphate  |
| AMPArs            | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors |
| ATP               | adenosine triphosphate   |
| BDNF              | brain-derived neurotrophic factor                              |
| CaMKII            | calmodulin-dependent protein kinase II                         |
| CIH               | chronic intermittent hypoxia                                   |
| CNS               | central nervous system   |
| CREB              | cAMP response element-binding protein                          |
| DMOG              | dimethylxaloglycine  |
| DPCPX             | 8-cyclopentyl-1,3-dipropylxanthine                             |
| EPSP              | excitatory post-synaptic potential                             |
| ERK               | extracellular signal-regulated kinases                         |
| HIF               | hypoxia inducible factors                                      |
| HRE               | hypoxic responsive element                                     |
| IL-1β             | interleukin-1beta  |
| iNOS              | inducible nitric oxide synthase                                |
| LPS               | lipopolysaccharide   |
| LTP               | long-term potentiation   |
| NFκB              | nuclear factor κB  |
| NMDAR             | N-methyl-D-aspartate receptors                                 |
| PHDs              | prolyl hydroxyl domains  |
| PI3K              | phosphatidylinositol-3 kinase                                  |
| ROCK              | Rho-associated protein kinase                                  |
| ROS               | reactive oxygen species  |
| TNF-α             | tumor necrosis factor alpha                                    |

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Article

# Neuroimmunology of the Interleukins 13 and 4

Simone Mori <sup>1</sup>, Pamela Maher <sup>2</sup> and Bruno Conti <sup>1,\*</sup>

<sup>1</sup> Department of Chemical Physiology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA; smori@scripps.edu

<sup>2</sup> Cellular Neurobiology Laboratory, Salk Research Institute, La Jolla, CA 92037, USA; pmaher@salk.edu

\* Correspondence: bconti@scripps.edu; Tel.: +1-858-784-9069

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**Abstract:** The cytokines interleukin 13 and 4 share a common heterodimeric receptor and are important modulators of peripheral allergic reactions. Produced primarily by T-helper type 2 lymphocytes, they are typically considered as anti-inflammatory cytokines because they can downregulate the synthesis of T-helper type 1 pro-inflammatory cytokines. Their presence and role in the brain is only beginning to be investigated and the data collected so far shows that these molecules can be produced by microglial cells and possibly by neurons. Attention has so far been given to the possible role of these molecules in neurodegeneration. Both neuroprotective or neurotoxic effects have been proposed based on evidence that interleukin 13 and 4 can reduce inflammation by promoting the M2 microglia phenotype and contributing to the death of microglia M1 phenotype, or by potentiating the effects of oxidative stress on neurons during neuro-inflammation. Remarkably, the heterodimeric subunit IL-13R $\alpha$ 1 of their common receptor was recently demonstrated in dopaminergic neurons of the ventral tegmental area and the substantia nigra pars compacta, suggesting the possibility that both cytokines may affect the activity of these neurons regulating reward, mood, and motor coordination. In mice and man, the gene encoding for IL-13R $\alpha$ 1 is expressed on the X chromosome within the PARK12 region of susceptibility to Parkinson's disease (PD). This, together with finding that IL-13R $\alpha$ 1 contributes to loss of dopaminergic neurons during inflammation, indicates the possibility that these cytokines may contribute to the etiology or the progression of PD.

**Keywords:** Interleukin 13; Interleukin 4; neuron; microglia; Parkinson; brain; neurodegeneration; neuroinflammation; neurotoxic; neuroprotection

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## 1. Introduction

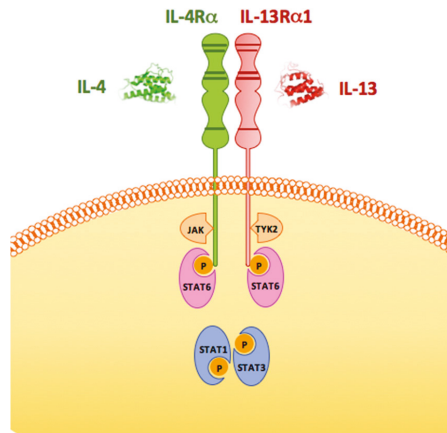
In this review we summarize the current body of knowledge on the role of IL-13 in the central nervous system. Although the study of this subject is in its infancy and only a limited amount of work has been done at this stage, it is likely that this will change in the near future. In fact, one of the interesting aspects of investigating the biology of IL-13 in the central nervous system (CNS) is that its canonical receptor, alpha type I (IL-13R $\alpha$ 1), appears to be expressed not only in glial cells during pathological conditions, but also in specific subsets of neurons in the healthy brain. Specifically, IL-13R $\alpha$ 1 has, so far, been found on dopaminergic neurons of the Substantia Nigra pars compacta (SNc) and the Ventral Tegmental Area (VTA) [1]. This finding indicates that its ligands, IL-13 and IL-4, could be important regulators of dopaminergic function and cell survival, and may provide a direct link between the immune system and the neurobiology of reward, addiction, or motor coordination.

## 2. What We Know about IL-13 Comes from Studies of Its Biology in the Immune System

The cytokines Interleukin 13 (IL-13) and interleukin-4 (IL-4) are two secreted proteins recognized for their role in promoting T-helper type 2 (Th2) lymphocyte-mediated allergic inflammation and

atopy in the periphery [2–17]. IL-13 and IL-4 also have the ability to downregulate the synthesis of T-helper type 1 (Th1) lymphocyte pro-inflammatory cytokines: for this reason they are normally listed as anti-inflammatory interleukins [8–10,15,18–20]. Both cytokines are produced by Th2, as well as by other cell types, including eosinophils and basophils [2,5,6,9,11–13] and IL-13 production is also stimulated in mast cells by lipopolysaccharides (LPS) [21–24].

IL-13 and IL-4 are often investigated together because they partially share a common receptor type: the IL-13 receptor alpha 1 chain (IL-13R $\alpha$ 1). IL-13R $\alpha$ 1 heterodimerizes with the IL-4R alpha chain (IL-4R $\alpha$ ) forming a complex capable of binding IL-13 or IL-4 (Figure 1) [25–32]. To date, this complex is the only known signal transducer for IL-13, while IL-4 can also signal through an IL-4R $\alpha$ /gamma chain complex. A high-affinity IL-13-binding protein (IL-13R $\alpha$ 2) also exists and is a specific inhibitor of IL-13 signaling, likely by functioning as a decoy receptor [28,33–36]. IL-13R $\alpha$ 2 is not found in the healthy brain and, so far, has only been shown to be expressed in the CNS on glioblastoma cells [37] making it one of the major targets of immunotherapy. Work on IL-13R $\alpha$ 2 in the CNS and its role as a therapeutic target will not be discussed here and is covered by recent excellent reviews [38].



**Figure 1.** Schematic representation of the heterodimeric receptor for IL-13 and IL-4 and its signaling. Interleukins 13 (IL-13) and 4 (IL-4) can bind to the same heterodimeric receptor composed of the IL-13 Receptor alpha 1 (IL-13R $\alpha$ 1) and the Interleukin 4 Receptor alpha (IL-4R $\alpha$ ). Binding of the receptor activates the Janus kinase (JAK) and leads to phosphorylation of members of the Signal Transducer and Activator of Transcription (STAT) family. The tyrosine-protein kinase 2 (TYK2) is a member of the JAK family. See the text for more details.

Binding of IL-13 to its cognate functional receptor allows the trans-phosphorylation of a specific tyrosine residue located in the Janus Kinase (JAK) activation segment [31,39] which promotes the kinase activity required for the phosphorylation of downstream substrates in its signaling cascades [39,40]. IL-13 activates two intracellular signaling cascades: the JAK-STAT and the insulin receptor substrate (IRS)-phosphatidylinositol 3'-kinase pathways [26,28,31]. While the IRS-phosphatidylinositol 3'-kinase pathway leads to cell proliferation, the JAK-STAT pathway induces the transcription of genes that contain the Stat6-responsive enhancer element N6-GAS located in their promoter [41–43]. Upon activation of IL-13R $\alpha$ 1, Stat1, 3, and 6 are phosphorylated and form a homodimer that migrates to the nucleus and binds to N6-GAS to drive transcription [31,42,44,45]. Reactive oxygen species (ROS) also play a role in the IL-13/IL-4 cellular transduction signaling. In intestinal epithelial cells upon IL-13R $\alpha$ 1 activation both the JAK-STAT pathway and Mitogen Activated Protein Kinase (MAPK) stimulate nicotinamide adenine dinucleotide phosphate oxydase to produce intracellular ROS that, in a positive feedback loop, facilitate the phosphorylation of STAT6 and ERK [46]. Moreover, IL-13/IL-4-driven

ROS production has been recently shown in alternatively-activated monocytes/macrophages through activation of monoamino oxydase A (MAO-A) [44].

### **3. Expression of IL-13 and IL-4 in the CNS**

As mentioned above, IL-13 and IL-4 were demonstrated to be produced peripherally. To date, there is no evidence that these two proteins, both with molecular weights in the range of 15 kDa, can cross the blood-brain barrier. However, experimental work shows, instead, their local production in the CNS. Expression of IL-13 in the rodent brain was described in microglia, where its production was enhanced by peripheral injection of LPS or the neurotoxin 1-metil-4-fenil-1,2,3,6-tetraidropiridina (MPTP) [47–51].

Evidence also exists that both IL-13 and IL-4 can be produced by neuronal cells of the hippocampus and the cortex in experimental models of ischemic insult [52,53]. In this context it has speculated that the production of IL-4 and IL-13, inducing alternative activation of microglia—known as the M2 state—can exert a protective effect against neuronal damage [53–55]. Neuronal production of IL-4 has been described lately in the noradrenergic neurons of the locus coeruleus, in which its release appears to be sensitive to behavioral stress [56]. Preliminary work in our laboratory also showed that IL-13 can be produced in neurons [57].

### **4. What Is the Role of IL-13 and IL-4 in the CNS?**

Few studies have tested the effects of IL-13 and IL-4 in the CNS. Most of these have investigated a possible action on neuronal survival with some studies finding that IL-13 and/or IL-4 potentiate the effects of LPS and Interferon gamma (IFN- $\gamma$ ), increasing oxidative damage and contributing to neuronal death [47–50,58–61]. On the other hand, other studies indicated that IL-13 and/or IL-4 could be neuroprotective either by directly reducing inflammation or by inducing the death of microglia cells that are considered to be cellular mediators of neuronal damage [47–50,59–65]. Notably, both IL-13 and IL-4 can potentiate LPS-induced sickness behavior when co-injected centrally with LPS, whereas only IL-4, and not IL-13, attenuates LPS-induced sickness behavior when administered several hours before LPS [47,66]. Recently, our laboratory collected evidence that IL-13 and IL-4 are not toxic when administered alone but can greatly increase the susceptibility of neurons to oxidative damage and contribute to their demise if they express IL-13R $\alpha$ 1 [1].

### **5. IL-13 and IL-4 in Multiple Sclerosis**

Multiple sclerosis (MS) is an autoimmune disorder affecting the CNS with a relapsing-remitting time course. IL-13 seems to exert a protective role in this context, as it is believed that in the development of the disease, a crucial role is played by the imbalance between pro-inflammatory cytokines (IL-1 $\beta$ ; TNF; INF- $\gamma$ ; IL-17) and anti-inflammatory cytokines (IL-4, IL-5, IL-10 and IL-13) [67,68]. IL-13 polymorphisms are associated with autoimmune diseases and also increase susceptibility to MS [69].

A study in humans with MS found that high levels of IL-13 in the cerebral spinal fluid (CSF) might exert a neuroprotective effect by enhancing Gamma Aminobuthirric Acid (GABA) over glutamate transmission [64]. Interestingly, an earlier report describes IL-4 having the same neural effect of increasing the GABA-induced inward current in neurons in a dose-dependent and reversible manner [70]. Moreover, the copolymer glatiramer acetate, an immunomodulatory drug currently used to treat MS, has shown to significantly increase the TH<sub>2</sub>-lymphocyte production of IL-13 in patients [71].

Consistently, using the mouse experimental model of MS, experimental autoimmune encephalomyelitis (EAE), Cash and colleagues showed that IL-13 exerts its anti-inflammatory action by inactivating macrophages and reducing oxidative stress [72]. In the same model, an increase in circulating and spleen IL-13 prevented axonal injury [73] alone or in synergy with IL-4 [74], whereas IL-13 reduction was associated with loss of protection [75].



Sex difference can play a role in affecting the role of IL-13 in the MS model. While autoimmune diseases, including MS, are more common in women [76], the incidence and severity of EAE in mice, null for IL-13, was lower in females compared to males, suggesting the possibility that the contribution of IL-13 to EAE/MS may be gender specific [77]. To this end, it is interesting to note that the expression of IL-13 mRNA can be decreased by estrogen in a mouse model of inflammatory intestine disease [78] and that the gene encoding for IL-13R $\alpha$ 1 is located on the X chromosome in both humans and mice.

Together, these studies suggest that IL-13 may have a neuroprotective role in MS. Although this may be different in other neurodegenerative diseases that, unlike MS, are not characterized by a severely-compromised blood-brain barrier, and are not primarily mediated by peripheral immune cells, IL-13 and IL-4 also showed protection in a mouse model of Alzheimer's disease (AD). Specifically, intracerebral injection of a mixture of IL-13 and IL-4 reduced amyloid deposition and improved spatial learning and memory in an AD transgenic mouse model when applied to young mice but did not show protective effects when administered in adult animals [79].

## 6. Parkinson's Disease

The IL-13 system may have a specific role in the pathogenesis and/or the progression of Parkinson's disease (PD). Data mining using the Online Mendelian Inheritance in Man (OMIM) database [80] showed that IL-13R $\alpha$ 1 lies within the PARK12 region of susceptibility to PD. Although PARK12 comprises a large portion of DNA, it is located on the X chromosome, an observation that may be of interest in that PD has a higher incidence in men than in women. Even more intriguing was the finding that expression of IL-13R $\alpha$ 1 in the brain appeared to be specific to the dopaminergic neurons of the VTA and of the SNc, the region affected by PD. Double-immunostaining studies also revealed that approximately 80% of the SNc neurons expressing the dopaminergic marker tyrosine hydroxylase also expressed IL-13R $\alpha$ 1 [1].

The possible contribution of IL-13R $\alpha$ 1 to neuronal fate was measured using a pro-inflammatory experimental mouse model of PD. Animals received periodic peripheral intraperitoneal injections of bacterial LPS over a period of six months, a regimen previously demonstrated to induce central loss of dopaminergic SNc neurons [81]. Comparative analysis showed that mice lacking IL-13R $\alpha$ 1 were protected from neuronal loss when compared to their wild-type littermates, suggesting a neurotoxic action of its ligands, IL-13 and/or IL-4. *In vitro* experiments using a dopaminergic cell line showed, however, that neither IL-13 nor IL-4 had cytotoxic effects when administered alone. However, both cytokines increased the toxicity of non-toxic doses of oxidants in a dose-dependent manner.

Thus, activation of IL-13R $\alpha$ 1 may be one of the mechanisms whereby the vulnerability of dopaminergic neurons is increased during inflammation, when both cytokines and ROS are produced. On the other hand, the lack of neurotoxicity of IL-13 or IL-4 in the absence of ROS suggests that these cytokines may be capable of regulating neuronal function by affecting the neurobiology of those neurons that participate in reward, addiction, and motor control.

Investigating these phenomena is likely to provide important information on the mechanisms of how IL-13 and IL-4 and, more generally, the immune system, may be capable of influencing behavior or can contribute to neurodegeneration.

## 7. Conclusions

Although in its infancy, the investigation of the central role of the interleukins 13 and 4 has is an exciting area of research. What makes it so attractive is that these two cytokines can be produced locally in the CNS and are active on both microglia and neuronal cells. Of special interest is the fact that they act through a common heterodimeric receptor that is expressed in dopaminergic neurons. Although these two Th2 cytokines are considered anti-inflammatory, studies conducted so far show that they can have cytotoxic effects on both glia and neurons. Interestingly these actions are not due to an intrinsic toxicity of IL-13 and IL-4 but rather to their ability to increase the cellular susceptibility to oxidative stress. This suggest that under pathological conditions, such as neuroinflammation when

reactive oxygen species are produced, IL-13 and IL-4 can participate to tissue damage and thus to Parkinson's disease or other neurodegenerative disorders. Instead, under physiological conditions, these two cytokines can contribute to the regulation of neuronal function via direct action through neuronal IL-13R $\alpha$ 1. Thus, they have the requisites of being potential neuromodulators.

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Review

# Immunomodulators as Therapeutic Agents in Mitigating the Progression of Parkinson's Disease

Bethany Grimmig <sup>1</sup>, Josh Morganti <sup>2</sup>, Kevin Nash <sup>3</sup> and Paula C Bickford <sup>1,4,\*</sup>

<sup>1</sup> Center of Excellence for Aging and Brain Repair, Department of Neurosurgery and Brain Repair, Morsani College of Medicine, University of South Florida, Tampa, FL 33612, USA; bgrimmig@health.usf.edu

<sup>2</sup> Sanders-Brown Center on Aging, Department of Anatomy and Neurobiology, University of Kentucky, Lexington, KY 40508, USA; josh.morganti@uky.edu

<sup>3</sup> Byrd Alzheimer's Institute, Department of Molecular Pharmacology and Physiology, Morsani College of Medicine, University of South Florida, Tampa, FL 33613, USA; Knash@health.usf.edu

<sup>4</sup> James A Haley VA Hospital, 13000 Bruce B Downs Blvd, Tampa, FL 33612, USA

\* Correspondence: pbickfor@health.usf.edu; Tel.: +1-813-974-3238

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**Abstract:** Parkinson's disease (PD) is a common neurodegenerative disorder that primarily afflicts the elderly. It is characterized by motor dysfunction due to extensive neuron loss in the substantia nigra pars compacta. There are multiple biological processes that are negatively impacted during the pathogenesis of PD, and are implicated in the cell death in this region. Neuroinflammation is evidently involved in PD pathology and mitigating the inflammatory cascade has been a therapeutic strategy. Age is the number one risk factor for PD and thus needs to be considered in the context of disease pathology. Here, we discuss the role of neuroinflammation within the context of aging as it applies to the development of PD, and the potential for two representative compounds, fractalkine and astaxanthin, to attenuate the pathophysiology that modulates neurodegeneration that occurs in Parkinson's disease.

**Keywords:** Parkinson's disease; neuroinflammation; microglia; fractalkine; astaxanthin

## 1. Parkinson's Disease

Parkinson's disease (PD) is a debilitating condition that affects millions of people worldwide. With the development of drugs to treat complications associated with significant morbidity and mortality, patients are living up to 20 years after the diagnosis of PD. Although the use of medications has led to a relative improvement in quality of life, these patients are often substantially disabled, requiring significant health care and compensation for loss of wages. It has been projected that the prevalence of PD will double by 2040, indicating severe economic consequences to come as the incidence increases. There are currently no available medications that prevent or reverse the neurodegeneration that causes these disabilities [1].

Parkinson's disease is primarily characterized neuroanatomically by the degeneration of the neurons in the substantia nigra pars compacta (SN), resulting in a substantial loss of dopamine (DA) afferents to the striatum and subsequent motor impairment. It is estimated that nearly 50% of dopaminergic cells in the SN have been lost prior to clinical presentation of motor dysfunction. It is now understood that PD pathology extends to extra nigral regions including the locus coeruleus, nucleus basalis of Meynert, pedunculopontine nucleus, intralaminar thalamus, and lateral hypothalamus suggesting dysfunction and neurodegeneration in many areas of the brain [2]. It is histopathologically characterized by the formation of Lewy bodies, intraneuronal protein inclusions

comprised predominantly of  $\alpha$ -synuclein ( $\alpha$ -syn). These protein aggregates have been observed throughout the brain, and pathological  $\alpha$ -syn deposition is thought to begin in the medulla and spread throughout the midbrain to cortical regions in a manner that corresponds to the onset of clinical symptoms [2]. These protein aggregates are associated with microgliosis [3], and impaired cellular physiology, although the precise mechanisms leading to cytotoxicity are currently unknown.

The vast majority of Parkinson's disease cases are classified as idiopathic, but approximately 5% of PD cases are genetically linked. There are several gene mutations that confer susceptibility or are associated with the development of PD, including mutations in leucine-rich repeat kinase 2 (LRRK), PTEN Induced putative kinase 1 (PINK1), and the protein deglycase (DJ1). However,  $\alpha$ -syn has proven to have a very strong association and relevance to PD and similar disorders collectively known as synucleinopathies. Abnormalities in the SNCA gene that encodes the  $\alpha$ -syn protein are strongly correlated with the development of an autosomal dominant familial form of PD [4]. Multiplications of the SNCA gene are known to increase the expression of the  $\alpha$ -syn protein, whereas certain missense mutations of the gene (A53T, A30P, E46K) produce variants of  $\alpha$ -syn, both of which have known pathological attributes related to the increased propensity to aggregate.

The physiological role of  $\alpha$ -syn, along with the isoforms  $\beta$ - and  $\gamma$ -synuclein, is related to neurotransmission, and they are primarily located in the synapse. Increased concentrations of  $\alpha$ -syn protein or mutated forms of  $\alpha$ -syn make the protein susceptible to misfolding and polymerization by self-assembly. These misfolded aggregates have heterogeneous conformations that have not been clearly elucidated. These aggregates are associated with various deleterious biological activities including (1) the permeabilization of the cellular membranes [5], associated with an alteration of intracellular ion concentrations [6]; (2) disruption of energy production through interactions with the mitochondria [7]; and (3) interruption of intracellular transport via physical interference of motor proteins, or direct interaction with organelles and vesicles [8]. The formation of soluble oligomeric species is presumed to be relatively more toxic than fibrils, through the energetically favorable association within the phospholipids that forms pores in the cellular membrane [9]. The amount of LB formations throughout the brain reflects the severity of impairment [10]. However, it has been postulated that the organizing of fibrils into Lewy Bodies may serve as a protective mechanism to divert toxic aggregate species into less harmful formations to preserve normal cellular physiology [11].

## 2. The Interaction with Aging

Aging is a major risk factor for the development of PD [12], evidenced by the fact that the incidence increases for every decade over 50 years of age. While aging contributes to PD, it is also underrepresented in PD research, as it is common for the experimental models to be carried out using young animals. However, the impact of aging is essential to consider in terms of the disease process because many of the physiological changes that occur in aged animals can drive or exacerbate the pathological mechanisms that lead to PD and alter both the course and tempo of the disease.

The impact of aging on the incidence of PD is compounded by the fact that the dopaminergic neurons of the SN seem to have a unique vulnerability to cellular stressors in the microenvironment [12]. Although it is not completely understood what sets these cells apart from those of other DA pathways, the neurons of this nucleus are more susceptible to the homeostatic disturbances and degenerate significantly more compared to similar or adjacent regions [13]. This can be grossly attributed to high oxidative stress and inflammation. It is known that the SN is exposed to higher levels of oxidation compared to other dopaminergic centers [11]. This is partly due to an unusually high concentration of iron, which through the Fenton reaction, can generate free radicals, as well as nitric oxide released by microglia that densely populate this region [14,15]. In addition to the accumulation of reactive oxygen and nitrogen species, the SN is also ill-equipped to neutralize them, due to a low expression of glutathione, an important endogenous antioxidant molecule, leading to an inevitable increase in oxidative stress [16]. Aging is also associated with increased levels of oxidative stress and altered



microglial function and this combined with the SN's reduced resilience to reactive oxygen species (ROS) could promote neurodegeneration.

The role of neuroinflammation in PD is also particularly important in the context of aging. Inflammation is known to increase with age. This is largely attributed to the age related changes in microglial physiology. Microglia, myeloid derived macrophages, are the resident immune cell of the CNS and constitute 10% of the cell population. Their highly ramified processes are highly motile and constantly survey parenchyma, facilitating the detection and the cellular response to infection and injury. However, microglia are also involved in many homeostatic functions and are now known to be highly active during post-natal synaptic pruning and synaptic plasticity [17,18]. Microglia are known to undergo a phenomenon known as priming with age. This describes a propensity for microglia to attain a pro-inflammatory state and is characterized by 4 principal features: (1) primed microglia will exhibit higher levels of inflammatory mediators and surface makers even in the absence of immune stimulation; (2) microglia also demonstrate hyperactivity upon subsequent activation where they release exaggerated amounts of cytokines and reactive oxygen/nitrogen species; (3) they are also resistant to regulatory mechanisms that typically restore microglia back to their inactivated state, causing them to remain in an aggravated state for a longer period of time [19]; and (4) they do not respond to stimuli, such as IL4, that promote anti-inflammatory factors, angiogenesis, and neurotrophic factor secretion [19,20]. For example, Lee, Ruiz et al. (2013) induced microglial activation in the brains of young and old mice with the application of a cytokine cocktail designed to elicit an M1, pro-inflammatory phenotype (IL1 $\beta$  + IL12) or an anti-inflammatory, M2 phenotype (IL-4 + IL-13). These authors demonstrated that M1 cocktail elicits an increased response microglia from the aged brain, while the same aged cells were less responsive to the M2 cocktail. This phenomenon has been reproduced with isolated microglia and has been termed priming [21].

In order to elucidate the molecular underpinnings of the age-related alterations in microglial function, numerous researchers have begun to assess the differential expression of genes and protein in primary microglia. A recent study using RNAseq comparing the transcriptional profiles of isolated microglia to whole brain has identified the microglial sensome [22]. These authors further assessed the sensome in microglia isolated from aged animals and demonstrated that many of the genes related to sensing endogenous ligands were down regulated, whereas genes pertaining to host defense were up-regulated. Furthermore, another recent study using gene microarrays of isolated microglia identified an up-regulation of NF $\kappa$ B related genes in these aged cells [23]. Sustained microglial activation and microglial priming can perpetuate neurodegeneration by increasing cellular stress both from enhanced release of cytotoxic substances, but also from the loss of trophic support as a result of impaired microglial homeostatic function.

### **3. Role of Neuroinflammation**

The precise pathophysiology that precipitates the development of PD is unknown, although it is understood that a few key biological processes are often impacted in patients, including mitochondrial function, proteostasis, immune function leading to oxidative stress and inflammation. Neuroinflammation is critical factor in the disease process that clearly contributes significantly to the neurodegeneration seen in PD. However, it is difficult to ascertain if inflammation initiates pathological features of PD, or is triggered by the widespread protein aggregation and neuronal death that occurs during disease progression. McGeer et al. (1988) described the presence of increased reactive gliosis and infiltrating T-cells around the SN in post mortem analysis of PD brains [24]. Their observations provided some of the first indications that increased neuroinflammation is associated with DA cell death. Many other studies have reported features of inflammation in post-mortem samples, such as increased levels of inflammatory mediators like iNos and Cox-2 [25], supporting the contribution of increased neuroinflammation during advanced stages of the disease. Similar patterns of activated microglia have been detected in the brain areas associated with clinical symptoms of the disease [26]. Interestingly, this distribution of gliosis was evident in both newly diagnosed patients and those

with advanced pathology. Elevated levels of pro-inflammatory cytokines have also been detected in the cerebrospinal fluid and plasma of PD patients at early stages of the disease. These findings suggest that microglial activation may be initiated in early stages and remain an ongoing process throughout the disease. Furthermore, inflammatory insults and injuries are known to increase the risk of developing PD. Traumatic brain injuries (TBI) have been linked to an increased risk of developing the disease in a frequency and severity dependent manner; multiple injuries or injuries requiring hospitalization were more strongly correlated with PD onset [27]. This occurrence is largely attributed to the neuroinflammatory cascade that follows trauma. Certain infections causing neuroinflammation have been known to lead to a post-encephalitic Parkinsonism. This may be because the SN is densely populated by microglia [28], rendering it is especially susceptible to inflammatory stimuli. For example, intracranial injections of lipopolysaccharide (LPS), a bacterial antigen, dramatically activate microglia and leads to nigrostriatal degeneration and motor symptoms of PD [29]. Parkinsonism is a separate condition distinct from PD, but these observations of inflammatory insults leading to cell death may have important implications for PD itself. Taken together, these data suggest a potential role of neuroinflammation in ongoing cell death that occurs in PD.

Furthermore, pathological forms of  $\alpha$ -syn are associated with microglial activation in the brains of PD patients and this is consistently recapitulated in animal models. The presence of  $\alpha$ -syn aggregates modulates glial activity, often eliciting the release of inflammatory mediators. Codolo et al. (2013) treated monocytes with various species of  $\alpha$ -syn to demonstrate that the aggregated and pathogenic forms of the protein can facilitate the secretion of IL-1 $\beta$  through stimulation of the inflammasome [30]. The nitration of  $\alpha$ -syn is a common modification associated with pathology thought to be promoted in an oxidative environment. Stimulation of microglial cells with nitrated and aggregated  $\alpha$ -syn alters the cellular morphology and transcriptional profile to a pro-inflammatory phenotype, with increased transcription of IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  as well as induction of NF-K $\beta$  signaling [31]. Additionally, this  $\alpha$ -syn activation of microglia seems to be related to phagocytic capacity, as inflammatory cascades and activation of NADPH oxidase are initiated after the glial cells take up the aggregates [3,32,33]. Zhang et al. (2005) demonstrated that impeding phagocytosis of primary microglia attenuates the release of superoxide from these cells when exposed to  $\alpha$ -syn in vitro. This detection and engulfment of  $\alpha$ -syn seems to be dependent on the FC $\gamma$  receptor, as FC $\gamma$  deficient mice are protected the resultant neurodegeneration from AAV driven over expression of  $\alpha$ -syn [32,33]. It is thought that this inflammatory response to abnormal  $\alpha$ -syn will perpetuate neural dysfunction through the release of cytotoxic compounds that leads to cell death. Many of these studies were done using either glial cell lines or in young animals, neglecting the impact of the pro-inflammatory actions of  $\alpha$ -syn within the primed glial environment of an aging brain, thus causing even more damage. In fact, when  $\alpha$ -syn is introduced into an aged animal it is more cytotoxic compared to the young controls [34].

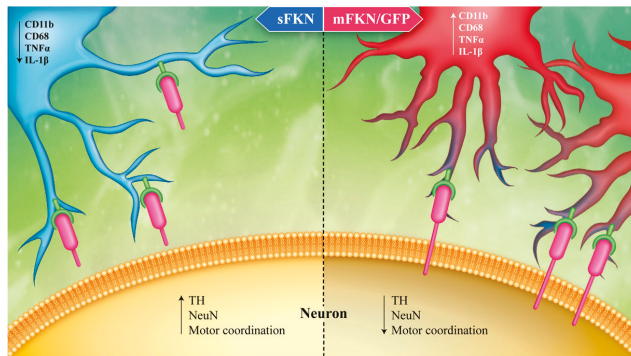
#### 4. Fractalkine as an Anti-Inflammatory Treatment

There is promising pre-clinical experimental evidence to support that reducing inflammation, specifically by suppressing microglial activity, can modify the progression of the loss of DA neurons [35–38]. Non-steroidal anti-inflammatory drugs have been suggested to reduce the risk of PD onset [39]. Also, the use of minocycline, a tetracycline with pharmaceutical actions that extend beyond the classical antimicrobial activity, has been shown to reduce cell death in a neurotoxic model of PD using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). These researchers attributed the neuroprotective effect to the down-regulation of iNOS expression and glial activation [40]. Minocycline was also shown to inhibit apoptosis through a reduction of related inflammatory mediators [41]. Because glial activation is associated with the release of pro-inflammatory factors capable of damaging neurons, this data suggests that mediating the excessive inflammation in PD is a viable therapeutic strategy. There are several signals produced by neurons that have an anti-inflammatory action on microglia, including CD200, CD22, CD47 and fractalkine (FKN, CX3CL1), which could be potential therapeutic targets.

Fractalkine is a protein expressed constitutively by neurons in a membrane bound form that can be cleaved by disintegrin and metalloproteinase (ADAM) 10 and 17. This proteolysis releases a soluble form of the protein, but both isoforms are thought to ligate the cognate receptor, CX3CR1, located on the surface of microglia in the CNS [42]. This neuron-glia interaction serves as an important endogenous mechanism to suppress microglial activation and regulate the output of inflammatory mediators and other damaging molecules [42]. It has been shown that increasing fractalkine levels using an AAV9 gene therapy approach can be neuroprotective [35,36], while the absence of this FKN-CX3CR1 signaling cascade confers susceptibility to neurodegeneration in rodent models of PD. Cardona et al. (2006) demonstrated that CX3CR1 deficiency lead to increase neurotoxicity to both peripheral LPS and MPTP injections. In these experiments, both heterozygous and homozygous mice exhibited increased neurodegeneration and IL-1 $\beta$  expression compared to the intact wild-type controls [43]. There is evidence indicating that fractalkine can regulate microglial function and subsequently reduce inflammation in the CNS. Multiple in vitro studies have established that enhancing fractalkine signaling through application of the ligand or stimulation of the receptor can protect against cell death in culture; FKN-ligand decreases microglial apoptosis and protects against neurotoxicity by both LPS and TNF- $\alpha$ . It has been demonstrated that maintaining or enhancing communication of FKN/CX3CR1 is neuroprotective in multiple rodent models of PD. Pabon et al. (2011) [37] attenuated the neurotoxicity of 6 hydroxydopamine (6OHDA) by delivering a chronic intrastriatal infusion of recombinant fractalkine ligand. This preservation of dopaminergic terminals in the striatum was also associated with decreased microglial activation around the lesion site, indicated by a reduced expression of the MHCII surface marker. To further examine the action of fractalkine in a model of PD where synuclein is introduced in the SN via AAV, Nash et al. (2015) corroborated the neuroprotective effects of FKN by using a viral vector to over express the different isoforms of FKN; membrane-bound (mFKN) or the soluble portion (sFKN). In this study, sFKN reduced DA cell death in young rats with overexpression  $\alpha$ -syn in the SN via AAV9. These findings may be due to altered FKN-CX3CR1 signaling [44] and a reduction in pro-inflammatory cytokines and modulation of microglial function into a more protective role (Figure 1). These results suggest that supraphysiological levels of the fractalkine are protective against the neurodegeneration occurring in two separate experimental models of PD. Morganti et al. (2012) [36] also illustrated the importance of neuron-glia communication by administering MPTP to animals deficient in the fractalkine ligand. These FKN knockouts were extremely susceptible to MPTP toxicity, and display both a dramatic loss of TH in the SN and the robust gliosis associated with the lesion site. Not only does this work indicate that FKN-CX3CR1 communication is necessary for moderating cell death and subsequent detrimental inflammatory events, but it also suggests that further enhancing or supplementing this signaling pathway above baseline activity is sufficient to achieve neuroprotection against these potent toxins (Figure 1). However, the action of fractalkine is quite complex; CX3CR1 knockout mice have been shown to increase phagocytosis of amyloid, but decrease phagocytosis of synuclein [44]. It is unclear if this action is mediated by membrane bound fractalkine or the cleaved, soluble form. Several studies have tried to clarify the roles of the differential processing of fractalkine and there does not appear to be a clear conclusion at this point. When an obligate soluble version of fractalkine is used, as described here with a gene therapy approach, it has been shown to be beneficial in models of PD and AD tau pathology [35,36,45]. However, in other models where an obligate soluble fractalkine mouse is used, opposite results have been observed, and this paper concludes that the membrane anchored fractalkine is associated with phagocytosis of A $\beta$  [46].

Much of the current data regarding the therapeutic potential of FKN in the treatment of inflammatory and neurodegenerative conditions stems from studies involving young animals. However, it is essential to note that aged microglia have been shown to be resistant to their regulatory signals [20,47]. Furthermore, communication via the FKN/CX3CR1 axis becomes dysregulated with age and can also contribute to microglial priming and dysfunction. There are age associated changes affecting both the ligand and receptor. It has been shown that FKN is reduced by in the aged hippocampus [48], although the extent of FKN downregulation in the SN has not yet been fully

characterized. Additionally, immune challenges in the aged CNS lead to a prolonged down regulation of the fractalkine receptor that is associated with a sustained inflammatory response, further compromising this protective neuron glia interaction [49]. Therefore, it is imperative to investigate the therapeutic potential of FKN in the treatment of PD in aged animal models; future studies are needed to examine the efficacy of FKN on aged or primed microglia.



**Figure 1.** A depiction of ligand/receptor binding for both the soluble and membrane bound isoforms of CX3CL1 (FKN) and their respective influences on the release of inflammatory mediators in the substantia nigra. As discussed above, sFKN when delivered via AAV into CX3CL1<sup>-/-</sup> mice following MPTP is associated with the suppression of cytokine production. However, when we delivered an obligate membrane bound form of CX3CL1 or a vector with GFP there was no rescue of TH neurons or a reduction in inflammatory mediators.

## 5. Therapeutic Potential of Astaxanthin

As discussed above, the SN is exposed to high levels of oxidative stress relative to other areas of the brain due to innate features of the neurons that comprise this region [50]. For example, there are low levels of glutathione and high concentration of iron leads to the production of free radicals through the Fenton reaction [51]. Glutathione activity in this region declines with age, further reducing the capacity to manage the accumulation of ROS in the SN. Taken together, these characteristics create an environment of high oxidative stress that can impair neuronal function.

One natural compound of particular interest is astaxanthin, a naturally occurring xanthophyll carotenoid. It is produced by the marine algae *Haematococcus Pluvialis* or synthetically derived from carotenoid precursors and used commercially to feed to farmed fish species to increase pigmentation. Astaxanthin has many suggested mechanisms of action that uniquely oppose pathophysiology that underlie Parkinson's disease including actions as an anti-inflammatory action and improvements in aspects of mitochondrial function, indicating a distinct and promising therapeutic potential in the treatment and management of symptoms in PD patients that are likely more important than its role to simply scavenge free radicals [52–54].

Astaxanthin has potent and diverse actions as an antioxidant and is reported to be several times more effective than other carotenoids in its class. The molecular structure of astaxanthin allows it to reduce free radicals in a variety of ways, including absorbing them into the carbon backbone, donating electrons and forming adducts with the reactive species. Although xanthophyll carotenoids are structurally similar, the presence of polar ionone rings at either end of the astaxanthin molecule makes it energetically favorable. The configuration allows the molecule to span across the phospholipid bilayer of cell membranes and protect the membrane against lipid peroxidation [55].

There is substantial evidence indicating that treatment with astaxanthin causes a reduction in the markers of cellular stress due to excess ROS production, such as 8-isoprostane, protein carbonyl

moieties and 8OHdG [56]. Additionally, astaxanthin has been shown to increase the efficacy of endogenous antioxidant mechanisms *in vivo* including increasing the expression or activity of glutathione, catalase, thioredoxin reductase and superoxide dismutase (SOD) [57,58]. It has also been shown to upregulate heme-oxygenase 1 (HO-1) through increase in NRF [59,60]. These findings suggest that astaxanthin treatment may help alleviate some of the ongoing oxidative stress that occurs during the progression of PD as it contributes to cellular dysfunction.

However, in addition to oxidative damage, there are a number of physiological changes that occur with age that exacerbate the cellular stress in the SN. Mitochondrial dynamics, proteosomal efficiency [61] and levels of synuclein [62] are all altered with age, likely rendering the DA cells more vulnerable to neurodegeneration. Aging also leads to microglial priming and may facilitate PD disease progression. Chronic microglial activation results in prolonged exposure to cytotoxic, pro-inflammatory cytokines, increasing cellular stress and ultimately leading to neurodegeneration [33]. Age-induced primed microglia are hyperactive upon subsequent stimulation and release exaggerated amounts of cytokines. They are resistant to reversion back to a state of tissue repair and maintenance of homeostasis, as they are less responsive to regulatory mechanisms [20,63,64]. As stated above, synuclein aggregation may also directly facilitate the release of these inflammatory mediators. It is thought that this inflammatory response to abnormal  $\alpha$ -syn will perpetuate neural dysfunction through the release of cytotoxic compounds that overwhelm the DA cells, inevitably leading to cell death.

Given the range of pathological mechanisms involved in neurodegeneration seen in PD, astaxanthin seems to have a unique potential for the treatment of this disorder. Many diverse biological activities have been described in the literature that are particular relevant to that pathophysiology of PD, as well as normal aging. Based on this knowledge, the interaction of aging and parkinsonian symptoms should be responsive to treatment with astaxanthin. For example, astaxanthin has also been implicated in modulating microglial activity. Experiments using astaxanthin to treat a transformed microglial cell line can reduce the expression of IL-6 and iNOS/NO *in vitro* when exposed to an immune stimulus such as LPS [65]. These results were corroborated by other studies using aged animals where astaxanthin reduced the release of nitric oxide [56]. These molecules are released in high amounts by activated microglia and are associated with neuronal damage; attenuating the output of inflammatory mediators with astaxanthin may offer some neuroprotection from the inflammatory cascades occurring in the SN.

Some authors have reported alterations in mitochondrial function after astaxanthin treatment. Although most of these studies were conducted *in vitro*, this is of great interest to the treatment of PD. Mitochondrial dysfunction has been implicated in the etiology of the disorder evidenced by the common toxins that induce Parkinsonism. Both MPTP and rotenone are used to produce Parkinson's models by selectively targeting mitochondria leading to the death of SN neurons. Multiple genetic mutations of proteins involved in mitochondrial dynamics have been clearly linked to the development of familial Parkinson's. Furthermore, some of these mitochondrial proteins are associated with a loss of function with age, and may contribute to the increased incidence of diagnosis over the lifespan.

Furthermore, it has been demonstrated recently that mitochondria are a significant source of oxidative stress not only in these DA neurons, but also in additional nuclei known to degenerate in PD. For example, both the locus coeruleus and SN express L-type calcium channels that allow for an extraneous calcium influx that taxes the mitochondria [66,67]. The presence of these channels and their associated calcium burden has been proposed to be a common physiological feature that contributes to the cellular vulnerability for the brain regions affected in PD. Attenuating this mitochondrial derived oxidative stress that results from calcium overload has led to the use of calcium channel antagonists for the treatment of PD [68]. These dihydropyridines, specifically israpidine, have been shown to be tolerable and safe among PD patients are now in Phase III clinical trial [69].

The success of these drugs lends support for the therapeutic use of AXT as well. There is substantial evidence to suggest that astaxanthin works at the level of the mitochondria. According to HPLC analysis of cellular fractions, astaxanthin will accumulate in mitochondria, and has the capacity

to increase mitochondrial activity as indicated by increased respiration and mitochondrial membrane potential (MMP) [70]. Mitochondrial dysfunction is a common pathophysiological observation in PD, and is recapitulated in the  $\alpha$ -synuclein model. It has been shown that treating isolated mitochondria with  $\alpha$ -synuclein oligomers induced mitochondrial dysfunction by inhibiting complex 1 and associated with reduced calcium retention time, release of ROS and induced mitochondrial swelling [7]. In specific studies related to PD, astaxanthin has been shown to protect SH-SY5Y cells from 6-OHDA [53]. In a similar experiment, astaxanthin treatment mitigated cytotoxicity in PC12 cells from MPP+ induced cytotoxicity. MPP+ is a toxic metabolite of the dopaminergic neurotoxin MPTP used in experimental animal models of PD [71]. These cell culture results were corroborated by an in vivo study using astaxanthin to prevent the neurodegeneration in the SN in response to dose of MPTP (1 i.p. dose 30 mg/kg daily for 28 days) [54]. This treatment regimen effectively protected against the loss of tyrosine hydroxylase in the SN and striatum after chronic exposure to the neurotoxin. However, it is important to understand that many drugs that have been successful in some pre-clinical models of PD have failed to translate to patients with PD. Developing and testing pre-clinical models involving disease relevant proteins such as  $\alpha$ -synuclein and the impact of aging must be considered for future studies.

## 6. Conclusions

Parkinson's disease is primarily characterized by degeneration of the dopaminergic neurons of the substantia nigra. The pathophysiology underlying this cell death is not yet clearly understood, although it is evident that many biological processes are impaired in this vulnerable brain region, explaining the rapid deterioration of the SN with age. Neuroinflammation is an integral factor perpetuating cellular damage during progression of the disease, and efforts to mitigate the inflammatory cascade have been successful in experimental settings, suggesting that anti-inflammatory treatments are a viable therapeutic strategy to employ in managing Parkinson's disease. Fractalkine signaling has proven to be a critical pathway in inflammation-mediated cell death that occurs in animal models of PD. Astaxanthin has diverse biological activities that have been reported in the literature, many of which seem to directly oppose the pathological mechanisms involved in neurodegeneration of the SN. Both fractalkine and astaxanthin represent two promising novel therapeutic agents for the treatment and management of PD.

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Article

# Prior Binge Ethanol Exposure Potentiates the Microglial Response in a Model of Alcohol-Induced Neurodegeneration

Simon Alex Marshall <sup>1</sup>, Chelsea Rhea Geil <sup>2</sup> and Kimberly Nixon <sup>2,\*</sup>

<sup>1</sup> Department of Psychology & Neuroscience; University of North Carolina-Chapel Hill, Chapel Hill, NC 27599, USA; simon.alexm@unc.edu

<sup>2</sup> Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY 40536, USA; chelsea.geil@uky.edu

\* Correspondence: kim-nixon@uky.edu; Tel.: +1-859-215-1025

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**Abstract:** Excessive alcohol consumption results in neurodegeneration which some hypothesize is caused by neuroinflammation. One characteristic of neuroinflammation is microglial activation, but it is now well accepted that microglial activation may be pro- or anti-inflammatory. Recent work indicates that the Majchrowicz model of alcohol-induced neurodegeneration results in anti-inflammatory microglia, while intermittent exposure models with lower doses and blood alcohol levels produce microglia with a pro-inflammatory phenotype. To determine the effect of a repeated binge alcohol exposure, rats received two cycles of the four-day Majchrowicz model. One hemisphere was then used to assess microglia via immunohistochemistry and while the other was used for ELISAs of cytokines and growth factors. A single binge ethanol exposure resulted in low-level of microglial activation; however, a second binge potentiated the microglial response. Specifically, double binge rats had greater OX-42 immunoreactivity, increased ionized calcium-binding adapter molecule 1 (Iba-1+) cells, and upregulated tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) compared with the single binge ethanol group. These data indicate that prior ethanol exposure potentiates a subsequent microglia response, which suggests that the initial exposure to alcohol primes microglia. In summary, repeated ethanol exposure, independent of other immune modulatory events, potentiates microglial activity.

**Keywords:** alcohol; ethanol; microglia; cytokines; TNF-alpha; alcoholism; microglial priming; neurodegeneration

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## 1. Introduction

Nearly 14% of the United States population meets the diagnostic criteria for an alcohol use disorder (AUD) in any given year [1]. Excessive alcohol consumption produces neurodegeneration in humans [2–4], an effect that has been confirmed in various pre-clinical models [5–8]. Due to its preventable nature, alcoholism traditionally has not been defined as a neurodegenerative disorder, but chronic, excessive consumption may cause damage in the temporal lobe on par with diseases such as Alzheimer’s [4]. Indeed, alcoholic-related dementia is the second leading cause of dementia in the United States only behind Alzheimer’s disease [9,10]. Even in the absence of dementia, cognitive deficits such as increased impulsivity and impaired executive decision-making are found in many with AUDs [11,12]. Alcohol-induced neurodegeneration and the associated cognitive deficits are thought to be critical factors in the development of AUDs [13–15].

Despite the number of reports in human and preclinical models describing the neurotoxic effects of alcohol, the mechanism of how alcohol produces neurodegeneration is unclear [16]. One such mechanism that has recently gained attention is the impact of excessive alcohol consumption on the neuroimmune system, and particularly, microglia [17,18]. Analysis of the brains of human alcoholics suggests that excessive alcohol consumption leads to microglial activation [19–21], but whether this activation is the cause or consequence of alcohol-induced neurodegeneration is an active debate [22]. This discussion is due, in part, to a lack of understanding of the effect of alcohol on microglia coupled with the recent appreciation of the role of microglia in both neurodegenerative and regenerative processes [22–25]. Although microglia have historically been discussed as the phagocytes of the central nervous system (CNS), these cells are far more complex, existing in a continuum of phenotypes or stages of activation [26]. Microglia are constantly surveying the parenchyma in non-pathological conditions; where in response to even a subtle change in their environment, microglia alter their morphological and functional characteristics, a process termed microglial activation [27]. The nomenclature for these stages or phenotypes vary. Terms like M1 and classical activation are applied when microglia have an amoeboid morphology and secrete pro-inflammatory cytokines, whereas M2 and alternative activation are used to describe microglia with bushier ramifications that secrete anti-inflammatory cytokines [26,28]. In neurodegenerative diseases where microglial activation drives neuronal loss, microglia are generally fully or classically activated (*i.e.*, M1 phenotype), secreting pro-inflammatory factors and undergoing uncontrolled phagocytosis [25,29]. How alcohol affects microglia is not well described and appears to vary depending on the model. Most reports of alcohol-induced microglia activation assume that all activated microglia are pro-inflammatory [19,23,30]. However, in the one model with alcohol-induced neurodegeneration, the Majchrowicz four-day binge model, only a low level of activation or alternative (M2) phenotype has been observed [22,24,31].

The variability of microglial phenotypes observed across different AUD models may be due to the pattern of alcohol exposure, specifically intermittent *versus* sustained intoxication. Interestingly, the intermittent exposure models show stronger evidence of pro-inflammatory microglia even with lower doses of ethanol [22,30]. These disparate findings across models led us to question whether the initial hit of alcohol exposure “primes” microglia such that intermittent exposure leads to a potentiated response. Primed microglia have similar morphology and cytokine/growth factor profiles as the M2/alternative microglia, but primed microglial activation is potentiated when subsequent neuroimmunomodulators are applied [28,32,33]. Ethanol’s ability to prime microglia and exacerbate the neuroimmune response to subsequent neuroimmune stimuli is suggested also by the enhanced microglia response to LPS following alcohol exposure [23,34,35]. However, the ability of a second “hit” or insult of ethanol to potentiate the neuroimmune response (independent of peripheral immunomodulators) has not been examined. Therefore, the current study determines whether a second binge ethanol exposure can potentiate the microglia response to binge alcohol exposure. Investigating whether repeated ethanol exposure differentially affects microglia is important considering that the majority of individuals suffering from an AUD drink in a binge pattern that produces periods of high BECs interspersed with periods of withdrawal and abstinence [36–38]. Specifically, this study examines both functional and morphological indices of microglial activation in the hippocampus and entorhinal cortex, regions consistently damaged in this model [7,8].

## 2. Materials and Methods

### 2.1. Alcohol Administration Model

A total of 33 adult male Sprague-Dawley rats (Table 1; Charles River Laboratories; Raleigh, NC, USA) were used in these experiments. Procedures performed were approved by the University of Kentucky Institutional Animal Care and Use Committee (protocol #2008-0321, approved 20/6/2008) and conformed to the Guidelines for the Care and Use of Laboratory Animals [39]. Animals weighed approximately 275–300 g at arrival and were pair-housed in a University of Kentucky AALAC

accredited vivarium with a 12 h light:dark cycle. Rats were allowed to acclimate to the vivarium for two days followed by three days of handling before any experimentation. Except during the binge periods, animals had *ad libitum* food and water access. Following acclimation, rats underwent a modified version of the Majchrowicz AUD model similar to previously published reports [40–42]; however, animals used in this study underwent the Majchrowicz 4-day paradigm twice separated by seven days. Rats were divided into four groups of comparable weights as summarized in Table 1. Briefly, rats were gavaged intragastrically with either ethanol (25% *w/v*) or control diet (isocaloric dextrose) in Vanilla Ensure Plus<sup>®</sup> (Abbott Laboratories; Chicago, IL, USA) every 8 h. Initially, each rat in an ethanol group received 5 g/kg of ethanol, but subsequent doses were titrated using the individual rat's behavioral intoxication score on a six-point scale identical to previous reports [40]. Control rats received an average of the volume given to the ethanol group. All rats were then given seven days of recovery with *ad libitum* access to food and water. A seven-day recovery period was chosen because microglial activation is elevated for a week after ethanol exposure [22], and seven days allowed animals to recover from withdrawal and regain body mass lost during the prior binge. Thus, on the 11th day, the Majchrowicz binge model was repeated with rats receiving either ethanol or control diet (Table 1). A separate group had *ad libitum* access to food and water throughout all periods. For all groups, body weights were assessed daily during the binge procedures. The percent difference in weight at the start and end of the 15-day treatment period was calculated.

**Table 1.** Experimental Design.

| Group                             | Binge 1 (4 Days) | Recovery (7 Days)      | Binge 2 (4 Days) |
|-----------------------------------|------------------|------------------------|------------------|
| Con/Con ( <i>n</i> = 10)          | Control Diet     |                        | Control Diet     |
| Con/EtOH ( <i>n</i> = 11)         | Control Diet     | <i>Ad libitum</i> chow | Ethanol Diet     |
| EtOH/EtOH ( <i>n</i> = 8)         | Ethanol Diet     |                        | Ethanol Diet     |
| <i>Ad libitum</i> ( <i>n</i> = 4) | N/A              |                        | N/A              |

## 2.2. Blood Ethanol Concentration Determination

To determine blood ethanol concentrations (BECs), tail blood was collected ninety minutes after the seventh session of ethanol dosing during Binge 1 and/or at euthanasia (Binge 2). Bloods were centrifuged for 5 min at  $1800 \times g$  to separate plasma from red blood cells and immediately stored at  $-20\text{ }^{\circ}\text{C}$ . BECs were determined from supernatant serum on an AM1 Alcohol Analyser (Analox; London, UK) calibrated against a 300 mg/dL external standard. Each sample was run in triplicate and the average of these runs was calculated and expressed in mg/dL  $\pm$  SEM.

## 2.3. Tissue Processing

Rats were euthanized within 2–4 h of their final gavage by rapid decapitation. Brains were extracted and dissected into two hemispheres on ice. The left hemisphere was fixed by immersion in 4% paraformaldehyde in phosphate buffer (pH = 7.4) for 2 h, rinsed and stored in phosphate buffered saline at  $4\text{ }^{\circ}\text{C}$  until use in immunohistochemical experiments. The right hemisphere was further dissected to remove the hippocampus and entorhinal cortex. Extracted regions were snap frozen on dry ice and stored at  $-80\text{ }^{\circ}\text{C}$  until use in enzyme linked immunosorbent assays (ELISAs).

## 2.4. Immunohistochemistry

Immunohistochemical procedures were similar to previous reports [22,31]. The left hemisphere was sectioned in a 1:12 series at 40  $\mu\text{m}$  thickness with a vibrating microtome (Leica VT1000S; Wetzlar, Germany) and sections were stored in cryoprotectant at  $-20\text{ }^{\circ}\text{C}$ . Adjacent series of every 12th section were processed for immunohistochemistry. Briefly, after a series of washes (TBS, pH = 7.5), quenching of endogenous peroxidases (0.6%  $\text{H}_2\text{O}_2$  in TBS) and blocking of nonspecific antibody binding (TBS, 0.1% triton X-100, and 3% horse or goat serum as appropriate), tissue series was incubated overnight in

one of the following primary antibodies at 4 °C: mouse anti-OX-42 (1:1000; Serotec MCA275; Raleigh, NC, USA), mouse anti-ED-1 (1:500; Serotec MCA341), mouse anti-OX-6 (1:500; Serotec, MC2687), or rabbit anti-Iba-1 (1:1000; Wako, 019-19741; Richmond, VA, USA). Primaries were chosen for their specificity for microglia phenotypes [26,43]. OX-42 was selected as a marker of microglial activation because it recognizes cluster of differentiation molecule 11b/c (CD11b/c) of complement receptor 3 (CR3), which is constitutively expressed in microglia; however, upregulation of CD11b/c is one of the first indications of microglial activation [44–46]. Both ED-1 and OX-6 are selective for more classical forms of microglial activation [26]. ED-1 recognizes the lysosomal membranes of microglia and is thought to be an indication of phagocytic activity [47]. OX-6, however, is an antibody against the major histocompatibility complex-II that elicits T-helper cell activation [26,48]. The Iba-1 antibody was selected because it recognizes a calcium binding protein expressed in all microglia [49]. Sections were incubated in secondary antibody (biotinylated horse anti-mouse, rat adsorbed, or biotinylated goat anti-rabbit, Vector Laboratories, Burlingame, CA, USA), avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories) and the chromagen, nickel-enhanced 3,3'-diaminobenzidine tetrahydrochloride (Polysciences; Warrington, PA, USA), as previously described [22,31]. Following the final wash, all processed sections were mounted onto glass slides, dried and coverslipped with Cytoseal® (Stephens Scientific, Wayne, NJ, USA).

During quantification, slides were coded to ensure the experimenter was blind to treatment condition. To determine OX-42 immunoreactivity, images of the hippocampus (Bregma –2.50 and –4.00 mm) or entorhinal cortex (Bregma –3.00 and –6.00 mm) were obtained with a 10× objective on an Olympus BX-51 microscope (Olympus, Center Valley, PA, USA) linked to a motorized stage (Prior, Rockland, MA, USA), microcator and DP70 digital camera (Olympus) [50]. OX-42 immunoreactivity was determined by optical density with Visiomorph™ (Visiopharm, Hørsholm, Denmark). Subregions of the hippocampus (dentate gyrus (DG), cornu amonis (CA1 and CA2/3)) and the entorhinal cortex were traced separately and the percent area of OX-42 immunopositive pixels within each region of interest was determined. Immunoreactivity was then normalized to the *ad libitum* control group and expressed as percent of control.

For ED-1 or OX-6 immunohistochemistry, sections were qualitatively assessed in the hippocampus and entorhinal cortex as in past reports [22]. To determine the impact of ethanol on microglia number, Iba-1+ cells were counted within the hippocampus and the entorhinal cortex. Iba-1+ cells within the subregions of the hippocampus were estimated by unbiased stereological methods as previously reported [22,51]. NewCAST™ Stereology software (Visiopharm version 3.6.4.0) coupled to the same Olympus BX-51 microscope system above applied a 70 μm × 70 μm counting frame and cells were randomly sampled using a 20 μm disector height with 2 μm guard zones within the CA1 (400 μm x,y step length), CA2/3 (250 μm x,y step length), and DG (250 μm x,y step length). Total Iba-1+ cells were calculated using the equation (1):

$$N = \sum Q \times 1/asf \times 1/tsf \times 1/ssf \quad (1)$$

where Q is the number of cells counted, asf is the area sampling fraction, tsf is the thickness sampling fraction, and ssf is the section sampling fraction [52]. Coefficients of error ranged from 0.011 to 0.039 and averaged  $0.023 \pm 0.001$ . For the entorhinal cortex, microglia number was determined using a profile counting method [53]. Images of the entorhinal cortex were collected with a 10× objective using a SPOT Advanced™ camera (SPOT Imaging Solutions, Sterling Heights, MI, USA). Iba-1+ cells were quantified in collected images by an automated counting system (Image Pro Plus 6.3; Media Cybernetics, Rockville, MD, USA) and expressed as mean Iba-1+ cells/section ± SEM as previously described [22].

### 2.5. Enzyme Linked Immunosorbent Assay

Hippocampus and entorhinal cortex from the right hemisphere were processed for ELISA as reported previously [22,54]. Briefly, tissues were homogenized in an ice-cold lysis buffer (1 mL of

buffer/50 mg of tissue; pH = 7.4), then tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; Invitrogen, #KRC3011C; Camarillo, CA, USA) and interleukin-10 (IL-10; Invitrogen, #KRC0101) cytokine protein was determined via ELISA according to the manufacturer's instructions. These two cytokines were used to assess pro or anti-inflammatory microglia, respectively [43]. Brain derived neurotrophic factor (BDNF) was measured in the hippocampus (Millipore, #CYT306; Billerica, MA, USA) as the hippocampus is more susceptible to alcohol-induced BDNF dysregulation [55,56]. All samples and standards were run in duplicate. Absorbance was measured at 450 nm on a DXT880 Multimode Detector plate reader (Beckman Coulter; Brea, CA, USA). Cytokine concentrations were normalized to the total protein content as determined by a Pierce BCA Protein Assay Kit (Thermo Scientific; Rockford, IL, USA) and reported as pg/mg of total protein  $\pm$  SEM.

## 2.6. Statistical Analyses

Data were analyzed and graphed using Prism (version 5.04, GraphPad Software, Inc. La Jolla, CA, USA). Effects were considered significantly different if  $p < 0.05$ . Behavioral scores were analyzed with a Kruskal-Wallis test. All other analyses used a one-way ANOVA with *post-hoc* Tukey's test to compare groups if an effect of treatment was observed. Where appropriate, each region of the hippocampus or entorhinal cortex was considered independent and therefore analyzed separately. Correlations were conducted to examine the relationship of microglial markers of activation and the animal model data as well as microglial activation and cytokine concentration. Correlations were only run within the Con/EtOH or EtOH/EtOH group if *post-hoc* analyses showed a significant difference to control groups. Spearman analyses were used for intoxication behavior scores (nonparametric), while Pearson's analyses were used for all other factors (parametric).

## 3. Results

### 3.1. Animal Treatment Data

For animal model data, each binge period was analyzed independently. For example, BECs from Binge 1 and Binge 2 for the EtOH/EtOH group were analyzed separately. No differences were detected between any groups in either intoxication score ( $H(3) = 5.60$ ,  $p = 0.07$ ; grand mean =  $1.6 \pm 0.1$ ) or in BECs ( $F(2,24) = 0.78$ ,  $p = 0.32$ ; grand mean =  $399.8 \pm 12.4$  mg/dL) as shown in Table 2. However, one-way ANOVA revealed differences in the average dose per day ( $F(2,24) = 4.235$ ,  $p = 0.03$ ). A *post-hoc* Tukey's test indicated that ethanol doses of Binge 2 in the EtOH/EtOH rats were significantly higher than ethanol doses of the single binge (Con/EtOH) rats (Table 2). Body weights were also assessed to determine whether restricted caloric intake affected microglia activation [57,58]. One-way ANOVA indicated that treatment affected weight change ( $F(2,24) = 4.235$ ,  $p = 0.03$ ) (Table 2). A *post-hoc* Tukey's test showed that the weight change differed between all of the liquid diet groups (Con/Con, Con/EtOH, and EtOH/EtOH) compared with the *ad libitum* group. There was a significant effect of receiving ethanol on weight loss compared with the Con/Con group, but no difference between the Con/EtOH and EtOH/EtOH groups was observed (Table 3).

**Table 2.** Alcohol Model Data.

| Group                        | Intoxication Behavior (0–5 Scale) | Dose (g/kg/day)  | BEC (mg/dL)      |
|------------------------------|-----------------------------------|------------------|------------------|
| Con/EtOH (15th Day)          | $1.8 \pm 0.1$                     | $9.6 \pm 0.2$    | $422.2 \pm 21.1$ |
| EtOH/EtOH Binge 1 (4th Day)  | $1.7 \pm 0.1$                     | $9.9 \pm 0.4$    | $378.7 \pm 17.7$ |
| EtOH/EtOH Binge 2 (15th Day) | $1.3 \pm 0.2$                     | $11.0 \pm 0.5$ # | $390.3 \pm 24.0$ |

#  $p < 0.05$  compared to Con/EtOH.

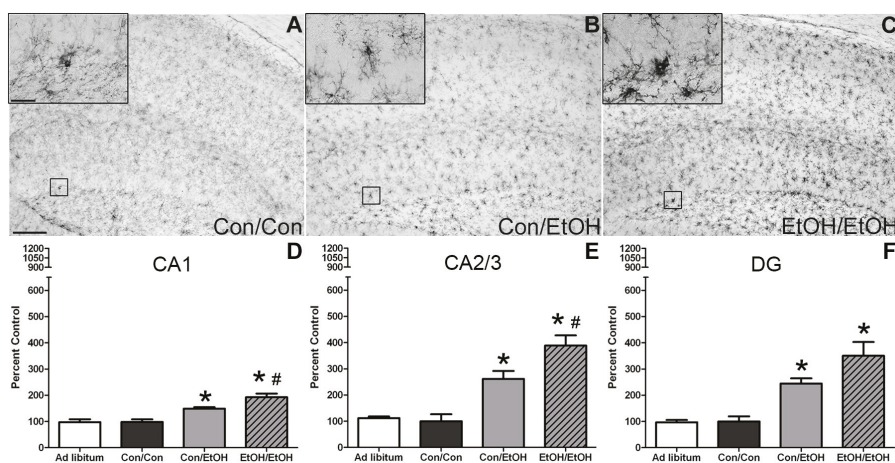
Table 3. Body Weight.

| Group                         | % Difference               |
|-------------------------------|----------------------------|
| Con/Con ( $n = 10$ )          | $+1.0\% \pm 1.4\%^\dagger$ |
| Con/EtOH ( $n = 11$ )         | $-6.6\% \pm 2.1\%^*$       |
| EtOH/EtOH ( $n = 8$ )         | $-8.7\% \pm 1.7\%^*$       |
| <i>Ad libitum</i> ( $n = 4$ ) | $+25.2\% \pm 1.7\%$        |

\*  $p < 0.05$  vs. Con/Con and *ad libitum*;  $^\dagger p < 0.05$  vs. *ad libitum* only.

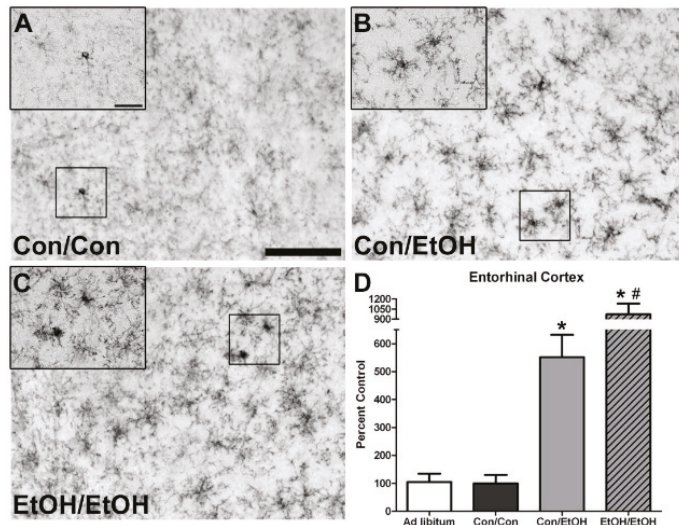
### 3.2. OX-42 Immunoreactivity Increased by EtOH Exposure

OX-42 expression was examined to determine whether microglia were further or differentially activated following a second binge exposure. OX-42 positive cells were apparent in all treatment groups, which is consistent with its constitutive expression in all types of microglia [59]; however, there was a visibly distinct increase in immunoreactivity in ethanol treated animals accompanied by an apparent morphological change. Microglia in ethanol animals appeared to have shorter but thickened ramifications compared with the control animals (Figures 1B,C and 2B,C). One-way ANOVAs indicated a significant effect of treatment in the CA1 ( $F(3,29) = 16.81, p < 0.0001$ ), CA2/3 ( $F(3,29) = 18.34, p < 0.0001$ ), and DG ( $F(3,29) = 14.43, p < 0.0001$ ) fields (Figure 1), as well as in the entorhinal cortex ( $F(3,28) = 19.01, p < 0.0001$ ) (Figure 2). As expected based on previous data [22], *post-hoc* Tukey's tests indicated a significant increase in OX-42 density in all ethanol treated groups in all subregions of the hippocampus compared with the control or *ad libitum* groups. Importantly, the EtOH/EtOH group showed greater immunoreactivity than Con/EtOH in all regions analyzed except the DG. Moreover, no difference in OX-42 was observed between *ad libitum* animals and the Con/Con group. Correlations between binge model parameters (intoxication behavior, dose per day, total dose, BEC, percent weight loss) and OX-42 immunoreactivity were run within the EtOH/EtOH and Con/EtOH group, but no significant correlations were observed (Table 4).



**Figure 1.** Potentiated Microglial Activation in the Hippocampus by Repeated Ethanol Exposure. OX-42 (CD11b/c) is upregulated in the hippocampus of ethanol-exposed rats as shown in representative photomicrographs of the (A–C) hippocampal dentate gyrus for (B) Con/EtOH and (C) EtOH/EtOH groups compared to (A) controls. Analysis of OX-42 immunoreactivity indicated that the EtOH/EtOH group had significantly more staining than the Con/EtOH group in the: (D) cornu amonis 1 (CA1) and (E) cornu amonis 2/3 (CA2/3) regions but not the (F) dentate gyrus (DG). Data expressed as a percentage of *ad libitum* control (not shown). Images were taken at 50 $\times$  magnification with insets at 600 $\times$  magnification. Scale bar = 200  $\mu\text{m}$ ; inset 30  $\mu\text{m}$ . \*  $p < 0.05$  compared to *ad libitum* and Con/Con groups; #  $p < 0.05$  compared to Con/EtOH.





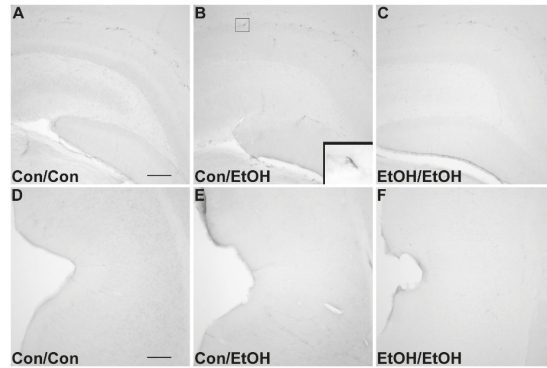
**Figure 2.** Potentiated Microglial Activation in the Entorhinal Cortex by Repeated Ethanol Exposure. OX-42 (CD11b) is upregulated in the entorhinal cortex of ethanol-exposed rats as shown in representative photomicrographs of the (A–C) entorhinal cortex for (B) Con/EtOH and (C) EtOH/EtOH groups compared to (A) controls. Analysis of OX-42 immunoreactivity indicated that the EtOH/EtOH group had significantly more positive pixels than the Con/EtOH group in the (D) entorhinal cortex. Data expressed as a percentage of *ad libitum* control (not shown). Images were taken at 200× magnification with insets at 600× magnification. Scale bar = 100 μm; inset 30 μm. \*  $p < 0.05$  compared to *ad libitum* and Con/Con groups; #  $p < 0.05$  compared to Con/EtOH.

**Table 4.** No Correlation between OX-42 and Model Parameters or Microglia Number.

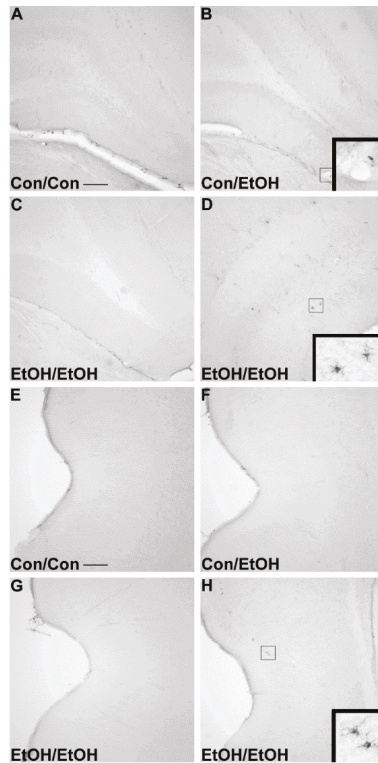
| Parameter             | Hippocampus  |               | Entorhinal Cortex |              |
|-----------------------|--------------|---------------|-------------------|--------------|
|                       | Con/EtOH     | EtOH/EtOH     | Con/EtOH          | EtOH/EtOH    |
| Intoxication Behavior | $S = 0.433$  | $S = 0.523$   | $S = 0.628$       | $S = 0.371$  |
| Dose/Day              | $p = -0.321$ | $p = -0.053$  | $p = -0.488$      | $p = -0.456$ |
| Total Dose            | $p = -0.303$ | $p = -0.0267$ | $p = -0.331$      | $p = -0.575$ |
| BEC                   | $p = 0.424$  | $p = -0.572$  | $p = -0.082$      | $p = 0.032$  |
| Percent Weight Loss   | $p = -0.222$ | $p = 0.249$   | $p = 0.029$       | $p = 0.319$  |
| Iba-1+ Cells          | $p = 0.161$  | $p = 0.539$   | $p = -0.136$      | $p = 0.357$  |

### 3.3. Lack of ED-1 or OX-6 Positive Cells

The ED-1 antibody was used to identify phagocytic microglia, whereas OX-6 was used to visualize the upregulation of MHC-II [26,29]. No ED-1 (Figure 3) positive cells were observed within the parenchyma of the hippocampus or entorhinal cortex of any animal in any group. No OX-6 (Figure 4) positive cells were observed within the parenchyma of the hippocampus or entorhinal cortex of any group, except for one EtOH/EtOH treated animal. This animal had several OX-6 cells in the more posterior regions of the hippocampus and entorhinal cortex (Figure 4D,H) but was not an outlier for any intoxication parameter including BEC, intoxication behavior, or ethanol dose per day. Interestingly, the morphology of these cells still appeared to be characteristic of the low grade, partial activation state of microglia as they are ramified and not amoeboid [26]. ED-1 and OX-6 positive cells were visible in blood vessels, the hippocampal fissure, and along the meninges in all treatment groups (Figures 3 and 4) similar to that observed previously following binge ethanol exposure [22,60]. Thus, repeated exposure to four-day binge ethanol treatment failed to significantly induce microglia to a phagocytic phenotype or state that expressed MHC-II in the brain parenchyma.



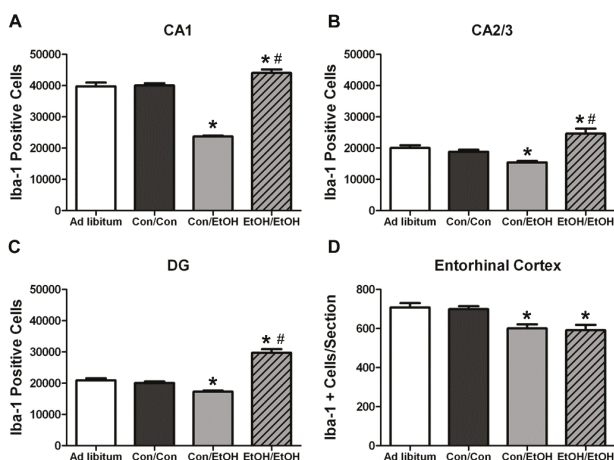
**Figure 3.** Lack of ED-1 Positive Cells. ED-1 was not visible in the parenchyma of the (A–C) hippocampus or (D–F) entorhinal cortex as seen in representative photomicrographs in (A,D) controls, (B,E) Con/EtOH (C,F) or EtOH/EtOH groups. ED-1 positive cells could be seen along the hippocampal fissure and blood vessels as shown in the inset of B. Scale bars = 200  $\mu$ m.



**Figure 4.** Lack of OX-6 Positive Cells. No OX-6 positive cells were observed regardless of treatment, except in one EtOH/EtOH rat as shown in representative photomicrographs of the (A–C) hippocampus or (E–H) entorhinal cortex in (A,E) controls, (B,F) Con/EtOH (C,G) or EtOH/EtOH groups. OX-6 positive cells could be seen along blood vessels as shown in the inset of B. One EtOH/EtOH animal showed upregulation of OX-6 in both the (D) hippocampus and (H) entorhinal cortex. Scale bars = 200  $\mu$ m.

### 3.4. Differential Effects of Treatment on Number of Microglia

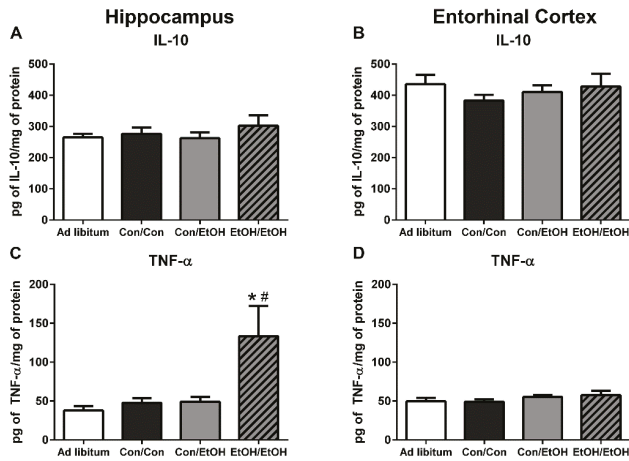
Stereology and profile counts were used to determine whether repeated ethanol exposure affected the number of microglia during ethanol exposure (Figure 5). One-way ANOVAs indicated a significant effect of treatment in the CA1 ( $F(3,29) = 161.6, p < 0.0001$ ), CA2/3 ( $F(3,29) = 17.99, p < 0.0001$ ), and DG ( $F(3,29) = 69.98, p < 0.0001$ ) fields, as well as in entorhinal cortex ( $F(3,28) = 6.78, p = 0.001$ ). *Post-hoc* Tukey's tests indicated a significant increase in the number of Iba-1+ cells throughout the hippocampus in the EtOH/EtOH group compared with all other groups (Figure 5A–C). However, in the entorhinal cortex microglia cells in the EtOH/EtOH group were decreased compared to the *ad libitum* and control groups but were similar to the number seen in Con/EtOH treated animals (Figure 5D). A *post-hoc* Tukey's test showed that Con/EtOH rats had decreased Iba-1+ cells in all regions measured as compared to Con/Con and *ad libitum* groups (Figure 5) [61]. Importantly, because the number of microglia can affect immunoreactivity, a correlation between the number of microglia versus OX-42 immunoreactivity was run, but no significant relationship was observed.



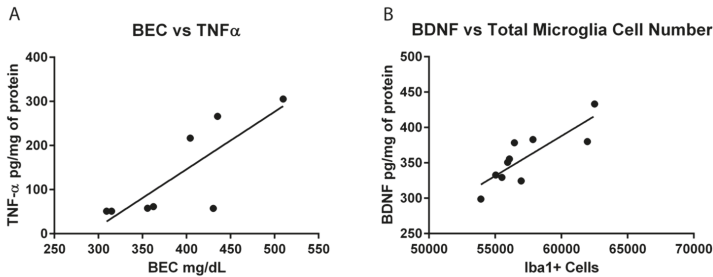
**Figure 5.** Microglial Cell Counts Differentially Altered by Ethanol Experience. Stereological estimates indicate an increase in the number of microglia in the EtOH/EtOH group in the (A) cornu amonis 1 (CA1), (B) cornu amonis 2/3 (CA2/3), and (C) dentate gyrus (DG) compared with all other groups. However, the number of microglia in the Con/EtOH group was decreased throughout the hippocampus. In the (D) entorhinal cortex, microglia were decreased in both the Con/EtOH and EtOH/EtOH groups compared to both the *ad libitum* and Con/Con groups. \*  $p < 0.05$  compared to *ad libitum* and Con/Con group; #  $p < 0.05$  versus Con/EtOH.

### 3.5. Increased Pro-Inflammatory Cytokine Expression in EtOH/EtOH Group

ELISAs were used to assess the functional state of microglia, specifically the anti-inflammatory cytokine, IL-10, and the pro-inflammatory cytokine, TNF- $\alpha$ . No changes were seen in IL-10 during intoxication among any groups in either the hippocampus ( $F(3,28) = 0.57, p = 0.64$ ) or the entorhinal cortex ( $F(3,24) = 0.50, p = 0.69$ ; Figure 6A,B). However, one-way ANOVAs of TNF- $\alpha$  protein concentrations indicated a significant effect of treatment in the hippocampus ( $F(3,28) = 4.658, p = 0.009$ ) but not the entorhinal cortex ( $F(3,24) = 0.99, p = 0.41$ ). *Post-hoc* Tukey's tests indicated a significant increase in TNF- $\alpha$  in the hippocampus in the EtOH/EtOH group compared to all other groups (Figure 6C). Correlations of binge parameters versus immunohistochemical results were run within the EtOH/EtOH group to further probe the distribution of TNF- $\alpha$  concentrations (Table 5). BECs correlated with TNF- $\alpha$  concentration ( $P(8) = 0.807, p = 0.016$ ; Figure 7).



**Figure 6.** Increased TNF-α in EtOH/EtOH Group. Concentrations of (A,B) interleukin-10 (IL-10) and (C,D) tumor necrosis factor-α (TNF-α) were determined by ELISA in both the hippocampus (A,C) and entorhinal cortex (B,D). No change in IL-10 was measured in either the hippocampus or the entorhinal cortex, but at least a 2.7-fold increase in TNF-α was measured in the (C) hippocampus in the EtOH/EtOH group compared with all other groups. However, no change in TNF-α was seen in the (D) entorhinal cortex. \*  $p < 0.05$  compared to *ad libitum* and Con/Con groups; #  $p < 0.05$  compared to Con/EtOH.



**Figure 7.** Correlations of Cytokines: (A) A positive correlation between blood ethanol concentration (BEC) and tumor necrosis factor-α (TNF-α) concentration. Animals with BECs over 400 mg/dL appear to have an increase in TNF-α. (B) A positive correlation between hippocampal estimates of microglia number and brain derived neurotrophic factor (BDNF) concentrations in the Con/EtOH group. A decline in the number of microglia cells correlated with decreases in BDNF concentrations.

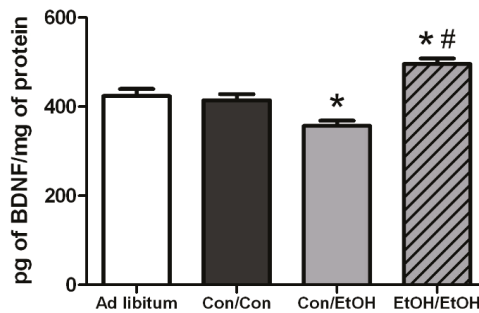
**Table 5.** Select Hippocampal Cytokine and Growth Factor Correlations.

| Parameter             | TNF-α        |               | BDNF          |              |
|-----------------------|--------------|---------------|---------------|--------------|
|                       | Con/EtOH     | EtOH/EtOH     | Con/EtOH      | EtOH/EtOH    |
| Intoxication Behavior | S = 0.451    | S = 0.371     | S = -0.421    | S = 0.216    |
| Dose/Day              | $p = -0.525$ | $p = -0.544$  | $p = 0.166$   | $p = -0.149$ |
| Total Dose            | $p = -0.496$ | $p = -0.355$  | $p = 0.160$   | $p = -0.144$ |
| BEC                   | $p = -0.081$ | $p = 0.807^*$ | $p = 0.166$   | $p = 0.298$  |
| Percent Weight Loss   | $p = 0.117$  | $p = 0.610$   | $p = 0.395$   | $p = -0.473$ |
| OX-42+ Density        | $p = 0.493$  | $p = -0.139$  | $p = 0.253$   | $p = -0.254$ |
| Iba-1+ Cells          | $p = -0.225$ | $p = -0.372$  | $p = 0.835^*$ | $p = 0.224$  |

\*  $p < 0.05$ .

### 3.6. Differential Effects of Treatment on BDNF Concentrations

Hippocampal BDNF concentrations were assessed to see the potential impact of microglia activity because alternative microglia, observed herein, are associated with neurotrophic support [62]. A one-way ANOVA on BDNF concentrations indicated a significant effect of treatment in the hippocampus ( $F(3,28) = 19.00, p < 0.0001$ ). *Post-hoc* Tukey's tests indicated a 20% increase in BDNF concentration in the hippocampus in the EtOH/EtOH compared with all other groups, but Con/EtOH rats had decreased concentrations of BDNF compared to both the Con/Con and *ad libitum* groups (Figure 8). Correlations between binge animal model data as well as markers of microglial activation were run *versus* BDNF concentrations for both the Con/EtOH and EtOH/EtOH groups (Table 5). The estimated total number of microglia ( $P(10) = 0.835, p = 0.003$ ) was correlated to BDNF concentrations only in the Con/EtOH group (Figure 7).



**Figure 8.** Ethanol Experience-Contingent Effects on BDNF. Concentrations of brain derived neurotrophic factor (BDNF) were determined by ELISA in the hippocampus. BDNF was decreased by approximately 15% in Con/EtOH treated animals compared with Con/Con or *ad libitum* groups but increased by 20% in the EtOH/EtOH group. \*  $p < 0.05$  vs. *ad libitum* and Con/Con groups; #  $p < 0.05$  vs. to Con/EtOH.

## 4. Discussion

These data collectively indicate that microglia previously activated by alcohol exposure can be further exacerbated by a second alcohol binge. This point was demonstrated by: (a) potentiated OX-42 immunoreactivity; (b) increased microglial number; and (c) increased TNF- $\alpha$  concentration in EtOH/EtOH (double binge) rats compared with Con/EtOH (single binge) rats. The alcohol model used produces a low-grade microglial activation state that is similar to an M2 phenotype [22,31]. However, as the subsequent binge produced more pro-inflammatory-like effects, these alcohol-activated microglia may also be primed. This enhanced response to a second binge aligns with the definition of microglial priming, which is where a stimulus changes microglia to be more susceptible to and over-respond to a second insult [33,63,64]. Primed microglia and/or an exacerbated microglial response could lead to abnormally increased cell death and is a hypothesized etiology of neurodegenerative disorders [28]. Furthermore, given that the majority of individuals with an AUD drink in an episodic binge pattern [38,65], the repeated cycles of binge drinking with periods of withdrawal, and therefore repeated microglial insult, may lead to even more dynamic microglial activation over time.

The first evidence of this potentiated microglia response in the double binge group was increased immunoreactivity to the OX-42 antibody. Increased OX-42 immunoreactivity, which labels CR3, is one of the earliest signs of microglial activation [22,46]. CR3 is associated with cell adhesion necessary for removing pathogens or damaged/dying neurons [45,66,67]. Increased OX-42 staining has been reported in a number of animal models of ethanol exposure [22,68–70]. The current study confirms those findings; but furthers that work by showing that a second hit of binge ethanol exposure

potentiates OX-42 immunoreactivity. A potentiated increase in OX-42 immunoreactivity, or CR3 density, by ethanol is particularly interesting because CR3 is intimately involved in microglial priming [33]. The increased upregulation of CR3 in the EtOH/EtOH (double binge) rats compared with Con/EtOH (single binge) rats suggests that binge ethanol exposure acts as a priming stimulus to microglia. Morphology, though not specifically quantified, appeared consistent with a low grade/phenotype of activation as cells were ramified and not amoeboid (e.g., Figure 1) [26]. A bushy, ramified microglial morphology is also consistent with that observed in other pathologies that report a primed microglia state [33,64,71]. Furthermore, despite the potentiation of CR3 receptor density, no changes in ED-1 or OX-6 expression were seen following the second binge. The lack of visible ED-1+ or OX-6+ cells concurs with other reports in this model that do not show signs of classical microglial activation following ethanol exposure [22,31,60].

Because multiple endpoints should be measured to understand the phenotype of microglia after insult, functional outputs such as hallmark pro- and anti-inflammatory cytokines were measured to better understand the type of microglial activation associated with a second “hit” of ethanol exposure. No change in the concentration of the hallmark anti-inflammatory cytokine, IL-10, was observed in either ethanol exposure group in the hippocampus or entorhinal cortex. The lack of IL-10 response during intoxication confirms previous findings in this model, although IL-10 is decreased in a mouse AUD model [22,23]. However, upregulation of TNF- $\alpha$  in the hippocampus in the EtOH/EtOH group compared with all other groups suggests that the second binge promoted a pro-inflammatory state. This finding is highly distinct from multiple previous reports using Majchrowicz-like models where no effect of ethanol was observed on TNF- $\alpha$  concentrations [22,24,31] and highlights the impact of repeated ethanol exposure on pro-inflammatory cytokine production and microglial activation. The potentiation of TNF- $\alpha$  expression by the second hit of ethanol, much like the morphological indices, is a common response for microglia that are primed and then hit with a secondary peripheral immune insult [28,64,72]. In fact, alcohol and other drugs of abuse have been shown to prime the TNF- $\alpha$  response to other immune stimulators [23,63], but these findings specifically suggest that alcohol exposure can act as both the priming and secondary stimulus resulting in an increase in TNF- $\alpha$ .

In the Majchrowicz model, microglia loss was observed during the last days of intoxication [61], whereas microglia proliferation occurs after the cessation of alcohol exposure, on the second day of abstinence [31,60]. Therefore, Iba-1+ cell number was assessed to determine how multiple cycles of ethanol affects microglia number. The single ethanol binge (Con/EtOH group) reduced the number of Iba-1+ microglia, in both the hippocampus and entorhinal cortex. Our recent work supports that this reduction is likely due to degeneration of microglia following 4-day binge exposure [61]. Interestingly, the second binge (EtOH/EtOH group) resulted in an increased number of Iba-1+ microglia in the hippocampus compared to either the control group or single binge (Con/EtOH) group. It is plausible that the increase in Iba-1+ cells in the EtOH/EtOH group observed in the hippocampus is due to microglial proliferation at two days following the first binge [22,31,60]. This effect also suggests that ethanol does not significantly reduce these newly proliferated microglial cells. It is of note that the effect of ethanol on microglia number varied by region: in the entorhinal cortex, both the single and double binge resulted in a decrease in the number of microglia consistent with our recent report [61]. The lack of increased Iba-1+ cells in the entorhinal cortex of EtOH/EtOH group is likely related to the finding that microglia neither proliferate dramatically at two days post-binge in the entorhinal cortex nor is there a significant increase in microglia number after seven days; however, both proliferation and increased Iba-1+ cells have been observed in the hippocampus at this same time point [22,60]. Why microglia proliferate in the hippocampus but not entorhinal cortex after binge ethanol exposure is puzzling. Neurons in the entorhinal cortex degenerate more robustly, peaking at four days of exposure [5,7,8], which is followed by other signs of reactive microgliosis [22]. More studies are necessary to fully understand the dynamic effects of alcohol on microglia number, especially considering the recent discoveries that microglia contribute to synapse refinement and plasticity [25]. These data support the hypothesis that a second binge alcohol exposure exacerbates the microglial

response, since an increase in the number of activated microglia would likely result in a potentiated neuroimmune response during the second binge.

Hippocampal BDNF concentrations were determined in order to assess the impact of microglia reactivity and changes in microglia number on the surrounding environment. BDNF plays a pivotal role in neuronal integrity and its dysregulation is associated with neurodegeneration [73]. In the Con/EtOH group, BDNF was decreased, the number of microglia were decreased and there was a significant correlation between the number of microglia and BDNF protein expression. However, in the EtOH/EtOH treated animals, where microglia were more activated and their numbers were increased, a significantly higher BDNF concentration was observed, though this value did not correlate significantly to microglia number. It is possible that the increase in BDNF concentrations is due to cells other than microglia, such as astrocytes, neurons, and other CNS cells secreting BDNF [74]. In addition, the effect of ethanol on BDNF expression is quite complex [75]. Nevertheless, the interplay between the increased cytokine and neurotrophin production observed in the EtOH/EtOH group requires further study to understand its functional implications.

The experimental design to use the same animals for both immunohistochemical and ELISA experiments allowed for a series of correlations to help determine what aspect of ethanol exposure, in this AUD model, was associated with microglial reactivity. OX-42 immunoreactivity did not correlate to average dose per day or to the total dose of ethanol in either the Con/EtOH or EtOH/EtOH groups. This lack of correlation is important as immune modulators such as LPS have dose-dependent responses in microglia reactivity [76]. The lack of correlation between OX-42 and total dose of ethanol suggests that ethanol potentiates the OX-42 response by acting as a secondary stimulus rather than an additive effect of the accumulative dose. Moreover, no relationship between the number of Iba-1+ cells and OX-42 immunoreactivity were observed, supporting that increased OX-42 immunoreactivity was a result of microglial activation and not an artifact of the change in cell number [77]. Correlations were also used to examine the relationship between OX-42 immunoreactivity or Iba-1 cell number and functional indices (cytokine/neurotrophin production). However, neither CR3 receptor (OX-42) upregulation nor Iba-1+ cell number was significantly correlated with TNF- $\alpha$  expression. Interestingly, the bimodal distribution of TNF- $\alpha$  production observed in the EtOH/EtOH group did map on to BECs. Although the mechanism by which BECs are related to TNF- $\alpha$  were not measured, at minimum, this correlation suggests that as BECs increase with repeated exposure, a primed microglial state may cause increased pro-inflammatory cytokines. Finally, in relation to BDNF, only microglia cell number in the Con/EtOH group showed a significant correlation with BDNF concentrations supporting the idea that microglial dysfunction and subsequent loss of trophic factors may contribute to neurodegeneration, especially alcoholic brain damage [61]. Correlations are not being interpreted as causation, but they do provide direction for what aspects of alcohol-exposure impact microglia reactivity leading to a primed microglial state.

Some evidence of classical activation has been observed in other AUD models, an effect that may be attributable to species differences and/or variations in the duration and pattern of exposure [30,69]. While previous reports suggested that the difference in microglia reactivity was due to these aforementioned variations in AUD models, the current data in this report more definitively indicates that it is the repeated insult that may drive the greater microglial response. For example, a model of alcohol exposure with lower total doses of alcohol dispersed over a longer period of time produced more OX-6 positive cells than the exposure used herein, where OX-6 expression may have been an anomaly in a single animal [30]. The appearance of the OX-6+ cells, however, in both models still appeared to be the bushy, ramified morphology associated with a low-level or M2-like activation. Indeed, the only alcohol study, human or animal, where ED-1+ microglia have been observed, is from a study in which rats underwent four cycles of a Majchrowicz-like model with three days between binges. However, the high mortality rate and severe weight loss of rats in that report make interpretations difficult. One interpretation is that microglial activation may have occurred due to the stress of repetitive gavage and/or weight loss [57,78–80]. Thus, the current study specifically

used a seven day abstinence period to allow rats to recover from four days of intoxication and the significant withdrawal sequelae that occurs in this model [40]. Moreover, because some weight loss is observed in the Majchrowicz model [40], repetitive gavage may be stressful [81], and both of these aspects modulate microglial reactivity [78,82], a group with *ad libitum* access to food and water was included. None of the measures of microglia activation were different between the *ad libitum* group and the Con/Con group despite their slight weight loss and experience with gavage. Moreover, weight loss did not correlate with any measure of microglial activation in animals receiving ethanol.

The potentiated microglia activation seen in this double binge AUD model suggests that the microglial response can be altered by ethanol alone and supports the idea that chronic ethanol exposure can elicit a more pro-inflammatory state than a single bout of binge exposure. The lack of expression of ED-1 and morphology of OX-42+ and Iba-1+ cells support that even with the two binges, cells are not fully or classically activated. However, microglia are “further” down the spectrum towards classical activation than a single binge alone. These data coupled with the lack of evidence for classically activated microglia in human alcoholic brain—whether the markers are not expressed or no one has examined those particular markers—supports that initially microglia activation is likely a consequence of alcoholic neuropathology and not a cause. Whether this increased response causes microglia to over-respond to insult or if it makes the brain more susceptible to ongoing neuroinflammation should be considered in future experiments. In addition, how these effects relate to changes in neurodegeneration, specifically neuronal or volume loss, is an important area for future study. Because microglia have the capacity to maintain low grade activation or a primed state for extensive periods following insult, including alcohol exposure [22,83], the episodic nature of binge drinking would lead to a cycle of repeated priming and over-response in individuals suffering from an AUD [18,38,65]. Understanding the mechanisms that underlie or contribute to alcohol-induced neurodegeneration may provide a novel therapeutic target to ameliorate damage and prevent the downward spiral into an AUD [14,84].

## 5. Conclusions

In summary, these studies present a novel view of the impact of alcohol abuse on microglial activity. Specifically, data presented herein indicate that alcohol causes a shift in microglial phenotypes to a primed state. Although this study focuses on how later bouts of alcohol can exacerbate the microglial response, the implications of an alcohol-induced primed microglial state also extend to how the microglia of alcoholics may respond to infections or other alcohol related immune responses in the peripheral system. This research provides a context in which to consider the implications of microglia on alcohol-induced neurodegeneration and further indicates that targeting the neuroimmune system may alleviate deficits caused by excessive alcohol consumption.

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**Author Contributions:** Alex Marshall and Kimberly Nixon conceived and designed the experiments; Alex Marshall and Chelsea Geil performed the experiments; Alex Marshall analyzed the data; and all authors contributed to the interpretation of the data, writing and editing the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviations

The following abbreviations are used in this manuscript:

|       |  |
|-------|--|
| AALAC | Association for the Assessment and Accreditation of Laboratory Animal Care |
| ANOVA | Analysis of Variance   |
| AUD   | Alcohol Use Disorder   |
| BBB   | Blood Brain Barrier  |
| BEC   | Blood Ethanol Concentration  |



|               |  |
|---------------|--|
| BDNF          | Brain Derived Neurotrophic Factor          |
| CA            | Cornu Amonis                               |
| CNS           | Central Nervous System                     |
| Con           | Control                                    |
| CR3           | Complement Receptor 3                      |
| DG            | Dentate Gyrus                              |
| ELISA         | Enzyme-Linked ImmunoSorbent Assay          |
| EtOH          | Ethanol                                    |
| Iba-1         | Ionized calcium-Binding Adapter molecule 1 |
| IL-10         | InterLeukin-10                             |
| ip            | Intraperitoneal                            |
| TBS           | Tris-Buffered Saline                       |
| TNF- $\alpha$ | Tumor Necrosis Factor alpha                |

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Article

# Stress and Withdrawal from Chronic Ethanol Induce Selective Changes in Neuroimmune mRNAs in Differing Brain Sites

Darin J. Knapp<sup>1,2,†,\*</sup>, Kathryn M. Harper<sup>1,†</sup>, Buddy A. Whitman<sup>1,3,†</sup>, Zachary Zimomra<sup>1</sup> and George R. Breese<sup>1,2,3,4,5</sup>

<sup>1</sup> Bowles Center for Alcohol Studies, The University of North Carolina at Chapel Hill, CB7178, Chapel Hill, NC 27599-7178, USA; Kathryn\_harper@med.unc.edu (K.M.H.); bawhitman@gmail.com (B.A.W.); zzimomra@kent.edu (Z.Z.); george\_breese@med.unc.edu (G.R.B.)

<sup>2</sup> Department of Psychiatry, The University of North Carolina at Chapel Hill, CB7178, Chapel Hill, NC 27599-7178, USA

<sup>3</sup> Curriculum in Neurobiology, The University of North Carolina at Chapel Hill, CB7178, Chapel Hill, NC 27599-7178, USA

<sup>4</sup> Department of Pharmacology, The University of North Carolina at Chapel Hill, CB7178, Chapel Hill, NC 27599-7178, USA

<sup>5</sup> UNC Neuroscience Center, The University of North Carolina at Chapel Hill, CB7178, Chapel Hill, NC 27599-7178, USA

\* Correspondence: Darin\_knapp@med.unc.edu; Tel.: +1-919-966-0505; Fax: +1-919-966-5679

† These authors contributed equally to this work.

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**Abstract:** Stress is a strong risk factor in alcoholic relapse and may exert effects that mimic aspects of chronic alcohol exposure on neurobiological systems. With the neuroimmune system becoming a prominent focus in the study of the neurobiological consequences of stress, as well as chronic alcohol exposure proving to be a valuable focus in this regard, the present study sought to compare the effects of stress and chronic ethanol exposure on induction of components of the neuroimmune system. Rats were exposed to either 1 h exposure to a mild stressor (restraint) or exposure to withdrawal from 15 days of chronic alcohol exposure (i.e., withdrawal from chronic ethanol, WCE) and assessed for neuroimmune mRNAs in brain. Restraint stress alone elevated chemokine (C–C motif) ligand 2 (CCL2), interleukin-1-beta (IL-1 $\beta$ ), tumor necrosis factor alpha (TNF $\alpha$ ) and toll-like receptor 4 (TLR4) mRNAs in the cerebral cortex within 4 h with a return to a control level by 24 h. These increases were not accompanied by an increase in corresponding proteins. Withdrawal from WCE also elevated cytokines, but did so to varying degrees across different cytokines and brain regions. In the cortex, stress and WCE induced CCL2, TNF $\alpha$ , IL-1 $\beta$ , and TLR4 mRNAs. In the hypothalamus, only WCE induced cytokines (CCL2 and IL-1 $\beta$ ) while in the hippocampus, WCE strongly induced CCL2 while stress and WCE induced IL-1 $\beta$ . In the amygdala, only WCE induced CCL2. Finally—based on the previously demonstrated role of corticotropin-releasing factor 1 (CRF1) receptor inhibition in blocking WCE-induced cytokine mRNAs—the CRF1 receptor antagonist CP154,526 was administered to a subgroup of stressed rats and found to be inactive against induction of CCL2, TNF $\alpha$ , or IL-1 $\beta$  mRNAs. These differential results suggest that stress and WCE manifest broad neuroimmune effects in brain depending on the cytokine and brain region, and that CRF inhibition may not be a relevant mechanism in non-alcohol exposed animals. Overall, these effects are complex in terms of their neuroimmune targets and neuroanatomical specificity. Further investigation of the differential distribution of cytokine induction across neuroanatomical regions, individual cell types (e.g., neuronal phenotypes and glia), severity of chronic alcohol exposure, as well as across differing stress types may prove useful in understanding differential mechanisms of induction and for targeting select systems for pharmacotherapeutic intervention in alcoholism.

**Keywords:** restraint stress; chronic ethanol withdrawal; cytokine mRNAs; CRF; alcohol; CP154,526

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## 1. Introduction

Acute withdrawal from chronic ethanol (WCE) exposure is associated with increased anxiety-like behavior [1–3]. Breese et al. [4] also found in a three-withdrawal protocol that stress substituted for the initial two withdrawals such that withdrawal from a single five-day cycle of chronic ethanol induced anxiety. Subsequently, Breese et al. [5] found that the anxiety-like response to restraint stress was facilitated when the stress was applied after WCE—a finding in agreement with other reports that stress after WCE can enhance negative effects [6,7].

Breese et al. [8] and Knapp et al. [9] also reported that administration of lipopolysaccharide (LPS) or a cytokine into the brain substituted for the initial intermittent ethanol exposures applied prior to a single CE exposure to induce negative effects. This latter outcome was comparable to the change observed with prior exposure to stress [4,5] or after WCE [2,3,10]. More recently, Whitman et al. [9] reported that WCE increased cytokine mRNAs in the cortex—a cytokine immune response that was not related to infection [11–13]. Whitman et al. [9] also observed increases in mRNAs for toll-like receptor 4 (TLR4) and High Mobility Group Box 1 Protein (HMGB1) which serve as an endogenous system that activates neuroimmune function [11,12,14–16]. The induction of cytokine mRNAs increases after WCE was blocked by a CRF1 receptor (CRF1R) antagonist [9]—a finding possibly linking the cytokine mRNA changes to CRF involvement in the anxiety-like behavior that accompanies WCE and stress [3–5].

Various studies have linked stress to increases in cytokine mRNAs in various brain sites [17–25]. Collectively, these reports provided new information about the effects of various stressors (social stress/defeat, footshock or tailshock, restraint, forced swim, glucose/insulin challenge, or cold stress) on neuroimmune mRNA responses of the hypothalamus, hippocampus, cerebellum, posterior cortex, and nucleus of the solitary tract. Relatedly, work from our group had shown that restraint stress could substitute for the initial repeated exposures to chronic alcohol to induce a negative emotional state following a future withdrawal as inferred from anxiety-like behavior, (e.g., [4]). To explore the potential relevance of stress effects on neuroimmune responses in a chronic ethanol and withdrawal model, Breese et al. [8] administered LPS or a pro-inflammatory cytokine into brain to substitute for the initial intermittent ethanol withdrawals or mild stress to induce anxiety-like behavior following a single ethanol withdrawal that otherwise would be incapable of eliciting anxiety [4,5]. Missing from this strategy was an assessment of whether the restraint stress itself induced neuroimmune changes consistent with functional effects on behavior. Thus, a key new component of the current studies was to assess whether stress, which by itself has been shown in some studies to increase brain cytokines (e.g., [18,20,21,26]), produced changes comparable to those triggered by WCE. Additionally, comparisons were made of cytokine mRNA in different brain regions after stress or WCE. Finally, to complement earlier studies with WCE and cortical cytokines, the present study explored whether a corticotrophin-releasing factor receptor antagonist would attenuate stress-induced cytokines in the cortex. This line of inquiry is pertinent to understanding the relative overlap of neuroimmune effects of stress and the WCE model in relation to alcohol abuse, drug addiction, and several psychiatric disorders (see [6–8,27–35]).

## 2. Materials and Methods

### 2.1. Animals

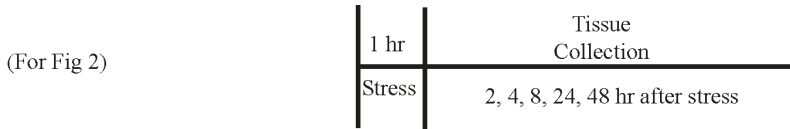
Adult male Sprague–Dawley (S–D) rats or Wistar Rats (Charles-River, Raleigh, NC, USA) weighing 180–200 g upon arrival were group housed and fed RMH3000 rat chow (Test Diets, Richmond, IN, USA) for 2–3 days prior to study to acclimate them to the new environment (temperatures 70–72 °F; humidity 40%–60%; and light/dark cycle 12 h:12 h with lights from 7:00 a.m. to 7:00 p.m.).

Subsequently, all rats were singly housed for the duration of experimental procedures. Methods used in this study were approved by Institutional Animal Care and Use Committee (IACUC, protocol number: 14-125) at the University of North Carolina (Chapel Hill, NC, USA).

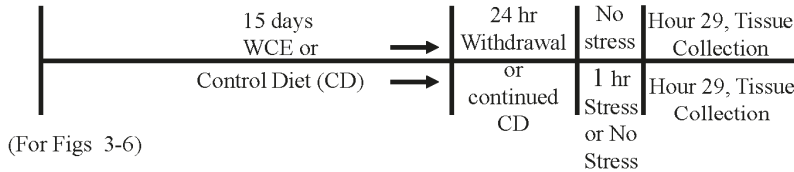
2.2. Liquid Diet for Controls and for Chronic Ethanol Exposure

In the initial experiment, rats that received an acute restraint stress were on chow diet before the stress challenge. For other experiments, a nutritionally-complete and calorically-balanced liquid diet was used for the rats that received a continuous 7% (*w/v*) ethanol diet followed by 24 h of withdrawal (WCE)—an approach previously utilized by our laboratory (e.g., [1,36,37]; see Figure 1) to administer daily ethanol doses of 9–13 g/kg. The liquid control diet (CD) was calorically balanced to the WCE diet by adjustments of the amount of dextrose. Rats were fed either control diet or the ethanol diet with a modified pair-feeding strategy [1].

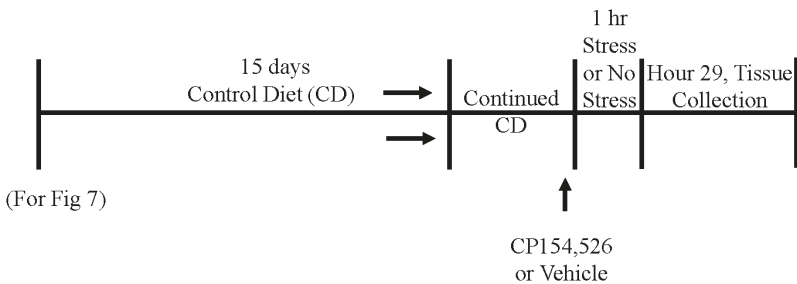
A Acute Restraint Stress Time Course



B Stress or WCE (Withdrawal from Chronic 7% Ethanol diet) Challenge Paradigms



C Stress Challenge Paradigm with CRF1R Antagonist Administration



**Figure 1.** Schematics depicting experimental protocols. **A:** represents the acute restraint stress time course, while **B:** represents the stress and withdrawal from chronic alcohol and **C:** represents the CRF1R antagonist study.

2.3. Restraint Stress in Controls and after Withdrawal from Chronic Ethanol Exposure

Initial efforts determined the time course of stress effects on brain cytokine mRNAs. Stress consisted of 60 min of restraint in plastic decapicones. These rats were sacrificed 2, 4, 8, 24, or 48 h following the stress (see schematic in Figure 1A).

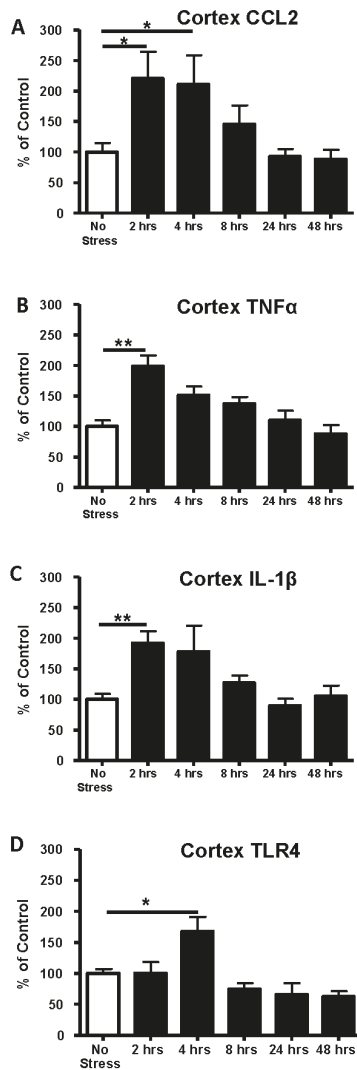


## 2.4. Brain Tissue Collection and Real-Time PCR Analysis for Tissue mRNA

Following experimental procedures, rats were rapidly decapitated and brain tissue stored at  $-80^{\circ}\text{C}$  for subsequent extractions for PCR. To initiate PCR procedures, total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) from homogenized dissected brain regions from control and ethanol-treated experimental brain sections followed by use of the SV total RNA isolation system (Promega, Madison, WI, USA). This tissue was then used for reverse transcription PCR using the Superscript First Strand or Superscript III First Strand Synthesis Super mix (Life Technologies, Grand Island, NY, USA) [38]. The primer sequences used for the cortex were chemokine (C-C motif) ligand 2 (CCL2) = 5'-TCACGCTTCTGGGCTGTG-3' (forward) and 5'-CAGCCGACTCATTGGGATC ATC-3' (reverse); Interleukin-1 $\beta$  (IL-1 $\beta$ ) = 5'-GAAACAGCAATGGTCGGGAC-3' (forward) and 5'-AA GACACGGGTTCCATGGT-3' (reverse); tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) = 5'-ATGTGGAAGTGGCAG AGGAG-3' (forward) and 5'-ACGAGCAGGAATGAGAAGAGG-3' (reverse); Toll-like-receptor-4 (TLR4) = 5'-GCCGAAAGTTATTGTGGTGGT-3' (forward) and 5'-ATGGGTTTATGGCGCAGAGTT T-3' (reverse);  $\beta$ -actin, 5'-ATGGTGGGTATGGGTCAGAAGG-3' (forward) and 5'-GCTCATTGTAG AAAGTGTGGTGCC-3' (reverse). SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) was used for real-time PCR analysis of the cortex on the Bio-Rad MyiQ (Bio-Rad, Hercules, CA, USA). For other brain regions (and the CP154,526 study), mRNA analyses were optimized with TaqMan<sup>®</sup> (Thermo Fisher Scientific, Waltham, MA, USA) expression assays—CCL2 (Rn00580555\_m1), TNF $\alpha$  (Rn01525859\_g1), IL-1 $\beta$  (Rn00580432\_m1), TLR4 (Rn00569848\_m1), and  $\beta$ -actin (Rn00667869\_m1)—and samples were run on a StepOnePlus real time PCR machine (Life Technologies, Grand Island, NY, USA). For all data, the cycle time (Ct) values were normalized with  $\beta$ -actin to assess the relative differences in expression between groups. Ct values of  $\beta$ -actin never differed across groups therefore  $\beta$ -actin was an appropriate choice as a housekeeping gene. Calculated values were expressed as relative change to a designated control set as 100%.

## 2.5. Enzyme-linked Immunosorbent Assay (ELISA) for Cytokines

Because changes in levels of cytokine proteins in the S-D rats have been found to correlate poorly with mRNA changes (see [38,39]) initially only expression of mRNAs for cytokines was assessed. Nonetheless, because of interest in the relationship between mRNA and proteins induced by stress, ELISA assays for cytokine proteins in cortex were first performed 4 h after stress in the time course determination in the S-D rats (Figure 2). Subsequently, assays of cytokine proteins were performed 4 h after an acute restraint stress to Wistar rats (Charles River, Raleigh, NC, USA). Each cortical sample was homogenized in Iscove's Modified Dulbecco Medium (Invitrogen, #12440046, Carlsbad, CA, USA) containing 1 tablet per 50 mL of the complete protease inhibitor cocktail (Roche Diagnostics #11697498001, Indianapolis, IN, USA). Homogenized specimens were then centrifuged at  $12,000 \times g$  for 10 min at  $4^{\circ}\text{C}$  and the supernatants collected and stored at  $-80^{\circ}\text{C}$  until the ELISA determination was made. ELISA kits were purchased for IL-1 $\beta$  and TNF $\alpha$  from R & D Systems, (Minneapolis, MN, USA), and for the CCL2 from BD Bio-Sciences (San Jose, CA, USA). ELISA procedures were performed according to the manufacturer's instructions. Standards for IL-1 $\beta$  and TNF $\alpha$  were serially diluted 4 times to concentrations of 1.95 pg/mL and 0.78 pg/mL, respectively, and the standard for CCL2 was used as supplied. All tissue cytokine levels were corrected for protein using Pierce<sup>®</sup> BCA Protein assay (Thermo Scientific, Rockford, IL, USA).



**Figure 2.** Time course of changes in cerebro-cortical mRNAs for CCL2, IL-1 $\beta$ , TNF $\alpha$ , and TLR4 following 1 h of restraint stress. Stress elevated CCL2 mRNA in cortex ( $F(5,32) = 3.82, p = 0.008$ ), an effect that peaked at 2 and 4 h. A similar effect was found for IL-1 $\beta$  mRNA ( $F(5,48) = 5.13, p < 0.01$ ) and TNF $\alpha$  ( $F(5,53) = 8.51, p < 0.0001$ ). TLR4 mRNA was elevated at 4 h ( $F(5,38) = 8.5, p < 0.0001$ ). In all cases, the cytokine mRNA levels gradually returned to control levels by 24 h. \*\*  $p < 0.01$  \*  $p < 0.05$  compared with controls that received no stress (open bars). A, B, C, and D delineate data for CCL2, TNF $\alpha$ , IL-1 $\beta$  and TLR4 mRNAs, respectively, ( $n = 5-8$  per time point)

## 2.6. CRF Receptor Antagonist Administration

Subgroups of CD rats were injected once with the CRF1R antagonist CP154,526 [38,40] or vehicle 15 min prior to the start of stress (Figure 1C). The drug was prepared as a microfine suspension in 0.5% carboxymethylcellulose and administered intraperitoneally at a dose of 15 mg/kg in a volume of 2 mL/kg.

## 2.7. Statistical Analysis

Data (expressed as mean  $\pm$  standard error of mean (SEM)) were evaluated for statistical significance with ANOVA with Fisher's least significant difference (LSD) tests for individual comparisons of group pairs as appropriate. Individual data points that were three standard deviations from their respective group means were removed from the group prior to analysis.  $p$ -values  $< 0.05$  were considered statistically significant.

## 3. Results

### 3.1. Time Course of Expression of Cytokine and TLR4 mRNAs in Cortex Following 1-Hour of Restraint Stress in Sprague–Dawley (S–D) Rats

Previous studies have shown that acute stress can affect neuroimmune function in brain [13,14,18–22,24–26,41]. Therefore, our initial investigation was to determine if the restraint stress utilized in previous behavioral studies [5] would induce a neuroimmune response. Figure 1A shows the experimental protocol for determining cytokine mRNA changes after 60 min of restraint stress in the absence of WCE. As shown in Figure 2, each mRNA assayed was increased 2–4 h after the restraint stress. The expression of CCL2 mRNA after stress was significantly increased above control by 121% at 2 h and by 111% at 4 h ( $p < 0.05$ ). The expression of TNF $\alpha$  mRNA after stress was increased above control by 2 h (99%) ( $p < 0.01$ ). Likewise, IL-1 $\beta$  mRNA was elevated by 92% above control by 2 h ( $p < 0.01$ ). Cytokine mRNAs gradually returned to control levels by 8 h and remained there for up to 48 h after the acute-stress exposure (Figure 2). Because TLR4 has been implicated in induction of cytokines [11,15,41–45], we also examined whether mRNA for TLR4 would be altered by the acute restraint-stress. Figure 2D shows that TLR4 mRNA expression was significantly elevated by 68% above control 4 h following the stress challenge ( $p < 0.05$ ) with return to control levels by 8 h.

### 3.2. Determination of Cytokine Protein Levels in Cortex after Restraint Stress

To determine whether increases in cytokine proteins accompanied the increases in CCL2, IL-1 $\beta$ , and TNF $\alpha$  mRNAs induced by restraint stress, proteins were measured in cortex 4 h after the restraint stress challenge to the S–D rats (Table 1). Cytokine protein levels, unlike cytokine mRNAs, in the controls were not statistically altered in the S–D rats (Table 1). Subsequently, this same assessment was performed for Wistar rats to determine if this rat strain might express a change in cytokine proteins following the 60 min of restraint stress. In the Wistar rats, as in the S–D rats, cytokine proteins were not increased by stress ( $p > 0.05$ ). Because increases in cytokine protein levels were not observed in either rat strain [38], only S–D rats were used in the experiments assessing expression of cytokine mRNAs induced by stress or WCE.

**Table 1.** Effect of acute stress on cytokine proteins in brain.

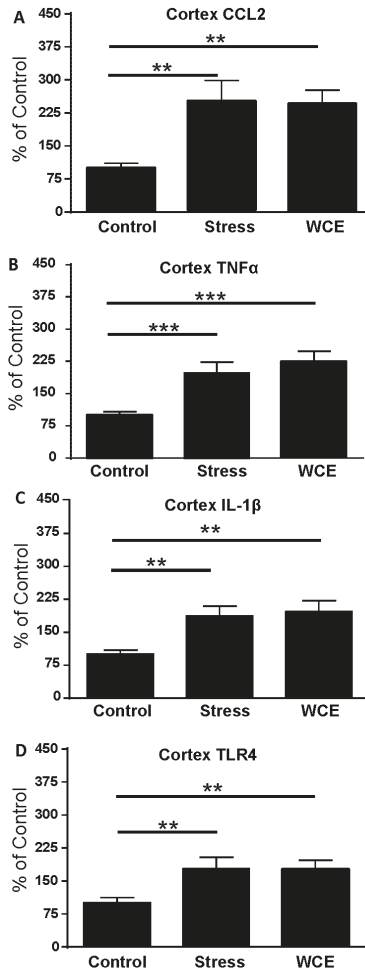
| Group               | CCL2        | IL-1 $\beta$ | TNF $\alpha$ |
|---------------------|-------------|--------------|--------------|
| S-D Non-Stressed    | 12.9 (0.3)  | 0.37 (0.05)  | ND           |
| S-D Stressed        | 13.7 (0.6)  | 0.28 (0.02)  | ND           |
| Wistar Non-Stressed | 42.2 (2.3)  | 1.48 (0.22)  | 0.11 (0.03)  |
| Wistar Stressed     | 58.1 (15.4) | 1.05 (0.84)  | 0.12 (0.03)  |

Data are mean  $\pm$  standard error of mean (SEM) protein/mg total protein from cerebral cortex of rats that were restrained for 1 h and sacrificed 4 h later. CCL2: Chemokine (C–C motif) ligand 2; IL-1 $\beta$ : Interleukin-1-beta; TNF $\alpha$ : Tumor Necrosis Factor alpha; ND: not detectable; S–D: Sprague–Dawley.

### 3.3. Effect of Stress or WCE on Selected Cytokine and TLR4 mRNAs in Cortex

In prior work, WCE increased anxiety-like behavior [46] and elevated cortical cytokines [38]. This study directly compared the magnitude of the WCE effect on cytokine mRNA with that produced by stress. Figure 3 shows that an acute 60 min restraint stress increased cortical cytokine mRNAs

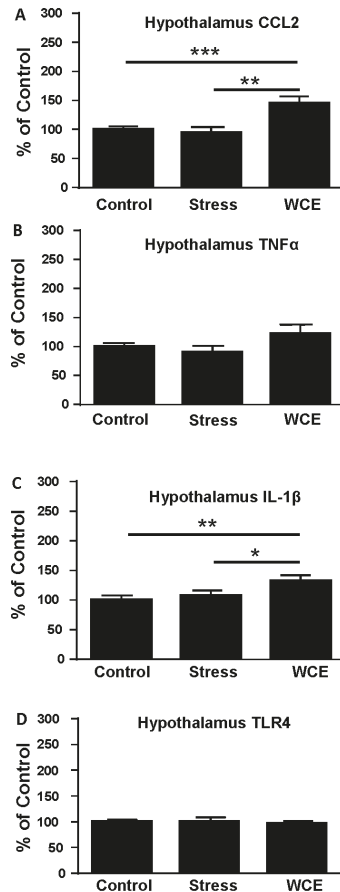
4 h later to a degree comparable to that observed in Figure 2. Likewise, in accord with previous work [38], Figure 3 confirms that CCL2, IL-1 $\beta$ , TNF $\alpha$ , and TLR4 mRNAs were significantly increased over controls in cortex 29 h after WCE ( $p < 0.05$ ). These cytokine mRNA increases following WCE were comparable in magnitude (80%–150% over control) to the increases induced by stress (Figure 2).



**Figure 3.** Effects of restraint stress or withdrawal from chronic ethanol (WCE) on cerebro-cortical neuroimmune mRNAs. Control and stress rats received non-ethanol containing liquid diets and 1 h restraint stress or no stress, respectively. WCE rats received chronic ethanol liquid diet for 15 days followed by 29 h of withdrawal. Overall, a significant effect was also noted across the groups for CCL2 ( $F(2,23) = 6.47, p < 0.01$ ) with individual comparison of groups revealing significant effects of stress or WCE relative to controls. A similar profile was noted for TNF $\alpha$  ( $F(2,32) = 10.2, p < 0.001$ ), IL-1 $\beta$  ( $F(2,27) = 7.00, p < 0.01$ ), and TLR4 ( $F(2,31) = 4.97, p < 0.05$ ) and individual group comparisons to the respective controls. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  versus Controls. **A, B, C,** and **D** delineate data for CCL2, TNF $\alpha$ , IL-1 $\beta$  and TLR4 mRNAs, respectively, ( $n = 8$ –12 per group).

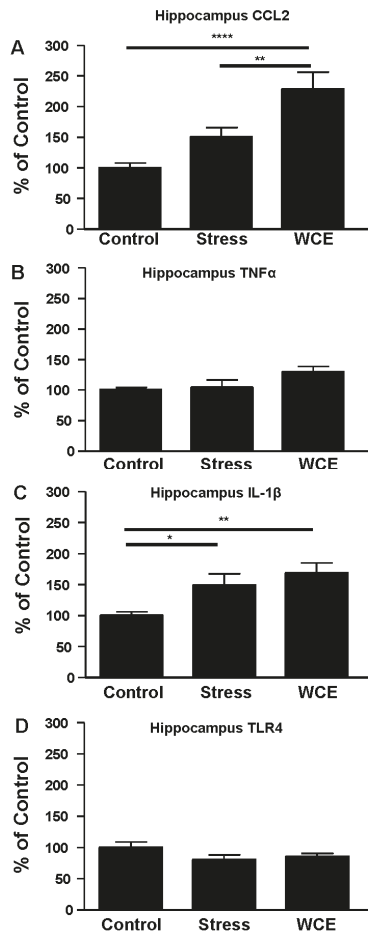
### 3.4. Effect of Stress or WCE on Selected Cytokine mRNAs in Hypothalamus, Hippocampus, and Amygdala after WCE

To examine the generalizability of the cortical neuroimmune mRNA response, the effects of stress or WCE were studied in additional brain regions of known importance in chronic ethanol effects. Figure 4 illustrates that, whereas stress did not increase CCL2 or IL-1 $\beta$  mRNA in the hypothalamus ( $p > 0.05$ , Figure 4A,C), WCE increased both of these cytokine mRNAs significantly (by about 30%–45%,  $p < 0.01$ ). Further, hypothalamic TLR4 and TNF $\alpha$  mRNAs were not significantly altered by either challenge ( $p < 0.05$  vs. control).

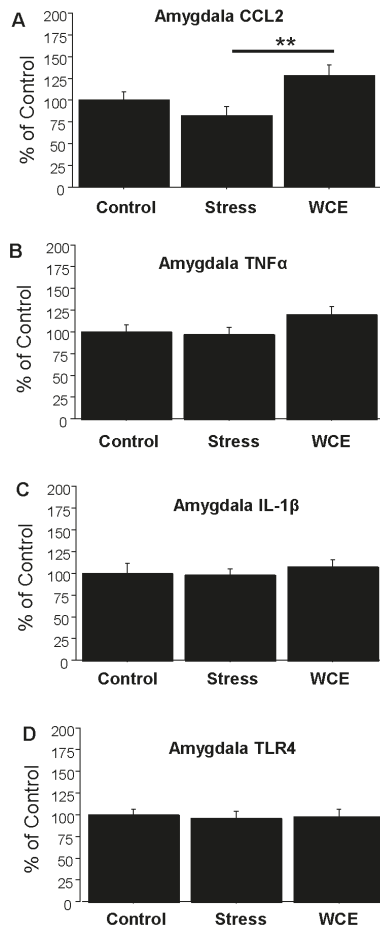


**Figure 4.** Effects of restraint stress or WCE on hypothalamic neuroimmune mRNAs. For CCL2, there was a significant overall effect of treatments ( $F(2,35) = 9.13$ ,  $p < 0.001$ ) with a significant group comparison relative to controls revealed only with the WCE treatment. For TNF $\alpha$ , there was no overall effect of treatment ( $p > 0.05$ ) despite a trend toward a WCE effect. There was an overall effect of treatment on IL-1 $\beta$  ( $F(2,38) = 4.59$ ,  $p < 0.05$ ) with the WCE group being higher than controls or stressed rats. Finally, there were no effects on TLR4. Group designations are the same as those in Figure 3. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  versus controls. A, B, C, and D delineate data for CCL2, TNF $\alpha$ , IL-1 $\beta$  and TLR4 mRNAs, respectively, ( $n = 8$ –13 per group).

In the hippocampus, CCL2 (Figure 5A) increased with stress (by approximately 50%), but the effect of WCE was more dramatic (129% of control) and significantly higher than that for stress. In contrast, while WCE tended to increase TNF $\alpha$  in this region, no significant effects were found in response to stress (Figure 5B), with a similar result for TLR4 (Figure 5D). With regard to IL-1 $\beta$  (Figure 5C), both stress and WCE comparably induced this cytokine. Finally, in the amygdala (Figure 6), there was no effect of stress alone on any measure ( $p > 0.05$ ), although WCE significantly increased CCL2 relative to stress levels.



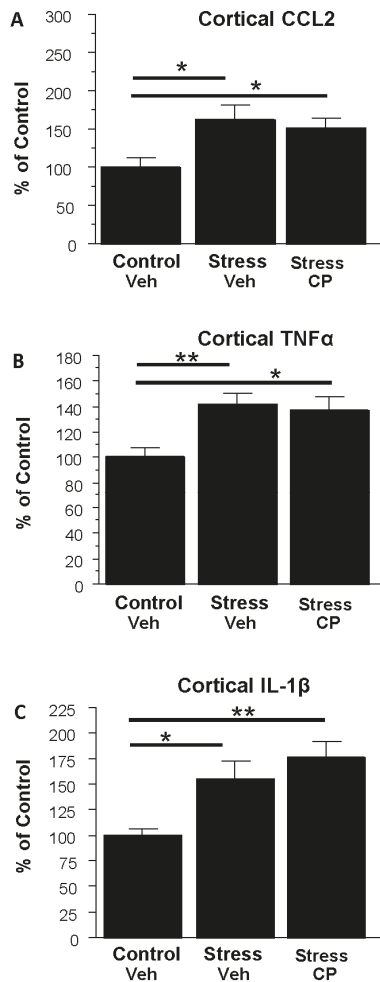
**Figure 5.** Effects of restraint stress or WCE for hippocampal neuroimmune mRNAs. For CCL2, there was an overall effect of the treatments ( $F(2,34) = 10.54, p < 0.001$ ) and a significant effect of WCE relative to stress or control. The trend toward a stress effect was not significant. Similarly, an overall trend toward a significant effect of treatments for TNF $\alpha$  was not significant ( $p = 0.07$ ). For IL-1 $\beta$ , there was an overall significant effect of treatments ( $F(2,34) = 4.98, p < 0.05$ ) with significant effects of stress and WCE relative to controls. Finally, there were no significant effects found with TLR4 mRNA. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$  versus Controls. **A, B, C,** and **D** delineate data for CCL2, TNF $\alpha$ , IL-1 $\beta$  and TLR4 mRNAs, respectively, ( $n = 9$ –13 per group).



**Figure 6.** Effects of restraint stress or WCE on amygdala neuroimmune mRNAs. For CCL2, there was a modest, but significant, overall effect of treatments ( $F(2,27) = 4.450, p < 0.05$ ) with a significant group difference between WCE and stress. There were no other effects of treatments for TNF $\alpha$ , IL-1 $\beta$ , or TLR4 mRNAs. \*\*  $p < 0.01$  compared with the stressed group. **A, B, C,** and **D** delineate data for CCL2, TNF $\alpha$ , IL-1 $\beta$  and TLR4 mRNAs, respectively, ( $n = 7$ – $11$  per group).

### 3.5. Effect of the CRF1R Antagonist CP154,526 on Cortical Cytokine mRNAs Following Stress

Having twice shown previously that a CRF1R antagonist blocks cytokine induction arising during ethanol withdrawal in the cortex [38], the final experiment focused on determining whether the drug would also block the induction due to stress. Figure 7 illustrates the effect of CP154,526 on cytokine mRNAs in the cortex. Figure 7A shows that stress increased CCL2 (63%,  $p < 0.05$ ) and this effect was not blocked by CP154,526. Similarly, Figure 7B shows that TNF $\alpha$  was increased by stress (41%,  $p < 0.01$ ) and again the stress effect was unaltered by CP. Finally, the profile of action on IL-1 $\beta$  was similar as shown in Figure 7C which shows that IL-1 $\beta$  was significantly increased by stress (55%,  $p < 0.05$ ) and CP154,526 failed to block this induction.



**Figure 7.** Effects of the corticotropin-releasing factor 1 (CRF1R) antagonist CP154,526 (CP) on cerebro-cortical neuroimmune mRNAs following restraint stress in rats. Overall there was a significant effect among the groups for CCL2 ( $F(2,16) = 4.00, p < 0.05$ ) with CP failing to block stressed-induction. There also was an effect of treatments for TNF $\alpha$  ( $F(2,16) = 4.87, p < 0.05$ ) with stress inducing and CP failing to block induction. Finally, as with CCL2 and TNF $\alpha$ , cortical IL-1 $\beta$  effects were significant overall ( $F(2,16) = 6.34, p < 0.01$ ), but there was no blockade of the stress effect by the drug. \*  $p < 0.05$ , \*\*  $p < 0.01$  versus control-vehicle treated rats. Veh = 0.5% carboxymethylcellulose. **A, B,** and **C** delineate data for CCL2, TNF $\alpha$ , IL-1 $\beta$  and TLR4 mRNAs, respectively, ( $n = 5$ – $7$  per group).

#### 4. Discussion

In the present investigation, restraint stress increased the mRNAs for CCL2, IL-1 $\beta$ , and TNF $\alpha$  and the receptor TLR4 in cortex of S–D rats (Figures 2 and 3)—a result supporting a growing body of reports of acute stressor-induced increases in neuroimmune mRNAs in various brain sites [19,20,23,24,26,47]. The restraint stress-associated increase in cytokine and TLR4 mRNAs in cortex peaked by around 4 h after exposure and then returned to control levels within 1 day. However, stress did not always affect these mRNAs in other brain sites. Even though mRNAs were increased



4 h after stress exposure in controls, corresponding cytokine proteins were not altered at this time point in either S–D or Wistar Rats (Table 1). Neuroimmune responses to WCE sometimes followed the response to stress and sometimes did not. While CCL2, TNF $\alpha$ , IL-1 $\beta$ , and TLR4 responses in the cortex were generally comparably high with either challenge, responses in the amygdala were comparably minimal. However, in the hippocampus, responses varied by type of challenge and across neuroimmune markers with a robust CCL2 response to withdrawal and a comparable IL-1 $\beta$  response to either challenge. In the hypothalamus, stress was inactive while withdrawal elevated CCL2 and IL-1 $\beta$ . Finally, the results showed that CRF1R inhibition did not alter stress-induced neuroimmune responses in the cortex, a result inconsistent with the earlier findings that CRF1R inhibition blocked cytokine responses to chronic withdrawal [38].

The reason that stress increased select cytokine mRNAs but not corresponding cytokine proteins is unknown. This pattern of mRNA versus protein results is similar to that observed in studies in other areas of rat brain. For example, Hueston et al. [21] demonstrated an increase in IL-1 $\beta$  mRNA without an accompanying increase in IL-1 $\beta$  protein in the paraventricular nucleus (PVN) to restraint stress. Deak et al. [19] found that IL-1 $\beta$  protein did not increase in the hypothalamus of S–D rats after restraint stress alone but did observe an increase in IL-1 $\beta$  to stress in the hypothalamus after applying a combination of restraint and shaking (i.e., a more severe stress). Additionally, Porterfield et al. [39] found that 2 h of restraint increased expression of IL-1 $\beta$  mRNA in the hypothalamus and caused a corresponding increase in this cytokine protein in the more stress-responsive Fischer 344 rats, but not in S–D rats [39]. However, Whitman et al. [38] found that an acute LPS challenge increased both selected cytokine proteins and corresponding cytokine mRNAs in the cortex of S–D rats [38]. While a focus on the hypothalamus and IL-1 (e.g., [19,23]) has been a fruitful focus of prior research so far as consistent effects of stress are concerned, reports of combinations of stressors may be particularly worthy of follow up. In addition to the work of Deak et al. [19] and Porterfield et al. [39] noted above, prior cold stress rendered animals' neuroimmune systems responsive to future LPS treatment [20], as shown by elevated hypothalamic and prefrontal cortical neuroimmune markers). Considered in aggregate, such studies suggest that genetic background and/or the degree and combinations of stress or challenge to the neuroimmune system may be at least in part responsible for the presence of a neuroimmune response and possibly for differences in protein versus mRNA responses as well. It may be particularly interesting in this context to examine the possibility that differentially engaged molecular mechanisms of mRNA versus protein processing across time may account for asynchrony of these constructs. That is, the observation of a stress-associated increase in cytokine mRNAs without corresponding changes in cytokine proteins could also be explained by release, utilization, and degradation of the protein during the stress challenge. Relatedly, habituation or exhaustion of mRNA generation could also be a factor in some cases. In this context, Minami et al. [23] showed that the IL-1 $\beta$  mRNA response in the hypothalamus declined over four hours despite continued immobilization stress. Such an effect could conceivably have influenced our amygdala and hypothalamic findings, but would be harder to extend to our cortical and hippocampal results. Also relevant is the more recent report of Vecchiarelli et al. [26] who found that increasing the length of restraint stress in Sprague–Dawley rats to two hours elevated protein levels of amygdala TNF $\alpha$ , decreased IL-6, while monocyte chemoattractant protein-1 (MCP-1/CCL2) remained unchanged. Based on our data, it is unlikely that any such diminished cortical response would apply to the cortex globally. Differential responses across subregions of the cortex might be critical with the net effect of global cortical responses being an increased response that must be dependent on some region(s) being particularly sensitive. Extending this logic to the hippocampus or hypothalamus may be premature, as Vecchiarelli did not see changes in these regions. The single hour of restraint stress in the present work did not alter amygdala or hypothalamic TNF $\alpha$  mRNA but did increase cortical IL-1 $\beta$ , TNF $\alpha$  and CCL2, and hippocampal IL-1 $\beta$ . Perhaps the length or severity of stress, along with a focus on cortical subregions represent key prerequisites to examinations of either individual or combinatorial challenges. Further research to

explore these various possibilities is warranted, including combinations of different intensities, times, and types of stress with chronic alcohol challenges, circadian rhythms, and genetic background.

To assess the possible contribution of other components to neuroimmune activation pathways that result in the effects observed here, TLR4 mRNA was also assessed after restraint stress. A significant increase in TLR4 mRNA, was observed only at 4 h in cortical tissue following stress, returned to control level by 8 h, and remained at this level through 48 h after the stress (Figure 2D). This fairly tight time-response relative to the other mRNAs was one of the reasons for focusing on 29 h of withdrawal (4 h post stress relative to non-stressed alcohol withdrawn rats) as the target for comparison. A consideration here is that prior assessments [38] focused mostly on alcohol withdrawal-derived mRNA data from the 24th hour of withdrawal although they showed that mRNAs can remain elevated for longer periods, thus it seems unlikely that mRNA levels would be meaningfully different across these two time points within the present study. Regardless, these results agree with Blandino et al. [18] who found no change in cortical TLR4 mRNA at their 2 h time point post footshock or LPS treatment. It is notable that stress or WCE elevated TLR4 mRNA only in the cortex. The reasons for this specific effect on TLR4 are unknown, but suggests differential neuroimmune regulation and possibly lower thresholds for activation across brain regions. The TLR4 receptor complex is a prominent driver in neuroimmune processes in general and in alcoholism (e.g., see [48]) and animal models in particular (e.g., [38,49,50]). In fact, the TLR4 receptor may play a role in a positive feedback loop that amplifies the intensity of overall neuroimmune activation in alcoholism [51]. Such reduced thresholds for neuroimmune activation may be represented most profoundly following endotoxin where activation progresses to neurodegeneration in the substantia nigra with corresponding behavioral deficits [52].

While WCE alone generally elevated cortical cytokines (and see [38]) and hippocampal CCL2 and IL-1 $\beta$ , WCE or stress alone did not consistently do so for some mRNAs in some regions (e.g., the hippocampus and hypothalamus, Figures 4 and 5). The limited TNF $\alpha$  response in these regions contrasts with the effects of pain-associated stress such as footshock reported by Blandino et al. [18] and thus supports the idea that the type of stress may be important in cytokine induction. Again, the pattern of results suggests that unique mechanisms are operating across brain regions and it would likely be productive to further examine neuroimmune mRNAs more generally on a region by region basis and thus speak to the relatively understudied yet critical issue of how neuroimmune changes across regions or networks could produce neuropathology. The potential differential induction of anti-inflammatory cytokines such as IL-10 could also be informative. These future studies should elucidate how stress induction of some cytokine mRNAs, but not others, contributes to the profile of neuroimmune activation in models of alcoholism [3–5,9].

One potentially important focus in identifying mechanisms of cytokine regulation in this experimental context relates to the corticotropin-releasing factor (CRF) system. The data herein show that the CRF1R antagonist CP154,526 was inactive against stress induction of neuroimmune mRNAs. This effect provides an interesting comparison with previous work [38] that focused on the effect of this drug on cytokine mRNAs elevated by WCE. The mechanisms that explain how the drug comes to exert an effect on one challenge and not the other are unknown, but differential engagement of adaptive mechanisms might be one possibility. That is, the drug may affect a recruited process unique to rats experiencing WCE. This interesting possibility is reminiscent of the work of Koob and colleagues who noted that CRF receptor antagonist effects were generally not manifest unless rats were dependent on alcohol (e.g., [53]). It is important to note that the CP154,526 study herein was limited in its scope and could be expanded in future studies to examine related questions. For example, the drug could be employed in examining the effect on cytokine mRNAs in the context of combined stress and WCE which is arguably a very relevant experience in some alcohol abusers.

While the current research corroborates the demonstration by Whitman et al. [38] that cytokine mRNAs are increased in the cortex 24 h after WCE and extends our inquiry into other brain sites and to the effects of stress, the studies do not address this potentially interesting combinatorial effects of the two challenges. What our results do show is that effects of these challenges are each themselves

complex and perhaps more nuanced in their consequence on the neuroimmune system than may have previously been appreciated. Such findings prompt considerations of the work of others where it was shown that the neuroimmune system may respond differently to stress depending on the degree and nature of prior challenges [17,20]. In related research, Buck et al. [54] reported that a single dose of ethanol before foot-shock stress had no effect on immune function and did not enhance the stress-induced increase in IL-1 $\beta$  mRNA in the PVN. In general, a second challenge before or after the WCE would seem appropriate strategy to gain new evidence for the possibility that an initial challenge to the neuroimmune system may permit or alter induction of select neuroimmune mediators by a second challenge. Thus, sufficient previous activation of immune function by chronic ethanol exposure might render stress capable of further increasing cytokine mRNAs, as previously noted behaviorally [2,4,10,55]. Thus, identification of the conditions under which prior stress or chronic alcohol exposure alters future responses to either challenge would seem to be a productive avenue for research.

Whatever future combinatorial stress studies might reveal, the present results nonetheless do provide an interesting contrast with Whitman et al. [38], who demonstrated that a CRF1R antagonist prevented the cytokine mRNA increases induced by the WCE alone. It would also be of interest to identify differential physiological effects of the drug in context of the two challenges. For example, in considering the idea that cytokines may have specific neurophysiological and behavioral actions manifest in select brain regions (e.g., [9,56]), it would seem likely that broadening the neuroanatomical focus of these CRF/cytokine interactions would very likely be a productive endeavor (see also [57]). Collectively, these studies implicate CRF involvement in the increased expression of cytokine mRNAs during the 24 h withdrawal from the WCE and suggest that there may be functional consequences. In this regard, amygdala CRF-amplified CCL2 regulation of alcohol self-administration [58] and elevated CRF-dependent amygdala CCL2 in human alcoholics [48] are consistent with a role of cytokines and CRF interacting to regulate alcohol consumption. These findings considered in the context of chronic alcohol dependent CCL2 induction within the central amygdala and robust elevations of the TNF receptor (Tnfrsf1a) in rats, support the idea that neuroimmune mechanisms in the amygdala are potentially critical in the behavioral pathology in alcoholism [59]. Thus, a future experiment should be undertaken to further examine the interactions of stress and WCE across additional relevant brain regions and to further isolate relevant mechanisms. Likewise, based upon the report by Johnson et al. [22] that norepinephrine-receptor antagonists blocked the stress increase in hypothalamic IL-1 $\beta$  protein induced by inescapable tail shock (i.e., a severe stress), and the finding that the beta-adrenergic agonist isoproterenol can enhance IL-1 production in the amygdala following chronic stress (Porterfield et al., [39]), the effects of this drug class on the cytokine mRNA changes across different brain regions after restraint stress in the presence and absence of WCE should also be explored.

## 5. Conclusions

The present findings provide additional evidence for neuroimmune involvement in brain function associated with stress or WCE and the differential induction of neuroimmune mRNAs and adds the novel observation that a CRF1R antagonist is inactive against a mild stress. The results herein show that some neuroimmune components are readily inducible in a brain-region-dependent manner while others are not. Such evidence adds to a growing literature that implicates neuroimmune dysfunction in alcoholism, other substance abuse disorders, and other neurobehavioral disorders associated with stress [7,9,27,28,32,60,61]. Our findings and others prompt questions about how some challenges exert specific neuroimmune effects within neuroanatomically limited areas and suggest further studies should be done to examine combinations of challenges/conditions thought to impact on alcoholism and associated neuropsychiatric conditions. Moreover, our findings, considered in the context of the documented roles of the neuroimmune system and stress in alcohol consumption and negative emotional symptoms due to chronic ethanol consumption [9,58,62–65], support the idea that specific neuroimmune processes are engaged in neurobehavioral processes fundamental to alcohol abuse.

It may be productive to ask whether risk of relapse in alcoholics relates to differential neuroimmune responses to stress as a consequence of prior chronic ethanol exposure history and to further develop animal models around this concept. These findings also have potentially broader implications in that neuroimmune system dysfunction has been implicated in other neurobehavioral disorders as well including insomnia [33], depression [31,34,66–68], and anxiety [6,35,60,69]. In particular, neuroimmune system regulation of anxiety associated with chronic alcohol and withdrawal are notable [8,9,49]. Identifying overlapping and independent neuroimmune processes across these pathologies would be worthy of further study.

While the current studies document that neural mechanisms associated with stress may at least partially overlap with the mechanisms that drive cytokine mRNA expression after WCE, the profile of effects shown herein for the two challenges do not completely overlap across neuroimmune marker or brain region. Further, the responses reported herein were elicited with relatively limited challenges (i.e., just 1 h of stress or 15 days of exposure to ethanol). Thus, it may be valuable to examine similar endpoints following exposure to the more chronic and/or severe challenges/stressors that define many neurobehavioral disorders. Of these effects, one could ask which are transient (yet perhaps behaviorally relevant), and which induce long term maladaptations that influence behavioral pathology. By understanding how stress and WCE engage the neuroimmune system, and worsens symptoms, therapeutic options by which to mitigate stress-associated neuroimmune dysfunction in drug addiction and other central nervous system disorders could emerge [29,32].

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## Abbreviations

|              |  |
|--------------|--|
| ANOVA        | analysis of variance                   |
| CCL2         | chemokine (C-C motif) ligand 2         |
| CRF          | corticotropin releasing hormone        |
| IL-1 $\beta$ | interleukin-1 beta                     |
| mRNA         | messenger ribonucleic acid             |
| S-D          | Sprague-Dawley                         |
| TLR4         | toll-like receptor 4                   |
| TNF $\alpha$ | tumor necrosis factor-alpha            |
| WCE          | withdrawal from chronic ethanol        |
| qPCR         | quantitative polymerase chain reaction |

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Article

# NLRP12 Inflammasome Expression in the Rat Brain in Response to LPS during Morphine Tolerance

Sulie L. Chang <sup>1,2,\*</sup>, Wenfei Huang <sup>1</sup>, Xin Mao <sup>1</sup> and Sabroni Sarkar <sup>1</sup>

<sup>1</sup> Institute of NeuroImmune Pharmacology, Seton Hall University, 400 South Orange Avenue, South Orange, NJ 07079, USA; wenfei.huang@shu.edu (W.H.); ninnamao@yahoo.com.cn (X.M.); Sabroni.Sarkar@gmail.com (S.S.)

<sup>2</sup> Department of Biological Sciences, Seton Hall University, 400 South Orange Avenue, South Orange, NJ 07079, USA

\* Correspondence: sulie.chang@shu.edu; Tel.: +1-973-761-9456; Fax: +1-973-275-2489

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**Abstract:** Morphine, an effective but addictive analgesic, can profoundly affect the inflammatory response to pathogens, and long-term use can result in morphine tolerance. Inflammasomes are protein complexes involved in the inflammatory response. The nucleotide-binding oligomerization domain-like receptor (NLR) Family Pyrin Domain Containing (NLRP) 12 (NLRP12) inflammasome has been reported to have anti-inflammatory activity. In this study, we examined the expression of NLRP12 inflammasome related genes in the adult F344 rat brain in response to the bacterial endotoxin lipopolysaccharide (LPS) in the presence and absence of morphine tolerance. Morphine tolerance was elicited using the 2 + 4 morphine-pelleting protocol. On Day 1, the rats were pelleted subcutaneously with 2 pellets of morphine (75 mg/pellet) or a placebo; on Days 2 and 4 pellets were given. On Day 5, the animals were randomly assigned to receive either 250 µg/kg LPS or saline (i.p.). The expression of 84 inflammasome related genes in the rat brain was examined using a Polymerase Chain Reaction (PCR) array. In response to LPS, there was a significant increase in the expression of the pro-inflammatory cytokine/chemokine genes interleukin-1 beta (IL-1β), interleukin-6 (IL-6), C-C motif chemokine ligand 2 (Ccl2), C-C motif chemokine ligand 7 (Ccl7), C-X-C motif chemokine ligand 1 (Cxcl1), and C-X-C motif chemokine ligand 3 (Cxcl3) and a significant decrease in the anti-inflammatory NLRP12 gene in both morphine-tolerant and placebo-control rats compared to saline-treated rats, although the changes were greater in the placebo-control animals. The Library of Integrated Network-Based Cellular Signatures' (LINCS) connectivity map was used to analyze the list of affected genes to identify potential targets associated with the interactions of LPS and morphine tolerance. Our data indicate that, in the morphine tolerant state, the expression of NLRP12 and its related genes is altered in response to LPS and that the Vacuolar protein-sorting-associated protein 28 (VPS28), which is involved in the transport and sorting of proteins into sub-cellular vesicles, may be the key regulator of these alterations.

**Keywords:** morphine tolerance; NLRP12 inflammasome; LPS

## 1. Introduction

Morphine is a potent analgesic that is widely used clinically for pain management. However, long-term use of morphine can lead to morphine tolerance and addiction [1]. In addition, morphine can profoundly and detrimentally affect the body's immune system, at both the cellular and molecular levels. Morphine suppresses lymphocyte trafficking; decreases natural killer cell activity; inhibits the production of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and

interleukin-1 beta (IL-1 $\beta$ ) [2–4]; and induces atrophy of immune organs, such as the spleen and thymus [5–9].

Morphine-induced immunosuppression significantly increases the risk of bacterial infection [10]. Although the immunosuppressive effects of morphine have been widely studied, the mechanisms involved in the body's inflammatory response to pathogens during morphine tolerance have not been fully investigated.

Inflammasomes are multi-protein complexes assembled from nucleotide oligomerization domain receptor proteins known as nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) [11]. They function as important mediators of innate immunity. Inflammasomes play a key role in the regulation of inflammation and immune responses by participating in the production of pro-inflammatory cytokines, including IL-1 $\beta$  and interleukin-18 (IL-18) [11–13]. Both IL-1 $\beta$  and IL-18 produce a wide variety of biological effects associated with infection, inflammation, and autoimmune processes.

There have been about 20 NLR inflammasomes identified in humans. The most commonly studied ones include NLR Family Pyrin Domain Containing 1 (NLPR1), NLR family apoptosis inhibitory protein 6 (NAIP2), NLR Family Pyrin Domain Containing 3 (NLRP3), NLR Family Pyrin Domain Containing 5 (NLRP5), NLR Family Pyrin Domain Containing 6 (NLRP6), NLR Family Pyrin Domain Containing 12 (NLRP12), NLR family member X1 (NLRPX1), and NLR Family CARD Domain Containing 4 (NLRC4) [14]. Inflammasomes have been sub-divided into two groups; pro-inflammatory inflammasomes and anti-inflammatory inflammasomes. The pro-inflammatory inflammasomes include NLRP3 and NLRC4, and their functions have been widely studied. The anti-inflammatory inflammasomes include NLRP12, NLRX1, NLRC3, and NLRC5. They appear to function by limiting or suppressing a pro-inflammatory response; however, this group has not been well studied to date.

There have been a few recent reports characterizing the anti-inflammatory properties of NLRP12 [15,16]. NLRP12 can inhibit NF- $\kappa$ B signaling through both the canonical and non-canonical pathways, which are important in the control and regulation of innate immune responses [15,16]. NLRP12 inhibits IL-1 receptor-associated kinase 1 (IRAK1), a downstream component of the pathogen activated Toll-like receptor (TLR) pathway, which, in turn, decreases the signaling of the canonical NF- $\kappa$ B pathway [17]. In the non-canonical NF- $\kappa$ B pathway, NLRP12 interacts with and rapidly degrades NF- $\kappa$ B-inducing kinase (NIK), thereby suppressing NF- $\kappa$ B signaling [18].

The Library of Integrated Network-Based Cellular Signatures (LINCS) [19] is a database which implements a biological network-based strategy to make assessments regarding the impact of drugs, genetics, and related biological perturbations (alterations induced by external or internal mechanisms) on cellular states. The library database is based on the philosophy that typical human pathology, biology, and pharmacology are most aptly understood using a systems-level approach. It was constructed to generate a robust approach for perturbing a diversity of cell types, measuring cellular responses, integrating and analyzing data, and visualizing and interrogating the database for a variety of biomedical research applications [19]. This library allows researchers to access a wide variety of data by using a matrix consisting of cell type by experimental treatment by phenotypic assay. Using LINCS, researchers are able to inquire about information regarding mechanism-based relationships among the effects of different drug responses and their targets (perturbants) as well as associations among responding cellular components, in the format of network interactions and structure-function relationships [20].

In this study, we used an inflammasome PCR array containing 84 inflammasome-related genes to investigate the expression of inflammasomes during an inflammatory response to the bacterial endotoxin, lipopolysaccharide (LPS), in the rat brain during morphine tolerance. LINCS was then used to project possible candidate targets from the mRNA gene expression profiles generated from the PCR array analysis.

## 2. Materials and Methods

### 2.1. Animals

Fisher/NHsd 344 (F344) rats were purchased from Harlan Laboratories (Indianapolis, IN, USA). The animals were housed in groups of 3–5 animals in a temperature controlled (21 °C–22 °C) animal holding room, under a 12-h light/12-h dark illumination cycle (lights on at 7:00 a.m.). Food and tap water were provided ad libitum. The Institutional Animal Care and Use Committee (IACUC) at Seton Hall University, South Orange, NJ, USA approved the experimental protocol.

### 2.2. Morphine and LPS Administration

A 2 + 4 regimen was used to produce morphine tolerance in 7–8 mo old (250–350 g) male F344 rats [5,21–24]. The rats ( $n = 16$ ) were randomly assigned into two groups. The morphine-tolerant group received two 75 mg morphine sulfate pellets (NIDA, Rockville, MD, USA) on Day 1 via subcutaneous (s.c.) implantation and four pellets on Day 2, whereas the control group received placebo pellets on both days. On Day 5, the two groups were randomly assigned to receive either LPS (250 µg/kg, Sigma, St. Louis, MO, USA) or saline (vehicle) [5,21–24]. Thus, the four experimental groups were placebo-control + saline, placebo-control + LPS, morphine-tolerant + saline, and morphine-tolerant + LPS. Two hours after the treatment with LPS or saline, the animals were euthanized and the brains were harvested.

### 2.3. RNA Isolation and Preparation of cDNA

Total RNA was extracted from the brain tissue using TRIZOL (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. To remove contaminating DNA, the total RNA samples were treated with RNase-free DNase (Qiagen, Valencia, CA, USA), followed by further purification using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The RNA quality and quantity were assessed using a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). An equal amount of RNA (400 ng) from each sample was then converted into first-strand cDNA using a RT<sup>2</sup> First Strand Kit (SABiosciences, Frederick, MD, USA) for a PCR array.

### 2.4. Real-Time PCR Array

The expression of 84 key genes involved in the function of inflammasomes, general NLR signaling, and cytokine and chemokine genes was quantified using a custom PCR array and RT<sup>2</sup> SYBR Green Fluorescein qPCR Master Mix (SABiosciences, Frederick, MD, USA), according to the manufacturer's protocol. Using an ABI Prism 7900HT Fast Detection System (Applied Biosystems, Foster, CA, USA), real-time PCR was performed by first denaturing the PCR mix at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min.

### 2.5. PCR Array Data Analysis

The expression of each gene was normalized to housekeeping genes and calculated using the  $\Delta\Delta C_t$  method. The threshold and baseline values were set manually, and the resulting threshold cycle values ( $C_t$ ) were analyzed using the PCR array data analysis template supplied on the manufacturer's website [25]. The mean fold change in mRNA expression from 3 to 5 biological replicates was considered significant at  $p < 0.05$ . The gene profile signatures were created for every two groups compared.

### 2.6. LINCS Analysis

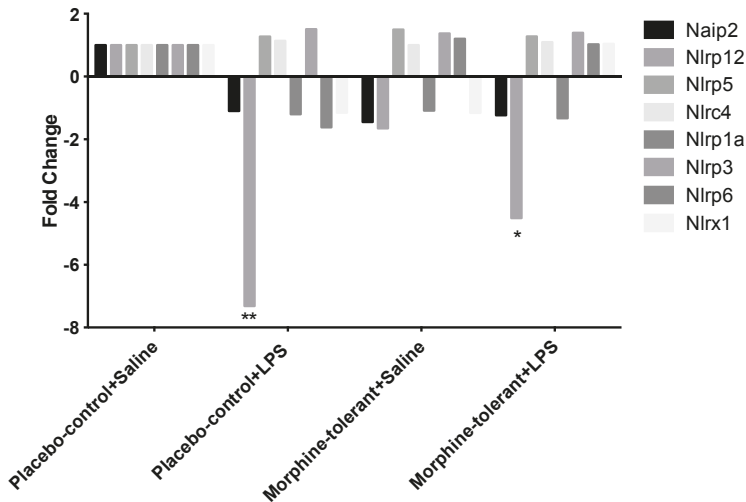
The differentially expressed genes were input into the Query App ([apps.lincscloud.org/query](http://apps.lincscloud.org/query)), as described previously [26,27]. Based on the LINCS database, LincsCloud utilized gene profile signatures generated from the PCR array to generate a report, including probability outcomes in terms of gene knockdown effects and drug mimics. The scores given in the report evaluated how much a

particular set of gene regulation features (named pertubagens) was likely to be connected with the genes listed in the LINCS report. Positive readings in the Consensus Knockdown Connections in the report indicate that knockdown of the genes listed in the LINCS report would match the gene changes input into the Query App, and thus the genes with high scores represent potential target genes for the experimental treatment.

### 3. Results

#### 3.1. Expression Profile of Inflammasome-Related Genes Following an LPS Challenge, with and without Morphine Tolerance

Alterations in gene expression were measured in rats challenged with LPS, with and without morphine tolerance, using a PCR array containing 84 genes related to inflammasome activation and function. NLRP12 expression was significantly decreased in response to LPS in both the morphine-tolerant (morphine-tolerant + LPS) and control (placebo-control + LPS) rats, compared to the rats given saline (morphine-tolerant + saline and placebo-control + saline) (Figure 1, Table 1). However, the decrease was greater in the placebo-control rats ( $-7.3$  fold;  $p < 0.01$ ) than in the morphine-tolerant rats ( $-4.5$  fold;  $p < 0.05$ ).



**Figure 1.** Inflammasome-related gene expression in the rat brain in response to lipopolysaccharide (LPS), with and without morphine tolerance. The expression of the inflammasome-related NOD-like receptor (NLR) genes (Naip2, Nlrp12, Nlrp5, Nlrc4, Nlrp1a, Nlrp3, Nlrp6, And Nlrp1) in the brains of rats given an i.p. injection of either 250  $\mu\text{g}/\text{kg}$  LPS or saline, with and without morphine tolerance ( $n = 3\text{--}5$  rats per group), was determined using a PCR array. Data were calculated using the  $\Delta\Delta\text{CT}$  method, relative to the control group (placebo-control +saline), and are represented as a fold change. \*  $p < 0.05$ , \*\*  $p < 0.01$ . Naip2: NLR family, apoptosis inhibitory protein 2; Nlrp12: NLR Family Pyrin Domain Containing 12; Nlrp5: NLR Family Pyrin Domain Containing 5; Nlrc4: NLR Family CARD Domain Containing 4; Nlrp1a: NLR family, pyrin domain containing 1A; Nlrp3: NLR Family Pyrin Domain Containing 3; Nlrp6: NLR Family Pyrin Domain Containing 6; Nlrp1: NLR family member X1.

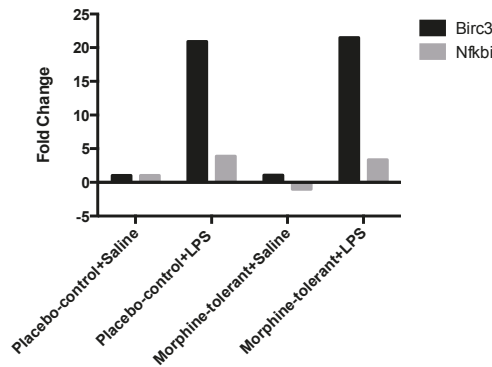
**Table 1.** Expression profile of inflammasomes and NLR genes in the rat brain in response to lipopolysaccharides (LPS), with and without morphine tolerance.

| Gene   | morphine-tolerant + saline/placebo control + saline |                   | placebo control + LPS/placebo control + saline |                   | morphine-tolerant + LPS/morphine-tolerant + saline |                   |
|--------|---|-------------------|--|-------------------|--|-------------------|
|        | Fold Change   | <i>p</i> -value * | Fold Change **                                 | <i>p</i> -value * | Fold Change **                                     | <i>p</i> -value * |
| Card6  | -1.2347   | 0.078926          | -1.5292  | 0.004159          | -1.714   | 0.017499          |
| Casp1  | 1.1505  | 0.299299          | 1.2132   | 0.276593          | 1.2764   | 0.02969           |
| Casp12 | 1.0972  | 0.462347          | 1.5422   | 0.003614          | 1.4249   | 0.092085          |
| Casp8  | -1.0402   | 0.612597          | -1.1363  | 0.391056          | -1.2652  | 0.139999          |
| Naip2  | -1.4483   | 0.067329          | -1.1031  | 0.619799          | -1.2316  | 0.25758           |
| Nlrp12 | -1.6504   | 0.674422          | -7.31  | 0.002731          | -4.5124  | 0.025372          |
| Nlrp5  | 1.494   | 0.054876          | 1.274  | 0.20786           | 1.2819   | 0.027352          |
| Nlr4   | 1.0028  | 0.915792          | 1.1349   | 0.159712          | 1.1003   | 0.445719          |
| Nlrp1a | -1.0898   | 0.470844          | -1.2027  | 0.001147          | -1.3308  | 0.1379            |
| Nlrp3  | 1.3773  | 0.134647          | 1.5114   | 0.054565          | 1.3956   | 0.02407           |
| Nlrp6  | 1.205   | 0.420019          | -1.6225  | 0.358997          | 1.0293   | 0.784547          |
| Nlr1   | -1.1574   | 0.24832           | -1.1494  | 0.191026          | 1.039  | 0.679959          |
| Nod2   | -1.6257   | 0.650152          | 1.0901   | 0.811946          | 1.2826   | 0.50395           |
| Pycard | -1.0575   | 0.610143          | -1.0285  | 0.797363          | -1.2363  | 0.030115          |

\* For *p*-value, letters in red mean  $p < 0.05$ . \*\* For Fold Change, letters in red mean fold change  $> 2$  and letters in blue mean the fold change  $< -2$ . For the color lines, █: 6–10 fold decrease; █: 2–5 fold decrease; █:  $< 2$  fold; █: 2–5 fold increase; █: 6–10 fold increase; █: 11–30 fold increase; █: 31–50 fold increase.

### 3.2. Expression Profile of Inflammasome-Related Downstream Signaling Genes Following an LPS Challenge, With and Without Morphine Tolerance

With a few exceptions, there were no significant changes in expression of the downstream signaling genes in response to LPS in either the morphine-tolerant rats (morphine-tolerant + LPS) or the control animals (placebo-control + LPS), compared to the rats given saline (morphine-tolerant + saline and placebo-control + saline) (Table 1). However, Baculoviral IAP Repeat-Containing 3 (Birc3), an important regulator gene involved in the downstream effects of inflammasomes, was significantly increased in response to LPS in both the morphine-tolerant (21.5 fold;  $p < 0.05$ ) and control (21 fold;  $p < 0.01$ ) groups, compared to the rats given saline (Figure 2, Table 2). In addition, NF-Kappa-B Inhibitor Alpha (Nfkbia), an inhibitor protein of NF- $\kappa$ B, was increased in both groups given LPS (morphine-tolerant + LPS, 3.4 fold,  $p < 0.01$ ; placebo-control + LPS, 3.9 fold,  $p < 0.01$ ) (Figure 2, Table 2).



**Figure 2.** Inflammasome-related downstream gene expression in the rat brain in response to Lipopolysaccharides (LPS), with and without morphine tolerance. The expression of the inflammasome-related downstream signaling genes Baculoviral IAP Repeat-Containing 3 (Birc3) and NF-Kappa-B Inhibitor Alpha (Nfkbia) in the brains of rats given an i.p. injection of either 250  $\mu$ g/kg LPS or saline, with and without morphine tolerance ( $n = 3$ –5 rats per group), was determined using a Polymerase Chain Reaction (PCR) array. The data were calculated using the  $\Delta\Delta$ CT method relative to the control group (placebo-control + saline) and are represented as fold change.

**Table 2.** Expression profile of inflammasome-related downstream signaling genes in the rat brain in response to lipopolysaccharides (LPS), with and without morphine tolerance. Full name of the genes were provided in Table A1.

| Gene      | morphine-tolerant + saline/placebo control + saline |                   | placebo control + LPS/placebo control + saline |                   | morphine-tolerant + LPS/morphine-tolerant + saline |                   |
|-----------|---|-------------------|--|-------------------|--|-------------------|
|           | Fold Change   | <i>p</i> -value * | Fold Change **                                 | <i>p</i> -value * | Fold Change **                                     | <i>p</i> -value * |
| Bcl2      | 1.0469  | 0.863233          | 1.0586   | 0.845254          | 1.2398   | 0.339246          |
| Bcl2l1    | -1.1256   | 0.01111           | 1.0572   | 0.476643          | -1.0291  | 0.671184          |
| Birc2     | 1.0509  | 0.586337          | -1.0281  | 0.622487          | 1.0699   | 0.531417          |
| Birc3     | 1.0648  | 0.587513          | 20.9356  | 0.011065          | 21.5025  | 0.001873          |
| Cflar     | 1.0226  | 0.697346          | 1.3021   | 0.004748          | 1.2155   | 0.140978          |
| Chuk      | 1.1867  | 0.119108          | -1.0233  | 0.754891          | 1.1593   | 0.073058          |
| Ciita     | -1.0617   | 0.750879          | 1.0619   | 0.873363          | 1.4678   | 0.39646           |
| Ctsb      | -1.0009   | 0.978499          | -1.0656  | 0.617885          | -1.1046  | 0.493204          |
| Fadd      | -1.1261   | 0.258884          | 1.1235   | 0.252486          | 1.0306   | 0.71174           |
| Hsp90aa1  | 1.2774  | 0.114349          | 1.0391   | 0.743375          | 1.189  | 0.149024          |
| Hsp90ab1  | 1.0752  | 0.376876          | -1.0968  | 0.305427          | 1.1579   | 0.18515           |
| Ikbkb     | 1.0718  | 0.550623          | -1.0011  | 0.962992          | 1.1799   | 0.201955          |
| Ikbkg     | 1.0924  | 0.324904          | -1.0402  | 0.492725          | 1.1419   | 0.079202          |
| Irak1     | 1.1549  | 0.062648          | 1.0287   | 0.755395          | 1.2213   | 0.242955          |
| Map3k7    | -1.2495   | 0.002404          | -1.1008  | 0.434596          | -1.2497  | 0.114056          |
| Map3k7ip1 | -1.0335   | 0.664499          | -1.1151  | 0.369226          | 1.02   | 0.864431          |
| Map3k7ip2 | -1.2869   | 0.000867          | -1.1169  | 0.456355          | -1.3427  | 0.007785          |
| Mapk1     | -1.0527   | 0.468581          | -1.0887  | 0.159097          | 1.0316   | 0.724595          |
| Mapk11    | -1.0673   | 0.530192          | -1.0706  | 0.578016          | -1.0633  | 0.571223          |
| Mapk12    | 1.1424  | 0.420684          | 1.1889   | 0.372287          | -1.0255  | 0.780819          |
| Mapk13    | -1.1629   | 0.924422          | -1.5423  | 0.355826          | 1.0504   | 0.651078          |
| Mapk14    | 1.0018  | 0.940725          | -1.0318  | 0.81795           | 1.0335   | 0.709962          |
| Mapk3     | -1.1294   | 0.224192          | -1.121   | 0.372958          | -1.0495  | 0.76638           |
| Mapk8     | 1.0383  | 0.883566          | -1.0215  | 0.798267          | 1.0037   | 0.911563          |
| Mapk9     | -1.1252   | 0.03886           | -1.0075  | 0.87718           | -1.0805  | 0.288977          |
| Mefv      | 1.2572  | 0.278807          | 1.2582   | 0.463496          | 1.9192   | 0.028283          |
| Myd88     | 1.0211  | 0.791137          | 1.0103   | 0.917416          | 1.2185   | 0.03802           |
| Nfkb1     | 1.1098  | 0.279116          | 1.2919   | 0.089881          | 1.3323   | 0.033357          |
| Nfkbia    | -1.0294   | 0.835021          | 3.885  | 0.001215          | 3.3744   | 0.001348          |
| Nfkbib    | -1.1473   | 0.330475          | -1.0153  | 0.762037          | -1.1167  | 0.643504          |
| P2rx7     | -1.1586   | 0.428492          | 1.0742   | 0.625549          | 1.0578   | 0.661519          |
| Panx1     | -1.076  | 0.275575          | 1.1455   | 0.406863          | 1.1101   | 0.334734          |
| Pea15a    | -1.0943   | 0.291531          | -1.043   | 0.625202          | -1.0802  | 0.492135          |
| Pstpip1   | -1.1041   | 0.527175          | -1.1018  | 0.680263          | -1.0232  | 0.87342           |
| Ptgs2     | -1.08   | 0.499364          | 1.5126   | 0.000571          | 1.1762   | 0.323717          |
| Rage      | 1.0729  | 0.478448          | -1.1273  | 0.17423           | 1.1854   | 0.16574           |
| Rela      | 1.0171  | 0.804744          | 1.1801   | 0.417806          | 1.2504   | 0.106174          |
| Ripk2     | -1.1057   | 0.237762          | 1.2913   | 0.028006          | 1.1199   | 0.389288          |
| Sugt1     | 1.0063  | 0.918273          | -1.1023  | 0.519396          | -1.1264  | 0.106262          |
| Tirap     | -1.3098   | 0.094434          | -1.0971  | 0.535721          | 1.0809   | 0.374146          |
| Hsp90b1   | 1.0475  | 0.680071          | -1.0334  | 0.69801           | -1.0877  | 0.314466          |
| Traf6     | -1.0344   | 0.915204          | -1.0171  | 0.943304          | 1.0988   | 0.437323          |
| Txnip     | -1.0761   | 0.70951           | -1.0718  | 0.699702          | -1.0187  | 0.789181          |
| Xiap      | 1.0572  | 0.503462          | -1.0558  | 0.471524          | 1.0089   | 0.93573           |

\* For *p*-value, letters in red mean  $p < 0.05$ . \*\* For Fold Change, letters in red mean fold change  $> 2$  and letters in blue mean the fold change  $< -2$ . For the color line, blue: 6–10 fold decrease; light blue: 2–5 fold decrease; light red: 2–5 fold increase; red: 6–10 fold increase; dark red: 11–30 fold increase; orange: 31–50 fold increase.

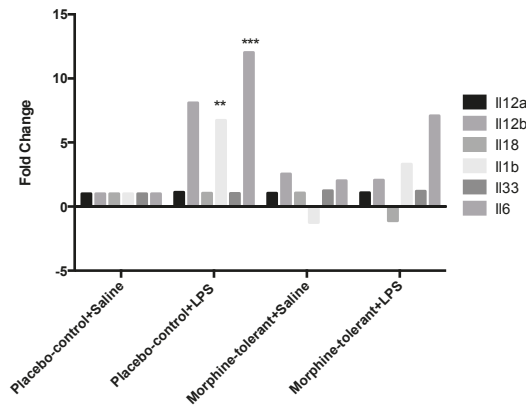
### 3.3. Expression Profile of Inflammasome-Related Chemokine and Cytokine Genes after an LPS Challenge, with and without Morphine Tolerance

Cytokine and chemokine gene expression in response to LPS was greater in the control rats (placebo-control + LPS) compared to the morphine-tolerant animals (morphine-tolerant + LPS) (Table 3). The cytokines IL-1 $\beta$  and IL-6 were significantly increased 7- and 12-fold, respectively ( $p < 0.01$ – $0.001$ ), in the control rats given LPS (placebo-control + LPS), whereas in the morphine-tolerant group (morphine-tolerant + LPS) the fold changes were not statistically significant (3- and 7-fold, respectively) compared to the rats given saline (Figure 3, Table 3).

**Table 3.** Expression profile of the cytokine and chemokine genes in the rat brain in response to LPS, with and without morphine tolerance.

| Gene    | morphine-tolerant + saline/placebo control + saline |           | placebo control + LPS/placebo control + saline |           | morphine-tolerant + LPS/morphine-tolerant + saline |           |
|---------|---|-----------|--|-----------|--|-----------|
|         | Fold Change **                                      | p-value * | Fold Change **                                 | p-value * | Fold Change **                                     | p-value * |
| Ccl11   | -2.4662   | 0.023104  | -2.8639  | 0.16576   | -2.7346  | 0.249229  |
| Ccl12   | -1.4362   | 0.646529  | 2.0214   | 0.219015  | 2.1108   | 0.399119  |
| Ccl2    | 1.1756  | 0.521412  | 23.7845  | 0.001354  | 9.1497   | 0.106064  |
| Ccl5    | -1.105  | 0.472434  | 1.2909   | 0.290174  | -1.0949  | 0.766893  |
| Ccl7    | -1.0841   | 0.688167  | 10.7188  | 0.000259  | 6.9528   | 0.163034  |
| Cxcl1   | 1.0131  | 0.944854  | 36.8609  | 0         | 14.4718  | 0.130008  |
| Cxcl3   | 1.6208  | 0.019673  | 8.1909   | 0.000076  | 3.6477   | 0.170486  |
| Cd40lg  | -2.2796   | 0.215753  | 1.1119   | 0.704126  | -2.4054  | 0.273798  |
| Ifnb1   | 1.2519  | 0.936419  | 1.6899   | 0.391302  | 1.5695   | 0.513801  |
| Ifng    | 1.5355  | 0.290413  | 1.1698   | 0.767882  | 2.2615   | 0.088346  |
| Il12a   | 1.05  | 0.57557   | 1.1178   | 0.33855   | 1.0837   | 0.456191  |
| Il12b   | 2.5587  | 0.26828   | 8.1055   | 0.102341  | 2.077  | 0.198782  |
| Il18    | 1.068   | 0.60015   | 1.0484   | 0.707002  | -1.1151  | 0.48359   |
| Il1b    | -1.2476   | 0.284501  | 6.7366   | 0.001383  | 3.3136   | 0.089312  |
| Il33    | 1.2342  | 0.054615  | 1.0407   | 0.583823  | 1.1978   | 0.072613  |
| Il6     | 2.023   | 0.45937   | 12.0303  | 0.008058  | 7.0943   | 0.083969  |
| Irf1    | 1.1299  | 0.521156  | 3.4575   | 0.000386  | 3.4104   | 0.01276   |
| Irf2    | -1.1481   | 0.050782  | 1.0027   | 0.916481  | 1.0556   | 0.593809  |
| Irf3    | -1.2824   | 0.024657  | -1.3225  | 0.225481  | -1.2478  | 0.088625  |
| Irf4    | -1.1491   | 0.43182   | -1.0557  | 0.846866  | 1.0263   | 0.858979  |
| Irf5    | -1.1024   | 0.38296   | -1.1468  | 0.376953  | 1.0391   | 0.841777  |
| Irf6    | 1.1055  | 0.413463  | 1.012  | 0.792025  | 1.118  | 0.272222  |
| Tnfsf11 | -1.6077   | 0.800779  | -1.362   | 0.519436  | -1.3266  | 0.965379  |
| Tnfsf14 | -1.396  | 0.243156  | -1.8445  | 0.126355  | -1.4278  | 0.629476  |
| Tnfsf4  | -1.0175   | 0.997398  | -1.0887  | 0.790199  | -1.3422  | 0.420711  |

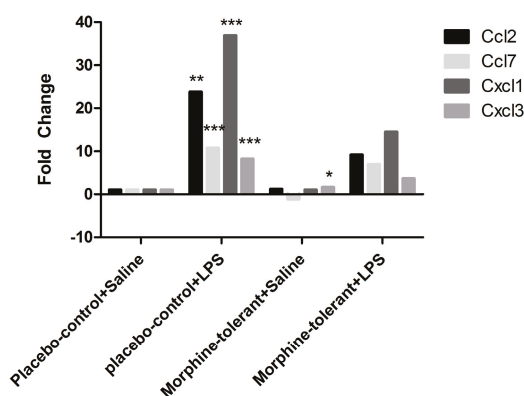
\* For p-value, letters in red mean  $p < 0.05$ . \*\* For Fold Change, letters in red mean fold change  $> 2$  and letters in blue mean the fold change  $< -2$ . For the color lines, blue: 6–10 fold decrease; light blue: 2–5 fold decrease; white:  $< 2$  fold; pink: 2–5 fold increase; red: 6–10 fold increase; dark red: 11–30 fold increase; orange: 31–50 fold increase.



**Figure 3.** Cytokine gene expression in the rat brain in response to lipopolysaccharides (LPS), with and without morphine tolerance. Gene expression of interleukins Interleukin (II)-1 $\beta$ , II-6, II-12a, II-12b, II-18, and II-33 in the brains of rats, with and without morphine tolerance, following an i.p. injection of either 250  $\mu\text{g}/\text{kg}$  LPS or saline ( $n = 3\text{--}5$  rats per group) was determined using a Polymerase Chain Reaction (PCR) array. Data were calculated using the  $\Delta\Delta\text{CT}$  method relative to the control group (placebo-control + saline) and are represented as a fold change. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

Similarly, Ccl2, Ccl7, Cxcl1, and Cxcl3 chemokine expression was significantly increased 24-, 11-, 37-, and 8-fold, respectively ( $p < 0.01\text{--}0.001$ ), in response to LPS in the control rats (placebo-control +

LPS), whereas in the morphine-tolerant (morphine-tolerant + LPS) group the fold changes (9-, 7-, 14-, and 3-fold, respectively) were not statistically significant (Figure 4, Table 3).



**Figure 4.** Chemokine gene expression in the rat brain in response to Lipopolysaccharides (LPS), with and without morphine tolerance. Gene expression of the chemokines C-C motif chemokine ligand (Ccl)2, Ccl5, Ccl7, Ccl11, Ccl12, C-X-C motif chemokine ligand (Cxcl)1, and Cxcl3 in the brains of rats with and without morphine tolerance, following an i.p. injection of either 250 µg/kg LPS or saline ( $n = 3\text{--}5$  rats per group), was determined using a Polymerase Chain Reaction (PCR) array. Data were calculated using the  $\Delta\Delta\text{CT}$  method relative to the control group (placebo-control + saline) and are represented as a fold change. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

### 3.4. LINCS Analysis of the Differentially Expressed Genes

Differentially expressed genes in the morphine-tolerant + saline versus morphine-tolerant + LPS rats and in the placebo-control + saline versus placebo-control + LPS rats as well as gene changes in rats the placebo-control + saline versus morphine-tolerant + saline rats were input into the Query App ([apps.lincscloud.org/query](https://apps.lincscloud.org/query)). One report was generated by LINCS for each set of genes input. The genes with a high positive score in Consensus Knockdown Connections were considered to be potential gene targets (Table 4). In the placebo-control + saline versus placebo-control + LPS report, VPS28, protein C receptor (PROCR), and charged multivesicular body protein 2A (CHMP2A) were the top three with the highest scores. VPS28 is an ESCRT-I complex subunit that functions in the transport and sorting of proteins into sub-cellular vesicles. PROCR is endothelial protein C receptor involved in the blood coagulation pathway. CHMP2A is a component of the endosomal sorting complex required for transport III, which is involved in the degradation of surface receptor proteins and the formation of endocytic multivesicular bodies.

In the placebo-control + saline versus morphine-tolerant + saline report, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1 (SMARCE1), aryl-hydrocarbon receptor repressor (AHRR), and glutathione peroxidase 7 (GPX7) were the most likely targets predicted by LINCS. SMARCE1 is required for the transcriptional activation of genes normally repressed by chromatin. AHRR mediates dioxin toxicity and is involved in the regulation of cell growth and differentiation. GPX7 is involved with cellular senescence and insulin secretion.

In the morphine-tolerant + saline versus morphine-tolerant + LPS group, AHR (aryl hydrocarbon receptor), UBE2L6 (ubiquitin-conjugating enzyme E2L 6), and PAFAH1B3 (platelet-activating factor acetylhydrolase 1b, Catalytic Subunit 3) were the top three candidates. AHR is involved in the regulation of biological responses to planar aromatic hydrocarbons; UBE2L6 targets abnormal or short-lived proteins for degradation; and PAFAH1B3 functions in brain development and is associated with mental retardation, ataxia, and atrophy of the brain.



The predicted potential targets in each group were different from those in other groups, both in targets and their possibility rankings. VPS28 was the only one that appeared in both the Top 100 lists of placebo-control + saline versus placebo-control + LPS (No. 1 in Table 4) and morphine-tolerant + saline versus morphine-tolerant + LPS (No. 4 in Table 4).

Table 4 shows the top three potential target genes from each set of gene comparisons. There was no similarity in the gene rankings in the three sets of gene comparisons.

**Table 4.** LINCS Consensus Knockdown Connections from differentially expressed genes in the rat brain in response to lipopolysaccharides (LPS), with and without morphine tolerance. (A) Top 10 Consensus Knockdown Connections in the three sets of gene comparisons; (B) Rankings of the top three Consensus Knockdown Connections in the three sets of gene comparisons. Full name of the genes were provided in Table A2.

|     | Rank      | placebo-Saline vs. Placebo-LPS | Placebo-Saline vs. Morphine-Tolerant-Saline | Morphine-Tolerant-Saline vs. Morphine-Tolerant-LPS |
|-----|-----------|--------------------------------|---|--|
| (A) | 1         | VPS28                          | SMARCE1                                     | AHR  |
|     | 2         | PROCR                          | AHRR  | UBE2L6   |
|     | 3         | CHMP2A                         | GPX7  | PAFAH1B3   |
|     | 4         | MB                             | ATP5F1                                      | VPS28  |
|     | 5         | ZNF768                         | CALR  | JUNB   |
|     | 6         | RBPJ                           | GPR110                                      | RYK  |
|     | 7         | WARS2                          | CHMP2A                                      | ARG1   |
|     | 8         | TBX2                           | ELF4  | PROC   |
|     | 9         | MRPS2                          | FCFR1                                       | ZNF324   |
|     | 10        | MAP3K14                        | F7  | ATP5D  |
|     | Gene Rank | Placebo-Saline vs. Placebo-LPS | Placebo-Saline vs. Morphine-Tolerant-Saline | Morphine-Tolerant-Saline vs. Morphine-Tolerant-LPS |
| (B) | VPS28     | 1                              | 42  | 4  |
|     | PROCR     | 2                              | 491   | 681  |
|     | CHMP2A    | 3                              | 7   | 177  |
|     | SMARCE1-1 | 675                            | 1   | 504  |
|     | AHRR      | 2383                           | 2   | 460  |
|     | GPX7      | 488                            | 3   | 2137   |
|     | AHR       | 46                             | 769   | 1  |
|     | UBE2L6    | 89                             | 545   | 2  |
|     | PAFAH1B3  | 95                             | 622   | 3  |

#### 4. Discussion

Inflammasomes recognize a variety of pathogen-associated molecular patterns (PAMPs), including endotoxins such as LPS. Depending on the NLR proteins that constitute inflammasomes, an inflammasome can be pro-inflammatory or anti-inflammatory in nature [28]. For pro-inflammatory inflammasomes such as NLRP3, *in vitro* studies have shown that the activation and release of pro-inflammatory cytokines requires two signals. The first signal, triggered by PAMPs, leads to the activation of inflammasomes, which then provide the second signal. The activated inflammasomes, through caspase 1 activation, promote the production of the pro-inflammatory cytokines, IL-1 $\beta$  and IL-18. However, the signaling pathways during infection or inflammation *in vivo* are not yet completely defined [29], and the characteristics of anti-inflammatory inflammasomes such as NLRP12 have not yet been extensively investigated. To our knowledge, our study is one of the first to report the modulation of NLRP12 expression in response to LPS and morphine *in vivo*.

Recently, NLRP12 was designated as an anti-inflammatory NLR inflammasome protein. It is believed to be a negative regulator of the NF- $\kappa$ B signaling pathway by inhibiting downstream signaling of TLRs, particularly IRAK-1 [28,30]. Our results showed that NLRP12 expression decreased in the brains of both the control (placebo-control + LPS) and morphine-tolerant (morphine-tolerant + LPS) rats in response to an LPS challenge, indicating that one of the mechanisms by which LPS induces an inflammatory response is by inhibiting the expression of the anti-inflammatory NLRP12 inflammasome.

Although NLRP12 expression was decreased in both groups given LPS, the decrease was significantly greater in the control rats than in the morphine-tolerant rats, which suggests that the LPS-induced NLRP12 decrease is countered during morphine tolerance. Hence morphine may also

modulate NLRP12 activity, directly or indirectly, thereby exerting its immunosuppressive effects and opposing the LPS-induced decrease in NLRP12 in the presence of morphine tolerance.

Birc3, a downstream regulator of inflammasome signaling, is essential for controlling the synthesis of cytokines and chemokines in the inflammatory Mapk and NF- $\kappa$ B pathways. It is also required for inflammasome activation, subsequent caspase 1 activity, and IL-1 $\beta$  formation [31]. In our study, Birc3 expression was significantly increased in both the placebo-control and morphine-tolerant rats in response to LPS, indicating that LPS is able to induce an inflammatory response through Birc3 activity, following inhibition of the anti-inflammatory NLRP12. However, in response to LPS, Birc3 expression in the morphine-tolerant rats did not change in comparison to the placebo-control rats. This indicates that morphine may not be able to modulate Birc3 expression, and therefore there is no change, increase or decrease, in its expression in the morphine tolerant state.

NF- $\kappa$ B is important in the activation of inflammatory mediators such as cytokines and chemokines [16]. Previous studies have reported that NLRP12 inhibits both canonical and non-canonical NF- $\kappa$ B activation [16,28] and that Nfkbia, a downstream regulator of inflammasomes, inhibits the activity of dimeric NF- $\kappa$ B/Rel complexes [32]. In our study, Nfkbia was significantly increased in response to LPS in both the placebo-control and morphine-tolerant rats. During an inflammatory response, one would expect the expression and activity of a positive regulator of inflammation to be increased, whereas that of a negative regulator would be decreased. However, from a physiological standpoint, there is a constant effort to balance pro- and anti-inflammatory activity [33,34]. This quest to balance the pro- and anti-inflammatory responses could be one of the reasons for an increase in Nfkbia, which is known to inhibit the activity of the pro-inflammatory dimeric NF- $\kappa$ B/REL, thus reducing the production of pro-inflammatory mediators.

As expected, we found that the expression of pro-inflammatory cytokines (IL-1 $\beta$  and IL-6) and chemokines (Ccl2, Ccl7, Cxcl1, and Cxcl3) was increased in response to LPS in the placebo-control rats [35–37]. In the morphine-tolerant rats, however, the LPS-induced cytokine and chemokine expression levels were lower, suggesting that NLRP12 inhibition in response to LPS may be opposed or subdued in the morphine tolerant state.

In a previous study, we observed that, in peripheral immune organs such as the spleen, NLRP3 expression, but not NLRP12 expression, is altered in response to LPS, with and without morphine tolerance [38], suggesting that the mechanism(s) of inflammasome activation in response to pathogens may be different in peripheral immune organs, compared to the central nervous system. During morphine tolerance, the LPS-induced expression of NLRP3, as well as that of cytokines and chemokines, is reduced in comparison to the placebo-control rats given LPS [38]. These observations are consistent with previous studies showing that immune activation, including an inflammatory response, is diminished during morphine tolerance [39]. Therefore, the data from the present study, as well as from our previous report [38], collectively indicate that morphine may exert its effects through both pro- and anti-inflammatory inflammasomes.

LINCS analysis is able to predict potential target genes based on a certain treatment and the gene profile signatures in its database. In our study, LINCS was able to generate a report of potential targets with a  $p$  value of  $<0.05$  from the list of genes with altered expression in response to LPS in control rats (placebo-control + saline versus placebo-control + LPS) but not from the other two comparisons (placebo-control + saline versus morphine-tolerant + saline, morphine-tolerant + saline versus morphine-tolerant + LPS), because there were not enough significant gene features in those two groups. When enlarging the set of gene features for the comparison of placebo-control + saline versus placebo-control + LPS to a  $p$ -value of  $<0.1$ , LINCS generated a report with similar potential targets. Thus, the gene features were then studied with a  $p$ -value of  $<0.1$  on all three sets of comparisons. VPS28, PROCR, and CHMP2A were the top three with the highest scores in LINCS report generated based on placebo-control + saline versus placebo-control + LPS gene alternations, suggesting that Vps28, Procr and Chmp2a were potential targets of LPS. In the report for placebo-control + saline versus morphine-tolerant + saline, SMARCE1, AHRR, and GPX7 were the most likely targets altered in

morphine tolerance predicted by LINCS. In the morphine-tolerant + saline versus morphine-tolerant + LPS report, AHR, UBE2L6, and PAFAH1B3 were the top three candidates that potentially responsible for the LPS-induced immune responses during morphine tolerance in rats. In the LINCS reports, the listed potential targets had different rankings using the different sets of gene features. This confirms that the response to LPS by those inflammasome-related genes could be affected by morphine tolerance.

Moreover, among the targets listed above, while VPS28 was No. 1 in the control rats in response to LPS, it was No. 4 in morphine-tolerant rats (Table 4). The VPS28 protein functions in transporting and sorting proteins into sub-cellular vesicles. In our study, LINCS analysis suggests that the actions of VPS28 in response to LPS could be dampened during morphine tolerance.

## 5. Conclusions

The results from our study indicate that, in the rat brain, LPS-induced inflammation involves both the inhibition of the NLRP12 anti-inflammatory inflammasome and the stimulation of downstream regulators such as Birc3, thereby increasing the expression of pro-inflammatory chemokines and cytokines. However, in the morphine tolerant state, the response to LPS is dampened, as indicated by the reduced expression of inflammasome-related genes. LINCS analysis confirmed that the response to LPS is altered during morphine tolerance and indicated that VPS28 may be one of the genes responsible for the alterations associated with morphine tolerance.

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**Author Contributions:** Sulie L. Chang designed the studies, participated in data collection, data analysis, and manuscript preparation, and approved the manuscript submission. Wenfei Wang conducted the LINCS analysis and participated in manuscript preparation. Sabroni Sarkar participated in the PCR array data analysis and in manuscript preparation. Xin Ma conducted the animal treatments, tissue collection, and PCR array analysis.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## Appendix A

**Table A1.** Genes analyzed in PCR Array.

| Gene Symbol | Full Name   | mRNA Entry     |
|-------------|---|----------------|
| Card6       | caspase recruitment domain family, member 6                                 | NM_001106413.1 |
| Casp1       | caspase 1   | NM_012762.2    |
| Casp12      | caspase 12  | NM_130422.1    |
| Casp8       | caspase 8   | NM_022277.1    |
| Naip2       | NLR family, apoptosis inhibitory protein 6                                  | XM_008760697.2 |
| Nlrp12      | NLR family, pyrin domain containing 12                                      | NM_001169142.1 |
| Nlrp5       | NLR family, pyrin domain containing 5                                       | NM_001107474.1 |
| Nlr4        | NLR family, CARD domain containing 4  | NM_001309432.1 |
| Nlrp1a      | NLR family, pyrin domain containing 1A                                      | NM_001145755.2 |
| Nlrp3       | NLR family, pyrin domain containing 3                                       | NM_001191642.1 |
| Nlrp6       | NLR family, pyrin domain containing 6                                       | NM_134375.3    |
| Nlr1        | NLR family member X1  | NM_001025010.1 |
| Nod2        | nucleotide-binding oligomerization domain containing 2                      | NM_001106172.1 |
| Pycard      | PYD and CARD domain containing  | NM_172322.1    |
| Bcl2        | BCL2, apoptosis regulator   | NM_016993.1    |
| Bcl2l1      | BCL2 like 1   | NM_001033670.1 |
| Birc2       | baculoviral IAP repeat-containing 2   | NM_021752.2    |
| Birc3       | baculoviral IAP repeat-containing 3   | NM_023987.3    |
| Clar        | CASP8 and FADD-like apoptosis regulator                                     | NM_001033864.2 |
| Chuk        | conserved helix-loop-helix ubiquitous kinase                                | NM_001107588.1 |
| Ciita       | class II, major histocompatibility complex, transactivator                  | NM_001278083.1 |
| Ctsb        | cathepsin B   | NM_022597.2    |
| Fadd        | Fas associated via death domain   | NM_152937.2    |
| Hsp90aa1    | heat shock protein 90, alpha (cytosolic), class A member 1                  | NM_175761.2    |
| Hsp90ab1    | heat shock protein 90 alpha family class B member 1                         | NM_001004082.3 |
| Ikbkb       | inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta  | NM_053355.2    |
| Ikbkg       | inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma | NM_199103.1    |
| Irak1       | interleukin-1 receptor-associated kinase 1                                  | NM_001127555.1 |
| Map3k7      | mitogen activated protein kinase kinase kinase 7                            | NM_001107920.2 |
| Map3k7ip1   | TGF-beta activated kinase 1/MAP3K7 binding protein 1                        | NM_001109976.2 |
| Map3k7ip2   | TGF-beta activated kinase 1/MAP3K7 binding protein 2                        | NM_001012062.1 |
| Mapk1       | mitogen activated protein kinase 1  | NM_053842.2    |
| Mapk11      | mitogen-activated protein kinase 11   | NM_001109532.2 |

Table A1. Cont.

| Gene Symbol | Full Name  | mRNA Entry     |
|-------------|--|----------------|
| Mapk12      | mitogen-activated protein kinase 12                          | NM_021746.1    |
| Mapk13      | mitogen activated protein kinase 13                          | NM_019231.2    |
| Mapk14      | mitogen activated protein kinase 14                          | NM_031020.2    |
| Mapk3       | mitogen activated protein kinase 3                           | NM_017347.2    |
| Mapk8       | mitogen-activated protein kinase 8                           | NM_053829.2    |
| Mapk9       | mitogen-activated protein kinase 9                           | NM_001270544.1 |
| Mefv        | Mediterranean fever  | NM_031634.1    |
| Myd88       | myeloid differentiation primary response 88                  | NM_198130.1    |
| Nfkb1       | nuclear factor kappa B subunit 1                             | NM_001276711.1 |
| Nfkbia      | NFKB inhibitor alpha   | NM_001105720.2 |
| Nfkbib      | NFKB inhibitor beta  | NM_030867.2    |
| P2rx7       | purinergic receptor P2X 7                                    | NM_019256.1    |
| Panx1       | Pannexin 1   | NM_001270548.1 |
| Pea15a      | phosphoprotein enriched in astrocytes 15                     | NM_001013231.1 |
| Pstpip1     | proline-serine-threonine phosphatase-interacting protein 1   | NM_001106824.2 |
| Ptgs2       | prostaglandin-endoperoxide synthase 2                        | NM_017232.3    |
| Rage        | MOK protein kinase   | NM_001010965.1 |
| Rela        | RELA proto-oncogene, NF-kB subunit                           | NM_199267.2    |
| Ripk2       | receptor-interacting serine-threonine kinase 2               | NM_001191865.1 |
| Sugt1       | SGT1 homolog, MIS12 kinetochore complex assembly cochaperone | NM_001013051.1 |
| Tirap       | TIR domain containing adaptor protein                        | XM_017596001.1 |
| Hsp90b1     | heat shock protein 90 beta family member 1                   | NM_001012197.2 |
| Traf6       | TNF receptor associated factor 6                             | NM_001107754.2 |
| Txnip       | thioredoxin interacting protein                              | NM_001008767.1 |
| Xiap        | E3 ubiquitin-protein ligase XIAP                             | NM_022231.2    |
| Ccl11       | C-C motif chemokine ligand 11                                | NM_019205.1    |
| Ccl12       | chemokine (C-C motif) ligand 12                              | NM_001105822.1 |
| Ccl2        | C-C motif chemokine ligand 2                                 | NM_031530.1    |
| Ccl5        | C-C motif chemokine ligand 5                                 | NM_031116.3    |
| Ccl7        | C-C motif chemokine ligand 7                                 | NM_001007612.1 |
| Cxcl1       | C-X-C motif chemokine ligand 1                               | NM_030845.1    |
| Cxcl3       | C-X-C motif chemokine ligand 3                               | NM_138522.1    |
| Cd40lg      | CD40 ligand  | NM_053353.1    |
| Ifnb1       | interferon beta 1  | NM_019127.1    |
| Ifng        | interferon gamma   | NM_138880.2    |
| Il12a       | interleukin 12A  | NM_053390.1    |
| Il12b       | interleukin 12B  | NM_022611.1    |
| Il18        | interleukin 18   | NM_019165.1    |
| Il1b        | interleukin 1 beta   | NM_031512.2    |
| Il33        | interleukin 33   | NM_001014166.1 |
| Il6         | interleukin 6  | NM_012589.2    |
| Irf1        | interferon regulatory factor 1                               | NM_012591.1    |
| Irf2        | interferon regulatory factor 2                               | NM_001047086.1 |
| Irf3        | interferon regulatory factor 3                               | NM_001006969.1 |
| Irf4        | interferon regulatory factor 4                               | NM_001106108.1 |
| Irf5        | interferon regulatory factor 5                               | NM_001106586.1 |
| Irf6        | interferon regulatory factor 6                               | NM_001108859.1 |
| Tnfsf11     | tumor necrosis factor superfamily member 11                  | NM_057149.1    |
| Tnfsf14     | tumor necrosis factor superfamily member 14                  | NM_001191803.1 |
| Tnfsf4      | tumor necrosis factor superfamily member 4                   | NM_053552.1    |

Table A2. Top 10 scored Genes in LINC report.

| Gene Symbol | Full name   |
|-------------|---|
| VPS28       | VPS28, ESCRT-I subunit  |
| PROCR       | protein C receptor  |
| CHMP2A      | charged multivesicular body protein 2A  |
| MB          | myoglobin   |
| ZNF768      | zinc finger protein 768   |
| RBPJ        | recombination signal binding protein for immunoglobulin kappa J region                            |
| WARS2       | tryptophanyl tRNA synthetase 2 (mitochondrial)  |
| TBX2        | T-box 2   |
| MRPS2       | mitochondrial ribosomal protein S2  |
| MAP3K14     | mitogen-activated protein kinase kinase 14  |
| SMARCE1     | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1 |
| AHR         | aryl-hydrocarbon receptor repressor   |
| GFX7        | glutathione peroxidase 7  |
| ATP5F1      | ATP synthase, H+ transporting, mitochondrial Fo complex subunit B1                                |
| CALR        | calreticulin  |
| GPR110      | G protein-coupled receptor 110  |
| ELF4        | E74 like ETS transcription factor 4   |
| FGFR1       | fibroblast growth factor receptor 1   |
| F7          | coagulation factor VII  |
| AHR         | aryl hydrocarbon receptor   |
| UBE2L6      | ubiquitin-conjugating enzyme E2L 6  |
| PAFAH1B3    | platelet-activating factor acetylhydrolase 1b, catalytic subunit 3                                |
| JUNB        | JunB proto-oncogene, AP-1 transcription factor subunit  |
| RYK         | receptor-like tyrosine kinase   |
| ARG1        | arginase 1  |
| ZNF324      | zinc finger protein 324   |
| ATP5D       | ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit                            |

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Review

# Oligodendrocyte Injury and Pathogenesis of HIV-1-Associated Neurocognitive Disorders

Han Liu, Enquan Xu, Jianuo Liu and Huangui Xiong \*

Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198-5880, USA; han.liu@unmc.edu (H.L.); enquan.xu@unmc.edu (E.X.); jnliu@unmc.edu (J.L.)

\* Correspondence: hxiong@unmc.edu; Tel.: +1-402-559-5140; Fax: +1-402-559-3744

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**Abstract:** Oligodendrocytes wrap neuronal axons to form myelin, an insulating sheath which is essential for nervous impulse conduction along axons. Axonal myelination is highly regulated by neuronal and astrocytic signals and the maintenance of myelin sheaths is a very complex process. Oligodendrocyte damage can cause axonal demyelination and neuronal injury, leading to neurological disorders. Demyelination in the cerebrum may produce cognitive impairment in a variety of neurological disorders, including human immunodeficiency virus type one (HIV-1)-associated neurocognitive disorders (HAND). Although the combined antiretroviral therapy has markedly reduced the incidence of HIV-1-associated dementia, a severe form of HAND, milder forms of HAND remain prevalent even when the peripheral viral load is well controlled. HAND manifests as a subcortical dementia with damage in the brain white matter (e.g., corpus callosum), which consists of myelinated axonal fibers. How HIV-1 brain infection causes myelin injury and resultant white matter damage is an interesting area of current HIV research. In this review, we tentatively address recent progress on oligodendrocyte dysregulation and HAND pathogenesis.

**Keywords:** HIV-1; dementia; oligodendrocyte; myelin sheath

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## 1. Introduction

With the introduction of combined antiretroviral therapy (cART), there was a significant decline in human immunodeficiency virus type one (HIV-1)-associated neurocognitive disorders (HAND). As HIV-1-infected patients live a longer lifespan with a cART regimen, it is becoming increasingly evident that the prevalence of milder forms of HAND seems to be on the rise [1–3]. Many studies have revealed a preferential damage to subcortical white matter (e.g., corpus callosum) in the HIV-1-infected brain, and such damage is prevalent even in the era of cART and more severe in patients with HAND [4,5]. HIV-1-related white matter damage includes demyelination and axonal dysfunction and injury. The demyelination occurs when myelin sheaths of neuronal axons are impaired in the central nervous system (CNS) or peripheral nervous system (PNS). Myelination, formation of myelin sheaths by oligodendrocytes wrapping neuronal axons in the CNS or Schwann cells in the PNS, is highly regulated by neuronal and astrocytic signals and maintenance of myelin sheaths is a complex process. The oligodendrocyte injury is a hallmark in demyelination and white matter damage. Such damage can be induced by an alteration of genetics, viral infections, inflammation, autoimmunity, and other unknown factors. HIV-1-associated oligodendrocyte/myelin damage has been observed both in cell culture [6] and patients [7].

The earlier studies demonstrate that human polyomavirus JC (JCV) primarily causes demyelination in HIV-1-infected brain. Compared to HIV-1 infection of astrocytes and microglia in the brain, JCV predominately infects oligodendrocytes and, thus, causes oligodendrocyte damage and further demyelination. Additionally, JCV is also the main causative factor for progressive multifocal

leukoencephalopathy (PML), a frequent opportunistic infection in the CNS and a common complication seen in AIDS patients [7,8]. Recent studies have shown that HIV-1 viral proteins per se can act on oligodendrocytes and produce detrimental effects, which are independent of JCV [6,9,10]. HIV-1 viral proteins, including the envelope glycoprotein 120 (gp120), trans-activator of transcription (Tat), and negative regulatory factor (Nef), have been implicated in HIV-1-associated oligodendrocyte injury [9,11–14]. Among these viral proteins, Tat has consistently been detected in both infected and uninfected oligodendrocytes in the brains of AIDS patients [7], and exhibited a synergistic detrimental effect with JCV or with addictive drugs, such as morphine. In this review, we tentatively address recent progress in HIV-1-associated oligodendrocyte pathophysiology, aiming at understanding the pathogenesis of milder forms of HAND.

## 2. Myelin/Oligodendrocyte Injury in HIV-1 Patients

The oligodendrocyte and myelin injury have been observed clinically from neurological imaging studies, serum biochemistry, and brain biopsies [15–18]. The diffusion tensor magnetic resonance imaging (DTI) promotes the investigations of white matter damage in early HAND and allows revealing the microstructures of myelin and oligodendrocytes. The changes of water molecules' diffusive parameters in brain white matter of HIV-1 patients, which indicate demyelination, have been detected in several DTI studies [15,19,20]. These findings were supported by a recent study on HIV-1-infected humanized mice that a decreased expression of myelin structural proteins was observed in whisker barrels, the corpus callosum, and the hippocampus, suggesting the loss of myelin elements [21]. In the sera and CSF of patients with HAND, antibody titers of myelin oligodendrocyte glycoprotein (MOG), an important myelin structural protein indicating CNS-specific autoimmune reaction for primary demyelination, are significantly higher compared with asymptomatic HAND patients and HIV-1-negative patients with other neurological diseases. In particular, the CSF anti-MOG antibodies exhibit a high sensitivity and specificity (85.7% and 76.2%) for discriminating patients with active HAND from those with asymptomatic HAND. The performance on HIV dementia scale tests is significantly worse and the viral loads in the CSF are higher in MOG immunopositive HAND patients than those in asymptomatic HAND patients [22], suggesting the dysfunction of oligodendrocytes is closely related with HIV-1 infection and HAND.

Compared to astrocytes that appear to promote recovery in response of injury, oligodendrocytes have a more passive role and tend to be damaged as a general response to insults [23]. In biopsy studies, the absolute number of nerve fibers and axons significantly decreased in HIV-1-infected brain, in particular in the frontal and occipital parts of the corpus callosum. The myelin sheath thickness diminished in corpus callosum as well [18]. Weighted gene co-expression network analysis showed that the oligodendrocyte-related genes are particularly elevated in the HIV encephalitis (HIVE) group, suggesting specific dysfunction of this cell type in those with HIVE [24].

In HIV-1 positive patients with PML, the myelin loss is apparent both macroscopically and microscopically [25]. Neuroimaging studies showed the myelin lesions were more frequently seen in the sub-cortical white matter areas [26]. PML is believed to be developed exclusively in immunosuppressive patients with significantly higher incidence in patients with AIDS, particularly in AIDS patients without cART and with a low CD4<sup>+</sup> lymphocyte count, than in patients with any other immunosuppressive conditions. Although cART has decreased the incidence of PML and improved patient survival [27], PML continues to occur in HIV-1-positive patients with good access to cART, and even with normal CD4<sup>+</sup> lymphocyte counts [28,29]. These findings suggest PML-related oligodendrocyte/myelin damage is often, but not necessarily, associated with severe immunosuppression or an immune reconstitution inflammatory syndrome (IRIS) in the cART era [30].

## 3. Fate of Oligodendrocytes in HIV-1-Infected Brain

Early publications reported that HIV-1 cannot be detected in oligodendrocytes [31,32] and this may due to the limitation of methodologies to identify oligodendrocytes. Dissenting results were found



in purified human oligodendrocytes from temporal lobe resections, HIV-1 (IIIB and BaL) infectivity was confirmed by detection of p24gag antigen and PCR amplification [33]. It is well-known that HIV-1 attaches and infects human host cells through CD4 receptors, along with CXCR4 and CCR5 as co-receptors. The oligodendrocytes are CD4- and CCR5-negative, but do express CXCR4 [31,34,35], which designedly promote the oligodendrocyte progenitor cell (OPC) migration and remyelination [36], and may provide the anchor for HIV-1-induced oligodendrocyte injury. However, most investigators agree that HIV-1 primarily infects microglial cells in the brain, but not oligodendrocytes. HIV-1-associated oligodendrocyte injury is believed to be mediated through viral proteins shed off from virions or released from infected other cells [9,11,12].

In HIV-1 patients with PML complication, Tat and JCV both are present in oligodendrocytes. Tat has been shown to synergize with JCV, and facilitate of JCV gene transcription and replication, leading to robust JCV infection [37,38]. Tat stimulates JCV gene transcription by cooperating with SMAD proteins, the intracellular effectors of TGF-beta, at the JCV DNA control region [37]. The effectiveness of Tat on facilitating JCV transcription and replication varies from different HIV-1 clades [38]. Since Tat is expressed in the brain at relative high levels while the viral load is controlled in blood, this may, at least in part, explain why some HIV-1 patients still develop PML despite having a good access to cART [39]. In addition to the synergistic effect of Tat and JCV in oligodendrocytes, cytotoxic CD8<sup>+</sup> T cells aggregate at demyelinated lesion sites in the brain to engage JCV-infected oligodendrocytes, which tend to control JCV dissemination, but at the cost of oligodendrocyte death and further demyelination in PML [40].

In addition to Tat, gp120 seems to be also involved in HIV-1-associated oligodendrocytes/myelin injury. It has been shown that gp120 inhibits myelination in rat cerebral cortex culture [12] and induces functional dysregulation and apoptosis in cultured oligodendrocytes [11,41], which is discussed in a subsequent section. In addition to the primary oligodendrocyte injury, which leads to secondary axonal injury (outside-in) to further exacerbate neurocognitive impairments, oligodendrocyte injury can be caused by primary axonopathy as well (inside-out) [42]. The recent study has shown that gp120-induced  $\beta$ -APP accumulation and axon injury in the corpus callosum was attenuated by a CXCR4 antagonist, exemplifying HIV-1 injury of oligodendrocyte/myelin via CXCR4 [35]. Although it is not clear whether gp120 causes such a detrimental effect through an “outside-in” or “inside-out” mechanism, or both, CXCR4 expressed in oligodendrocytes can be a potential target [42].

#### **4. Association between Blood-Brain Barrier (BBB) Disruption and Myelin Injury**

Increasing evidence indicates that myelin injury may be associated with a dysregulated blood-brain barrier (BBB) since myelin pallor is often observed in perivascular sectors during white matter edema [43,44]. The BBB is a critically-protective barrier for the brain and serves as a highly selective layer that separates the CNS from the rest of the body. In HIV-1-infected brains, the BBB disruption is believed to be mediated by both viral and cellular factors, released from HIV-1-infected and immune-activated mononuclear phagocytes and endothelial cells [45,46]. The reported direct mechanisms underlying HIV-1-associated BBB disruption are often related to alterations of vascular tight junctions, direct toxicity of brain endothelial cells, production of matrix metalloproteinases, and N-Methyl-D-Aspartate (NMDA) receptor activation [47,48]. The disruption of BBB is essential for HIV-1 entrance to the brain, resulting in brain white matter damage and consequent neurologic deficits in patients with neuroHIV. This notion is supported by an observation that HIV-1 patients with impaired BBB showed poorer neurologic status than those with intact BBB [49]. However, myelin damage may also be related to BBB disruption without HIV-1 brain invasion. In a case report, diffuse myelin pallor in white matter and massive perivascular dilatation were observed in an AIDS patient without evidence of brain HIV-1 infection, significant inflammation, or microglial activation [50]. Postmortem studies on the brains of AIDS patients revealed discrete myelin pallor areas always associated with capillaries or venules [44]. These findings suggest that BBB breakdown may contribute to the observed oligodendrocyte/myelin/white matter injury.

Under physiological conditions, the BBB endothelial cells and components of the extracellular matrix support OPC survival, and promote neural progenitor cells (NPC) differentiate to neurons, astrocytes, and oligodendrocytes [51–53]. The critical function of BBB and the consequences of BBB disruption imply its involvement in HIV-1-associated oligodendrocyte/myelin injury. It is not surprising that the disrupted BBB promotes the entrance of viruses, infected T cells, and toxic substances from the blood to the brain, resulting in OPC/oligodendrocyte/myelin injury. Inhibition of OPC proliferation can be caused by plasma, serum, thrombin, and plasmin in primary culture. Thrombin also suppresses the differentiation of OPCs into mature oligodendrocytes [54]. In addition, elevated levels of TNF- $\alpha$ , an inflammatory cytokine promoting oligodendrocyte death [55], were detected in blood mononuclear phagocytes in HIV-infected patients [56]. HIV-1 infection also induces interleukin (IL)-1 $\beta$  production from mononuclear phagocytes [57]. IL-1 $\beta$  promotes oligodendrocyte death through glutamate excitotoxicity [58]. These findings suggest that BBB disruption contributes to HIV-1-associated myelin/oligodendrocyte damage.

### 5. Cellular Mechanisms for Oligodendrocyte Injury in HIV-1-Infected Brain

Apoptotic signal activation of oligodendrocytes has been observed in HAND patients [59]. Such an apoptotic activation of oligodendrocytes could be caused directly by HIV-1 viral proteins or induced indirectly by immune and inflammatory factors.

It is well-known that tumor suppressor p53 induces apoptosis by activating transcription of various pro-apoptotic genes [60]. Activation of p53 was detected in the oligodendrocyte lineage cells in the brains of HAND patients, but not in control brains [59]. Due to the difficulties in distinguishing the differentiating stages of oligodendrocyte lineage on autopsy samples, the detected p53 reactivity reflects apoptosis of mature oligodendrocytes and OPCs. These suggest that, in addition to oligodendrocyte injury, proliferation of OPCs is also impaired in HAND.

Gp120 was shown to cause slow but progressive oligodendrocyte cytosolic Ca<sup>2+</sup> rise in a mixed culture of cerebellar cortex cells [41] and the rise of intracellular Ca<sup>2+</sup> concentration may trigger oligodendrocytic apoptosis. Exposure of oligodendrocytes to Tat also produced a rapid increase in intracellular Ca<sup>2+</sup> levels through NMDA and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, causing oligodendrocyte injury. It is worth mentioning that the roles of NMDA and AMPA receptors appear to be different and dependent on the stage of OPC differentiation. Tat-induced OPC death can be blocked by either NMDA or AMPA receptor antagonists. However, Tat's detrimental effects on mature oligodendrocytes can only be reversed by NMDA receptor antagonists, but not AMPA receptor blockade [6]. Tat was also found to cause oligodendrocyte apoptosis *in vitro* and myelin injury *ex vivo* by enhancing voltage-dependent K<sup>+</sup> channel (Kv) 1.3 activity [61]. The loss of K<sup>+</sup> ions may cause cell regulatory volume decrease (shrinkage), leading to cell apoptosis [62]. The involvement of Tat in oligodendrocyte apoptosis has been demonstrated in an HIV-1 Tat transgenic mouse model. The oligodendrocytes within the striatum exhibit a high sensitivity to morphine in HIV-1 Tat transgenic mice and they are the only apoptotic cell type in response to combined morphine exposure and Tat induction in Tat transgenic mice [9]. Tat also interacts with morphine to decrease the proliferation of OPCs [63]. Opioid abuse produces synergistic toxic activity in HIV-1-infected brains by direct actions on immature astrocytes and oligodendrocytes, which express  $\mu$ -opioid or  $\kappa$ -opioid receptors [64].

In other viral-induced demyelination, there is clear evidence that mouse hepatitis virus (MHV) can directly infect and activate microglia during acute inflammation, which eventually causes phagocytosis of the myelin sheath, leading to demyelination during the chronic inflammation stage [65]. A similar theory has been proposed for multiple sclerosis, which is the most prevalent demyelinating disease, that immune-activated microglia strip the myelin. Recent evidence has shown that microglia become phagocytic in response to HIV-1 Tat [66,67]. It might be possible that the infected and activated microglia phagocyte oligodendrocytes and myelin sheath lead to the myelin damage and consequent HAND pathogenesis, although there is no direct evidence indicating microglia phagocytosis of oligodendrocyte in neuroHIV [68].

## 6. Myelin Maintenance and Remyelination in HIV-1-Infected Brain

Repair of the damaged myelin sheath, which is termed remyelination, is physiologically required to maintain myelin homeostasis. The myelin injury in neuroHIV may also be induced by abnormalities of remyelination, in addition to the loss of existing myelin sheath. Remyelination requires proliferation and survival of OPCs, migration of OPCs to the damaged site, and development of OPCs from immature to mature myelinating oligodendrocytes. HIV-1 disrupts OPC development, migration, and remyelination processes.

### 6.1. Alteration of OPC Proliferation and Differentiation in HIV-1-Infected Brains

In HIV-1-infected brains, mild degrees of myelin damage were associated with an increase in oligodendrocyte numbers, an initial reactive hyperplasia which was believed to represent an attempt to repair myelin damage. Such a change was reversed in the presence of severe myelin damage [69]. In agreement with the aforementioned results, mRNA levels of transcription factor Olig2, a marker expressed with higher levels in OPCs and lower levels in mature oligodendrocytes [70], are elevated in the front cortex of patients with HIV-1 [24], indicating an increase of OPC proliferation needed for repairing the damaged myelin sheath. Mature oligodendrocyte defects are also observed in animal models of secondary degeneration, which represents additional loss of neurons, myelin, and glial cells through toxic events. Early onset of secondary degeneration triggers OPC proliferation, but the cell numbers decrease in a long-term degenerative condition [71]. However, Tat exposure reduces the population of undifferentiated Sox2<sup>+</sup> NPC (ancestor of OPC) and Olig2<sup>+</sup> OPCs, but progenitor survival is unaffected [63], suggesting the proliferation was interrupted. Tat may inhibit NPC proliferation by downregulating cyclin D1, which is an important cell cycle component interacts with cyclin-dependent kinase 4 and 6 [72]. Over all, HIV-1 infection or viral protein exposure appears to incline NPC fate toward production of glia/astroglia at the expense of neurons and/or oligodendrocytes [63,73,74]. Thus, OPC differentiation and maturation are likely the key processes affected during remyelination in neuroHIV.

### 6.2. Imbalance of OPC Differentiation and Remyelination in HIV-1-Infected Brain

It has been shown that differentiation of OPCs into post-mitotic oligodendrocytes is a major checkpoint in the myelination process and such an oligodendrocyte differentiation is controlled by a number of factors, many of which act to inhibit myelination, including leucine-rich repeat and immunoglobulin domain-containing-1 (LINGO-1) [75,76], Notch-1 [77,78], and Wnt [79,80], whereas p38 MAPK [81,82] and AKT [83] have been shown to be required for oligodendrocyte differentiation and myelination. While molecular mechanisms in the regulation of developmental myelination are discussed in excellent review papers published elsewhere [84,85], the direct interaction between HIV-1 and these molecules remains largely unknown. It is, however, believed that HIV-1 may disturb the complex regulating network leading to the remyelination imbalance based on the following findings: (1) HIV-1 alters the cell cycle by Wnt signaling pathways and further impacts the cell proliferation and differentiation in different cell types, including peripheral blood mononuclear cells [86], HEK293 cells [87], and astrocytes [88]; (2) HIV-1 infection of astrocytes altered the astrocytic Wnt profile by elevating Wnt family members 2b and 10b [88]; (3) elevation of secreted Wnt from astrocytes may negatively regulate oligodendrocyte differentiation in neuroHIV; (4) Notch-1 signaling is permissive for OPC expansion, but inhibit differentiation and myelin formation [89]; and (5) in Kaposi's sarcoma cells, which is a neoplasm in HIV-1-infected individuals, overexpression of activated Notch-1 signaling is detected [90]. However, to our knowledge, there is no report yet on how OPC/oligodendrocyte Notch-1 signaling in responding to HIV-1. Moreover, CD44, a predominant hyaluronan receptor widely expressed in the nervous system, plays a negative role in OPC differentiation and myelination. Overexpression of CD44 in precursor cells inhibits differentiation toward oligodendrocytes and promotes differentiation into astrocytes, cause progressive demyelination in conditional transgenic

mouse model [91,92]. In lymphocyte cell lines of Jurkat and U937 cells, HIV-1 infection-caused particle production is accompanied by CD44 upregulation [93]. In HIV-1-related diffuse large B-cell lymphoma patients, the CD44 levels significantly increased compared with HIV-1-unrelated diffuse large B-cell lymphoma patients (87% vs. 56%) [94]. These findings suggest CD44 may play a role in HIV-1-related remyelination failure.

Neurotrophins are important factors in the regulation of oligodendrocyte myelination and remyelination. The main cellular sources for neurotrophins in the brain are astrocytes, microglial cells, and neurons, in addition to lymphocytes' contribution through the blood circulation. Being aware of excellent reviews on the alteration of neurotrophins in HAND [95] and immunological communications between oligodendrocytes and microglia [96], we focus here on the HIV-1-induced alterations of neurotrophins that are potentially associated with oligodendrocyte abnormalities.

The platelet-derived growth factor (PDGF) is the most predominant mitogen for oligodendrocyte lineage cells. PDGF A and B chains both promote proliferation through activating PDGF receptor alpha (PDGFR $\alpha$ ) expressed on OPCs, whereas the PDGF B chain appears to be more important for early NPC expansion [97,98]. It has been shown that PDGF regulates OPC development via glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) signaling pathway, which is a negative regulator of OPC differentiation and remyelination [99,100]. PDGF-BB prevents NPC from Tat-mediated proliferating impairment by inactivating GSK-3 $\beta$ / $\beta$ -catenin pathways and, this effect is significantly inhibited by the p38 and JNK inhibitors [101]. The levels of fibroblast growth factor (FGF), which is an important pro-survival signal to stimulate OPC proliferation [102], increased in the sera of HIV-1-infected patients [103,104], but decreased in CSF [103]. FGF signaling complex is interrupted in HIV-1-infected brains, resulting in the abnormal activation of downstream signals, including GSK-3 $\beta$  [105,106], p38, ERK, and JNK cascades [107] in neurons through the surface receptors, such as NMDA receptor and CXCR4, which are also expressed on oligodendrocytes [36,108,109]. In addition, HIV-1 Tat and FGF-2 share a common core mechanism of unconventional secretion [110], although it is not clear whether they compete for the secretory routine. The brain-derived neurotrophic factor (BDNF), predominantly derived from astrocytes, has also been found to be essential for oligodendrocyte lineage development [68,111–113]. In rat primary neurons, gp120 promotes a time-dependent proBDNF accumulation at both intracellular and extracellular spaces by decreasing the expression level of intracellular furin, an enzyme required for cleavage and release of mature BDNF, leading to a reduction in mature BDNF. A similar imbalance in the ratio of proBDNF/mature BDNF was confirmed in postmortem brains of HAND patients [114]. These findings suggest that HIV-1 decreases the brain BDNF level by infecting astrocytes and gp120-associated neurotoxicity, resulting in downregulated remyelination. As BDNF is believed to protect neurons from HIV-1-induced apoptosis, thus, the reduction of BDNF may make the oligodendrocyte lose the support from neuronal axons that consequently cause myelin damage through the "inside-out" mechanism as proposed [42].

In addition to these signaling molecules, HIV-1 Tat interacting protein (TIP30), a co-factor that specifically enhances HIV-1 Tat-activated transcription [115], negatively regulates oligodendrocyte development. Overexpression of TIP30 dramatically inhibits the OPC differentiation, while knockdown of TIP30 enhances the differentiation of OPC remarkably [116]. The blockade of TIP30 may have dual benefits on inhibiting Tat-dependent gene transcription and promoting OPC differentiation, which is a potential therapeutic strategy for HIV-associated demyelination. Potassium channels are also involved in regulation of OPC development. Kv1.3 [117,118], Kv1.6 [117], Kv2.1 [119], and inward-rectified K<sup>+</sup> channel 4.1 [120,121] play crucial roles in regulation of OPC/oligodendrocyte proliferation and differentiation. Generally, channel expressions on oligodendrocyte lineage cells correlate with differentiating stages and are more complex in OPCs than in oligodendrocytes. Particularly, Kv1.3 channel plays an important role in G1/S transition in proliferating OPCs through regulating AKT signaling [118,122]. Moreover, L-type voltage-operated Ca<sup>2+</sup> channel 1.2 knockdown induces a decrease in the proportion of oligodendrocytes expressing myelin proteins, and an increase in the population of immature oligodendrocyte [123].

Most recent studies proposed that myelin injury in HAND is partially due to the effects of antiretroviral drugs on oligodendrocyte survival and differentiation. The common prescribed antiretroviral drugs, ritonavir and lopinavir, impair both the differentiation of OPCs into myelin-producing oligodendrocytes and the maintenance of myelin proteins *in vivo*. Ritonavir induces accumulation of reactive oxygen species, which arrest the oligodendrocyte differentiation process [124,125]. Controversial results were reported in HIV-1-infected children in Africa that significant myelin loss in cART-naïve children was observed in comparison with cART-treated children. However, cART-treated children also exhibited a significant myelin loss in the corpus callosum [126]. Interestingly, myelin-related genes encoding myelin-associated oligodendrocyte basic protein, myelin transcription factor 1, and myelin basic protein are downregulated in both cART-treated and untreated HAND patients [127]. Apparently, the impact of antiretroviral drugs on oligodendrocyte pathophysiology requires further investigation.

## 7. Summary and Prospects

HIV-1 persists in the brain despite cART. The cART-treated subjects are not able to purge the virus from their brains and show concomitant and persistent white matter abnormalities. There are increasing interests in understanding how HIV-1 causes myelin sheath loss and white matter damage in HIV-1-infected brains. In this article, we try to address the clinical and postmortem manifestations of myelin damage in HAND patients and possible involvement of BBB integrity disruption, oligodendrocyte apoptosis mechanisms, and OPC regulation imbalance in HIV-1-induced oligodendrocyte/myelin abnormalities. The studies on direct toxicity of HIV-1 viral proteins on oligodendrocytes and OPCs are emerging. As the transcription of HIV-1 viral protein continues in the CNS, even when the viral load is at a low level [128], the persistence of the virus and viral proteins in the brain has changed the pattern of HAND pathogenesis, by which inflammation, encephalitis, and neurodegeneration have been significantly decreased by the advent of cART.

The methods of regulation of oligodendrocyte lineage cell development are well-established, including the extracellular pathways, cell to cell contact, and intracellular pathways. As NG2<sup>+</sup> cells are the largest population of progenitor cells in the human adult brain, a decrease of absolute cell number and proliferation of NPC and OPC may contribute less to myelin deficits in HAND. In contrast, HIV-1-related OPC differentiation and remyelination imbalance may better correlate with an impaired remyelination in HAND patients. The strategies for promoting axonal remyelination have been introduced especially in those demyelinating disease like multiple sclerosis. It is anticipated that those strategies for promoting axonal remyelination in other neurodegenerative disorders can be applied for HIV-1-associated oligodendrocyte/myelin injury, though studies are needed to elucidate the underlying mechanisms for HIV-1-associated brain white matter damage.

Further studies on understanding the mechanisms underlying HIV-1-associated oligodendrocyte/myelin injury may be hampered by the following potential difficulties: first, oligodendrocytes share many common extracellular signals and intracellular signaling pathways with neurons, the proposed “inside-out” and “outside-in” mechanisms for virus-induced demyelination are indistinguishable under these conditions [42]; and, second, the pro-proliferation signals for OPC are sometimes anti-maturative [129–131]. This will be a significant challenge to identify the certain time window to access proper remyelination *in vivo*. Overall, promoting remyelination could be an important therapeutic strategy for HAND and other neurodegenerative disorders in the future.

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Review

# Overview of Traumatic Brain Injury: An Immunological Context

Damir Nizamutdinov <sup>1,2</sup> and Lee A. Shapiro <sup>1,2,\*</sup>

<sup>1</sup> Department of Surgery, Texas A & M University Health Science Center, College of Medicine, Temple, TX 76504, USA; dnizamutdinov@medicine.tamhsc.edu

<sup>2</sup> Department of Neurosurgery, Neuroscience Research Institute, Baylor Scott & White Health, Temple, TX 76504, USA

\* Correspondence: lshapiro@medicine.tamhsc.edu; Tel.: +1-254-724-6267

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**Abstract:** Traumatic brain injury (TBI) afflicts people of all ages and genders, and the severity of injury ranges from concussion/mild TBI to severe TBI. Across all spectrums, TBI has wide-ranging, and variable symptomology and outcomes. Treatment options are lacking for the early neuropathology associated with TBIs and for the chronic neuropathological and neurobehavioral deficits. Inflammation and neuroinflammation appear to be major mediators of TBI outcomes. These systems are being intensively studied using animal models and human translational studies, in the hopes of understanding the mechanisms of TBI, and developing therapeutic strategies to improve the outcomes of the millions of people impacted by TBIs each year. This manuscript provides an overview of the epidemiology and outcomes of TBI, and presents data obtained from animal and human studies focusing on an inflammatory and immunological context. Such a context is timely, as recent studies blur the traditional understanding of an “immune-privileged” central nervous system. In presenting the evidence for specific, adaptive immune response after TBI, it is hoped that future studies will be interpreted using a broader perspective that includes the contributions of the peripheral immune system, to central nervous system disorders, notably TBI and post-traumatic syndromes.

**Keywords:** traumatic brain injury; neuroimmunity; neuroinflammation

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## 1. Types of Traumatic Brain Injuries in Humans

### 1.1. Epidemiology of TBI in the United States

A traumatic brain injury (TBI) is an injury that disrupts the normal function of the brain and can be caused by a bump, blow or jolt to the head, rapid acceleration and deceleration of the calvarium, or a penetrating head injury [1]. In 2010, the Centers for Disease Control and Prevention estimated that TBIs accounted for approximately 2.5 million emergency department (ED) visits in the United States. Of these, approximately 87% (2,213,826) were treated and released, 11% (283,630) were hospitalized and discharged, and approximately 2% (52,844) died [2]. The leading causes of non-fatal TBI in the U.S. are falls (35%), motor vehicle-associated accidents (17%) and strikes or blows to the head from/against objects, including sport injuries (17%) [3]. The leading causes of TBI-related deaths are motor vehicle crashes, suicides and falls. In the United States, children aged 0–4 years, adolescents aged 15–19 years, and older adults aged >75 years have the highest rates of TBI-related hospitalizations and deaths among all age groups [3]. Approximately 145,000 children/adolescent (aged 0–19 years) and 775,000 older adults (>75 years) are estimated to be living with substantial and long-lasting limitations in social, behavioral, physical and/or cognitive functioning following a TBI [4]. In every age group, TBI-related ED visit rates are higher for males than for females, which were 800.4 vs. 633.7 cases per 100,000,

respectively [2]. Males aged 0–4 years have the highest rates for TBI-related emergency department visits, hospitalizations and deaths combined. Regarding the military, Department of Defense data revealed that from 2000–2011, 235,046 service members (4.2% of the 5,603,720 who served in the Army, Air Force, Navy and Marine Corps) were diagnosed with a TBI [5]. Thus, TBI afflicts millions of people each year, including civilian and military populations. It is pertinent to note that these statistics do not account for those people suffering from concussion/mild TBI who did not receive medical care or had outpatient/office-based visits, estimated by some to be hundreds of thousands, if not millions of people each year [3].

### *1.2. Classification of TBI*

The severity of TBIs is typically categorized using the Glasgow Coma Scale and can range from: (a) mild; (b) moderate; to (c) severe [6]. TBI outcomes are often determined by using the Glasgow Outcome Scale, which categorizes gross neurobehavioral ranges of recovery: (a) dead; (b) vegetative state; (c) severe disability; (d) moderate disability; (e) good recovery [7]. An alternative prognosis, using Russell and Smith's classification, is divided as severe or very severe [8]. Considering that detailed classification helps to determine the severity of injury, informs treatment options and is used to assess prognosis and functional recovery, recent suggestions have indicated that better diagnostic and assessment criteria are needed in the TBI field [9,10].

### *1.3. TBI Prognosis*

The effects of TBI can adversely affect quality of life, including cognitive, behavioral, emotional and physical deficiencies. Any one or more of these can negatively impact interpersonal, social and occupational functioning, as well as families, communities and the economy in general [11,12]. Impairment of cognitive function can lead to difficulties with memory, attention, learning, coordination and sleep disturbances [12] and can persist for days, months or even years following the initial injury. Other long-term deficiencies include: language and communication problems (19%), dysarthria (30%), dysphagia (17%) [13], mood disorders [14,15] and cognitive impairment, even six months after mild TBI [16]. Another post-traumatic syndrome that can have a relatively delayed onset is post-traumatic epilepsy [14,15,17]. While all epilepsies are seizure disorders, not all seizures are epilepsy. As such, the incidence of early post-traumatic seizures (seizures immediately following, up to the first few days after the TBI) is higher than the incidence of post-traumatic epilepsy. Notably, about 25% of brain contusion patients and 32%–53% of patients with penetrating TBI develop different degrees of early post-traumatic seizures. Post-traumatic seizures also seem to be more prevalent following severe TBIs, although mild and moderate TBIs can also result in seizures [18]. Considering the negative impact of these numerous disorders associated with post-traumatic deficiencies, as well as the significant numbers of people suffering from the chronic effects of TBIs, research efforts are underway in the hopes of better understanding the pathogenic progression, and developing successful treatments of and diagnostic criterion for TBI.

## **2. A Brief Review of Experimental TBI Animal Models**

In view of the heterogeneous clinical nature of TBI, numerous animal models have been developed for experimentation. Although larger animals are closer in size and physiology to humans, rodents are a valuable and commonly-used model in TBI research. Their modest cost, biological similarities, more manageable size and standardized outcome measurements are all advantageous. Such models have been incorporated for studies aimed at improving our understanding of the detrimental, complex molecular cascades that are initiated by head trauma, as well as the long-term neurological and behavioral consequences. Therefore, unless otherwise indicated, this review focuses on data from animal models (primarily rodents) of TBI. Among them, several models are widely used in research: fluid percussion injury (FPI) [19–22], cortical impact injury (CCI) [23–25], penetrating ballistic-like brain injury [26], weight drop/impact acceleration injury [27] and blast TBI injury [28,29]. Although

we will highlight the two most highly-cited animal models, FPI and CCI, it is important to note that many similarities with regard to the general neuroinflammatory responses are observed across rodent models of TBI, despite the different methods and modalities of the injuries.

### *2.1. Percussion Injury Animal Models*

In percussive injury models, there are two devices that are most commonly incorporated into experiments. In the FPI model, the insult is inflicted by a pendulum striking the piston of a reservoir of fluid, and this generates a fluid pressure pulse that is delivered to the intact dura, via a syringe secured over an opened midline or lateral craniotomy [30,31]. In the second percussive TBI, an injury is delivered by a piston that is controlled either pneumatically or via a piezoelectric mechanism [32–34]. The percussion produces brief displacement and deformation of brain tissue, and the severity of injury depends on the strength of the pressure pulse [31], as well as the location of the craniotomy/injury. FPI models replicate clinical TBI without skull fracture [35] and, despite the craniotomy, are considered a closed head injury model [36]. The FPI model is often considered to be of mild to moderate severity, rather than severe [30]. A number of studies have shown that FPI can reliably reproduce intracranial hemorrhage [30,31], swelling [31,37], neuroinflammation [37–39] and gray matter damage [19,30], all of which are pathophysiological changes observed in human TBIs [40]. Based on the position of the craniotomy, FPI models can be divided into midline (centered on the sagittal suture), parasagittal (<3.5 mm lateral to midline) and lateral models (>3.5 mm lateral to midline; LFPI) [31,41–43]. The midline FPI model of TBI was first developed for use in cats and rabbits [37,44], secondly adapted for rats [30] and subsequently modified for use in mice [19,31]. FPI has also been used for studying TBI pathophysiology and pharmacology in other species [37,45,46], although the volume of literature pales in comparison to that for rodents.

In rodents, FPI produces a rapid combination of focal cortical contusion and diffuse subcortical (such as hippocampus and thalamus) neuronal injury. These can occur within minutes of the impact, progress to a loss of neurons by 12 h and are accompanied by a rapid neuroinflammatory response that is initially focused in the peri-injury region [38,39]. Neuronal death and neuroinflammatory signaling seem to peak at around three days after FPI and in many other models [38]. This inflammation persists, albeit at levels lower than the peak, well into chronic time points ( $\geq 1$  month). In the days and months following the injury, progressive degenerative cascades that include chronic inflammation continue to be observed in a variety of brain regions implicated in higher cognitive functions. These include the hippocampus, thalamus, medial septum, striatum and amygdala [35,47,48]. It is often deduced that the neuropathology in these regions underlies the observed neurobehavioral and cognitive deficits that are commonly seen in the FPI model [36,49,50]. Of significance is the fact that analogous symptoms are often seen in patients with TBI-related injuries to corresponding brain regions [36,49].

### *2.2. Controlled Cortical Impact Injury Animal Model*

The CCI model uses a pneumatic or electromagnetic impact device to drive a rigid impactor onto the exposed intact dura and mimics cortical tissue loss, acute subdural hematoma, axonal injury, concussion, blood-brain barrier (BBB) dysfunction and even coma [23–25]. It has been applied to a number of animals, such as ferrets [24], swine and monkeys [51] and, most prominently, rodents [23,25]. CCI is delivered to the intact dura through a craniotomy and results in deformation of the underlying cortex [23]. The damage created is highly reproducible and includes a rapid and sometimes widespread neuropathological damage. This damage is most prominent in the peri-injury area, includes neurodegenerative and neuroinflammatory responses [52], and can also encompass cortical, hippocampal and thalamic degeneration [53]. The histopathological severity of CCI rises with increasing cortical deformation, as does the cognitive impairment that is likely related to the extent of damage [54–59]. Similar to the FPI model, the neuropathology and associated cognitive and behavioral deficits after CCI persist chronically, and diffuse neuropathology is evident [60,61].

Similarly, the neuroinflammatory response appears to play a major role in both the early and chronic deficiencies observed following TBI.

### 3. Mechanisms of Neuropathology Following TBI

It is now widely acknowledged that TBI is a complex multimodal disease process, not a single pathophysiological event [62]. It causes structural and functional damage, which lead to deficits resulting from both primary and secondary injury mechanisms [63]. The primary injury is the result of the immediate mechanical damage from direct contact and/or inertial forces to the brain that occurs at the moment of the traumatic impact. This damage can include direct neuronal, glial and other cellular damage, contusion, damage to blood vessels (hemorrhage) and axonal shearing [64,65]. Secondary injury evolves over minutes, to days, to months, to years after the primary injury and is the result of cascades of metabolic, cellular and molecular events. These occur concurrently with, and contribute to, alterations of endogenous neurochemical, inflammatory and neuroinflammatory mechanisms. Such mechanisms ultimately lead to brain cell death or rescue, plasticity, tissue damage and atrophy [35,66,67]. Many biochemical alterations responsible for secondary injury have also been identified. These include, perturbation of cellular calcium homeostasis, glutamate excitotoxicity, mitochondrial dysfunction, increased free radical generation, inflammation, neuroinflammation, increased lipid peroxidation, apoptosis and diffuse axonal injury (DAI) [68]. Interestingly, all of these alterations can be linked either directly or indirectly to neuroinflammation, and such inflammation has been implicated in the early and chronic components of TBI-induced neuropathology [69–71].

### 4. Inflammation Following TBI: An Immunological Perspective

#### 4.1. Innate, Non-Specific Immune Response to TBI

At present, the prevailing viewpoint in the TBI field has been that most, if not all of the inflammation that follows a TBI can be considered components of the innate immune response [72–74]. However, accumulating evidence using updated technology suggests that specific adaptive immune mechanisms are also at play. Thus, a working operational definition is needed to define immune specificity after TBI, and few authors have adequately separated innate from adaptive immune components after a TBI. The early neuroinflammatory response across injuries and injury models occurs in a relatively stereotypical manner and can largely be considered to consist mainly of innate immune mechanisms. When damage to the brain takes place during TBI, it triggers the release and production of cytokines and chemokines, which activate receptors, and results in local and systemic immune responses [72,75,76]. The net effect of these innate inflammatory mediators is aimed at limiting the spread of the injury and restoring homeostatic balance [77].

#### 4.2. Cytokines in TBI

Cytokines are categorized by structural and functional components, can be either pro- and/or anti-inflammatory and, in a classical immunological sense, are mediators of the cellular immune response, as well as of antibody synthesis and release. Cytokines can be synthesized and/or released by a wide variety of cells, including microglia, macrophages, T and B lymphocytes, endothelial and mast cells [78,79]. Although a full discussion of cytokine changes and functions after TBI is beyond the scope of this review, several reports indicate that interleukin (IL) IL1- $\beta$ , IL18 and tumor necrosis factor alpha (TNF $\alpha$ ) are involved in the onset and development of the inflammatory cascade after TBI in rodents and humans [72–74]. IL1- $\beta$  binds to IL1-receptors, primarily localized on microglia and astrocytes in the brain, but also to other cell types, including infiltrating immune cells [80,81]. Activation of the neuroglial and immune cell IL1-receptors initiates the production and release of inflammatory cytokines, including increased production of IL1- $\beta$  and IL18 [82]. This results in a self-perpetuating, pro-inflammatory environment, which may be damaging to the CNS parenchyma [75,76,83,84].



The damaging effects of IL1- $\beta$  can also be related to activation of other pro-inflammatory pathways, such as, TNF $\alpha$  [85] and IL18 [72].

Several studies support the evidence of rapid and sustained induction of TNF $\alpha$  in damaged brain tissue, within one hour after TBI in rodent models [75,76,86]. TNF $\alpha$  triggers the production of other cytokines (IL1- $\beta$ , IL6), chemokines [75] and nuclear factor kappa B (NF- $\kappa$ B) family (p50, p52 and p65) of transcription factors. Thus, TNF $\alpha$  is an important modulator of inflammation, at the transcriptional and translational levels, in the nervous system and in non-neural tissues [87,88]. IL18 also appears to play an important inflammatory role after TBI. IL18 has been shown to be elevated following a number of CNS inflammatory insults [89–91], including TBI [72]. In humans and rodents, the IL18 pathway can contribute to delayed neuronal injury, up to 14 days following TBI [72]. Activated neuroglial and immune cells in the area of injury secrete IL18, which binds to the IL18 receptor. Activation of the IL18 receptor initiates inflammatory signaling cascades [92]. Thus, cytokines, some of which have been mentioned here, are major contributors to the inflammatory and neuroinflammatory response.

#### 4.3. Chemokines in TBI

Chemokines (CCL) are chemotactic inflammatory proteins that mediate interactions among inflammatory cells and target cells. In general, chemokines are typically 10 kDa or smaller. CCLs are synthesized and/or released along with other mediator molecules by a variety of cell types that include: astrocytes, microglia, macrophages, eosinophils, neutrophils, dendritic cells, mast cells and natural killer cells (NK cells) [93–95]. Release of chemokines serves to chemotactically guide receptor-sensitive cells, primarily through activation of G protein-coupled receptors [96]. Similar to cytokines, chemokines can be either pro- and/or anti-inflammatory. After a TBI, chemokines contribute to the attraction of a wide range of immune cells to the site of damage [97,98]. The specific activities of classes of chemokines have been elucidated following TBIs [99–101]. Although a full discussion is beyond the scope of this review, one example, CXC chemokines, activates the migration of neutrophils to the site of the lesion [96]. Alternatively, chemokines CCL2, CCL3, CCL5, CCL7, CCL8, CCL13, CCL17 and CCL22 attract monocytes and macrophages [102–104]. Other chemokines, such as, CCL1, CCL2, CCL17 and CCL22, are involved in the recruitment of T-lymphocytes [103].

Another important role of cytokine and chemokine release is to activate pattern recognition receptors (PRRs). PRRs are proteins of the innate immune system and identify danger-associated molecular patterns (DAMPs) of cellular stress. This identification, and the ensuing response, helps defend against cell and/or tissue damage [105,106]. The PRRs are divided into several subgroups, depending on cell localization, type and function. One such group is the nucleotide-binding domain leucine-rich repeats (NLRs) [107], which are also called the nucleotide oligomerization domain (NOD)-like receptors. These receptors are located in the cytoplasm and help to regulate the host inflammatory, apoptotic and innate immune responses [108,109]. The NLR family of proteins can be activated by multiple types of cell/tissue damage that are seen in TBI and can form multi-protein complexes called “inflammasomes” [110]. The unique compositions of these inflammasomes depend on the extent and type of cell and tissue damage. Some reports suggested the specific contributions of NLR family proteins (NLRP3-inflammasome) after TBI [110,111]. The NLRP3-inflammasome has been detected in neurons, astrocytes and microglia in the cortex after TBI [110], and the NLRP3-inflammasome complex is associated with increases in the aforementioned IL1- $\beta$  and IL18 [108,110,112]. Interestingly, the NLRP3-inflammasome has also been demonstrated to associate with other CNS inflammatory disorders, including Alzheimer’s disease (AD) [113], which is an increased risk factor following TBI.

Thus, the overall contributions of the cytokines and chemokines released after TBI are mediated by the release, and subsequent recruitment of immune cells to the site of injury, and to coordinate the ensuing activity of these cells. These immune cells are an essential part of the innate immune response and also the putative transition to the adaptive immune response and will be discussed below.

#### 4.4. Cellular Immune Response to TBI

Several reports show that TBI and the associated neuroinflammation lead to local deposition of specific immune cells at the peri-injury area and beyond [97,114,115]. In addition, peripheral inflammation can also influence TBI outcomes [116]. There appears to be a rapid expansion and activation of peripheral immune cells after experimental TBI, as well as a significant extravasation of immune cells from the spleen, resulting in splenic hypotrophy following TBI [117,118]. It is also known that some of these immune cells that exit the spleen can contribute to the innate immune response, and some may also contribute to an ensuing adaptive immune response following a TBI [116,119,120]. Similarly, it is also known that some of these immune cells will infiltrate the CNS [121–123]. Immune cells involved in coordinating the innate immune response include: (1) monocytes, which develop into macrophages; (2) mast cells; (3) granulocytes (basophils, eosinophils and neutrophils); (4) dendritic cells (DC); and (5) natural killer cells.

Neutrophils and macrophages strongly infiltrate the brain in the early phase of TBI [121,123,124]. Neutrophils appear to be the most numerous type of granulocytes and possess high phagocytic potential. These cells are highly migratory and were reported to be involved in phagocytosing damaged elements within the brain parenchyma following a TBI [115,122]. The processes whereby neutrophils function are by: (1) secreting lysosomal enzymes; (2) releasing free radicals; (3) decreasing blood flow by direct physical microvascular occlusion; (4) increasing vascular permeability [125–127]. Interestingly, neutrophils can be found in the microvasculature lining the peri-injury region by as early as 2 h after injury and in brain parenchyma shortly thereafter [115,122]. Thus, neutrophils can contribute to the development of BBB breakdown and subsequent brain edema formation [122,128]. It is possible that neutrophils contribute to the secondary damage seen after TBI. Consistent with this notion, blocking neutrophil migration and adhesion decreases the total area of neuronal damage after a TBI in rabbits [129].

It has been hypothesized that accumulation of DCs at the site of damaged brain parenchyma can negatively contribute to brain tissue damage after the onset of TBI [122,130]. Infiltrating DCs may be activated by contact with the damaged cells at the site of injury. Antigen materials get processed by DCs, which can then travel to distant lymph nodes, present antigen and generate a local immune response. Once this response is initiated, antigen-specific T cells may migrate into CNS parenchyma and cause extended, chronic damage to the brain [131,132]. T cell accumulation at the site of injury, concurrent with DC accumulation, has also been indicated to negatively influence TBI outcomes [115].

Activated macrophages and/or microglia also contribute to neuronal damage [122,133]. It has been reported that different molecules released after TBI contribute to microglia/macrophage phenotype shifts [134,135]. Although the utility of classifying the M1/M2 phenotype *in vivo* has come into question [136,137], it is still useful to review the potential impact of the different phenotypes. For example, damaged endothelial cells can mediate microglia/macrophage polarization through secretion of cytokines [138], such as TNF- $\alpha$ , IL-6, IL-25, transforming growth factor beta (TGF- $\beta$ ), interferon-gamma (IFN-gamma), including substance-P and lipocalin-2 [139–141]. Additionally, infiltrating peripheral immune cells (T lymphocytes specifically) can induce macrophage/microglial phenotype transformation [142]. Recent studies suggest that such phenotype changes can alter neurogenesis after TBI, such that macrophage polarization from M2 toward M1 stimulates the release of soluble factors that impair basal neurogenesis [138], and this may impair functional recovery [134,143]. Thus, macrophages and/or microglial cells can be acted upon by a number of factors that ultimately determine the functional consequences of these cell types.

Macrophages, including both resident (microglia) and infiltrating (peripheral macrophages), are observed in relatively high numbers following TBIs [133]. Macrophages appeared to be abundant between 12 and 72 h and predominate in damaged cortical regions [122,144]. These cells accumulate near the area of injury [144], and this accumulation is a result of at least two mechanisms. One is through attractions by locally-secreted chemo- and cytokines released at the site of injury, as previously discussed. The other mechanism appears to occur via the T-cell activation. Once in the damaged

area, the T cells get activated by direct contact with antigen presentation on DCs, macrophages and microglia [145,146]. It is this latter activation which is the hallmark of a transition from a non-specific innate immune response, to a specific adaptive immune response; e.g. T cell recognition of presented antigen.

T lymphocytes play an important role in the development and maintenance of secondary brain injury after TBI, through engagement of different cell types and mechanisms. Steady increases in the number and composition of T cells at the site of injury are highly suggestive of a transition to an adaptive immune response following a TBI. The peak of brain tissue infiltration by T cells after TBI, seems to occur within a range of 1–5 days, although the data on this topic are inconsistent [147,148]. Despite these inconsistencies, it appears that gamma delta T cells ( $\gamma\delta$  T cells), which are a distinct class of T cells largely developed in thymus and express  $\gamma\delta$  receptors, rather than  $\alpha\beta$  T-cell receptor (TCR) on their surface, are early responders to the site of brain injury [149]. Combined with CD8+ T lymphocytes and CD4+ T-helper 1 (TH1) cells, these cells may worsen damage by cytotoxic and pro-inflammatory actions [150,151]. However, some suggest that the infiltration of immune cells into the CNS after TBI can also be neuroprotective [120,152,153]. This hypothesis is substantiated by the supposition that antigen-activated T cells provide protection, maintenance of neurological integrity and repair of tissue after a TBI [154]. Reports indicate that CD4+ T-helper 2 (TH2) cells specific for myelin basic protein (MBP) and CD4+ T cells may play such role of protection in neuronal survival [155,156]. Despite the pathogenic potential of anti-MBP T cells, they were also found in human immune system of healthy individuals [157,158]. It seems that regulatory T cells (CD4+ CD25+ Foxp3+) might also play important role in protection by suppressing autoimmune activity [159,160]. They derive from naive CD4 cells and are known for their immunosuppressive action, which downregulate the induction and proliferation of effector T cells [159,160].

Thus, it is possible that after TBI, the inflammatory sequelae result in antigen processing and presentation, as well as an eventual transition to an adaptive immune response. The implications of a transition to an adaptive immune response after a TBI are poorly understood and may have a positive and/or negative impact on the CNS. Here, we will summarize the existing data supporting an adaptive immune response after TBI, and we will provide a novel hypothesis to generate a foundation from which ensuing studies can occur in the context of adaptive immunity and TBI.

#### *4.5. Adaptive Immune Response to TBI*

Although scant, following TBI, some studies have provided strong evidence for a switch from a non-specific innate immune response, to a specific, adaptive immune response. As such, a paradigm shift in thinking may be necessary to fully understand and appreciate the sequelae that occur in the early and chronic stages after a TBI. A switch to an adaptive immune response has occurred, once antigen is processed and presented by professional antigen-presenting cells (APCs), and T cells recognize the presented antigen. The evidence supporting the transition to an adaptive immune response following TBI has been observed following retinal crush studies [161,162], fluid percussion injury in mice [116] and in human TBI patients [163]. The cellular components of the adaptive immune response may entail resident brain cells and/or infiltrating immune cells, as described above. In the case of infiltrating immune cells, they can gain access to the CNS via the compromised BBB, as well as the aforementioned chemotactic signaling. The humoral component of the adaptive immunity can be initially mediated by B cells and subsequently by T cells, which produce antigen-specific antibodies. Although the evidence is lacking for a direct connection between antibody production by T cells and neurodegeneration, accumulating evidence supports a role in the adaptive immune response in neurodegeneration. Once a transition from an adaptive immune response occurs, the possible outcomes have the potential to profoundly influence the outcomes for TBI.

In the case of most TBIs, the injuries are non-penetrating; thus, it would seem, if an adaptive immune response is occurring, then the antibody response is likely to be against self-antigen. Indeed, in human patients, evidence for this is found in the fact that antibodies to glial fibrillary acidic

protein (GFAP)-fragments have been observed in the cerebral spinal fluid (CSF), at various time points after a TBI [164]. Moreover, proteolytic fragments of MBP, neuron specific enolase and to ubiquitin D-terminal hydrolase-L-1 (UCH-L1), which is a highly specific protein to neurons and an essential component of the ubiquitin proteasome system, have also been observed in humans and animal models of TBI [165–168]. Moreover, auto-reactive T cell responses to MBP have been documented in humans after TBI [169], further supporting a switch to an adaptive immune response. Considering that white matter loss has been reported after TBI in humans and animals, the observation of antibodies and T cells to MBP could have a tremendous implication on the loss of white matter and axonal degeneration that is observed at chronic time points after a TBI. A potential consequence of antibody production and the implied transition to an adaptive immune response is that memory immune cells are formed. It is possible that reactivation of the memory T cells, by another injury or perhaps by other neuroinflammatory stimulus, such as bacterial or viral infections [170], might re-open the BBB and expose the memory immune cells to self-antigens, such as GFAP, UCH-L1 or MBP. In this scenario, the subsequent appearance of post-traumatic syndromes may only appear after appropriate spatial and temporal stimuli, hence explaining the wide variation in when, why and what types of post-traumatic syndromes appear. Therefore, the development of post-TBI auto-antibody response might be highly pathogenic and contribute to chronic neuropathology that persists for a relatively long duration (days, week, months, years) after injury, and there is evidence in support of this notion from a wide swath of neurological and immunological studies [171–174].

Resident brain immune cells, such as microglial cells, infiltrate the CNS very early during development [175,176] and do not seem as likely a candidate to present self-antigen, despite the fact that these resident microglial cells are highly competent, professional APCs, and they can also express major histocompatibility complex class II molecules (MHCII). Considering that resident microglial cells in the brain are continuously exposed to GFAP, UCH-L1 and MBP and there does not seem to be any auto-reactivity in normal conditions, one alternative scenario is that infiltrating immune cells are responding to the antigenic peptide fragments of GFAP, UCH-L1 and MBP [165–167,177–180].

Another important factor to consider in the context of an adaptive immune response after TBI is the recent reports of two distinct lymphatic portals that directly service the brain. First, sitting subjacent to the superior sagittal sinus is a lymphatic area that has recently been shown to have white blood cells that dip in and out of the CNS, even in normal conditions [181,182]. Immune signals from the CNS have been shown to directly signal through this lymphatic portal and to initiate a global immune response that can exacerbate the severity of an injury [147,183,184]. Second is the “glymphatic” system, which allows for clearance of CNS waste products and soluble proteins. This system also has been shown to provide a substrate for the signaling of CNS components to the more classically-defined peripheral lymphatic system [184–187]. In either of these two CNS “lymphatic compartments”, there can be a rapid communication between the CNS and the periphery, and this signaling can exacerbate injuries, such as stroke or TBI [184,188]. In the stroke literature, removing the spleen (splenectomy) and, therefore, reducing the number of B and T cells capable of responding to the TBI results in a significant improvement in lesion size and functional outcome measures. However, the data were inconsistent between humans and rodent models [189–191]; thus, the true functional implications require further examination. Interestingly, other studies that have blocked the expansion and activation of B and T cells in the spleen after a TBI have demonstrated significant neuroprotection [116]. Thus, the role of peripheral immune cells after TBI might provide novel targets for the development of therapeutic options to treat TBIs and post-traumatic syndromes.

## **5. Conclusions**

TBI remains a complex, multi-system pathology, with a wide-ranging potential for short- and long-term detrimental outcomes. Using the existing and newly-developed animal models, as well as clinical and translational studies, research continues to unravel the complex interactions between the

brain, the periphery and the immune system. New understanding of these interactions will lead to novel therapeutic targets, with the hope of improving the outcomes for millions of people each year.

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