Mediators of Inflammation

Microglia in Health and Disease: A Double-Edged Sword

Lead Guest Editor: Joana Gonçalves
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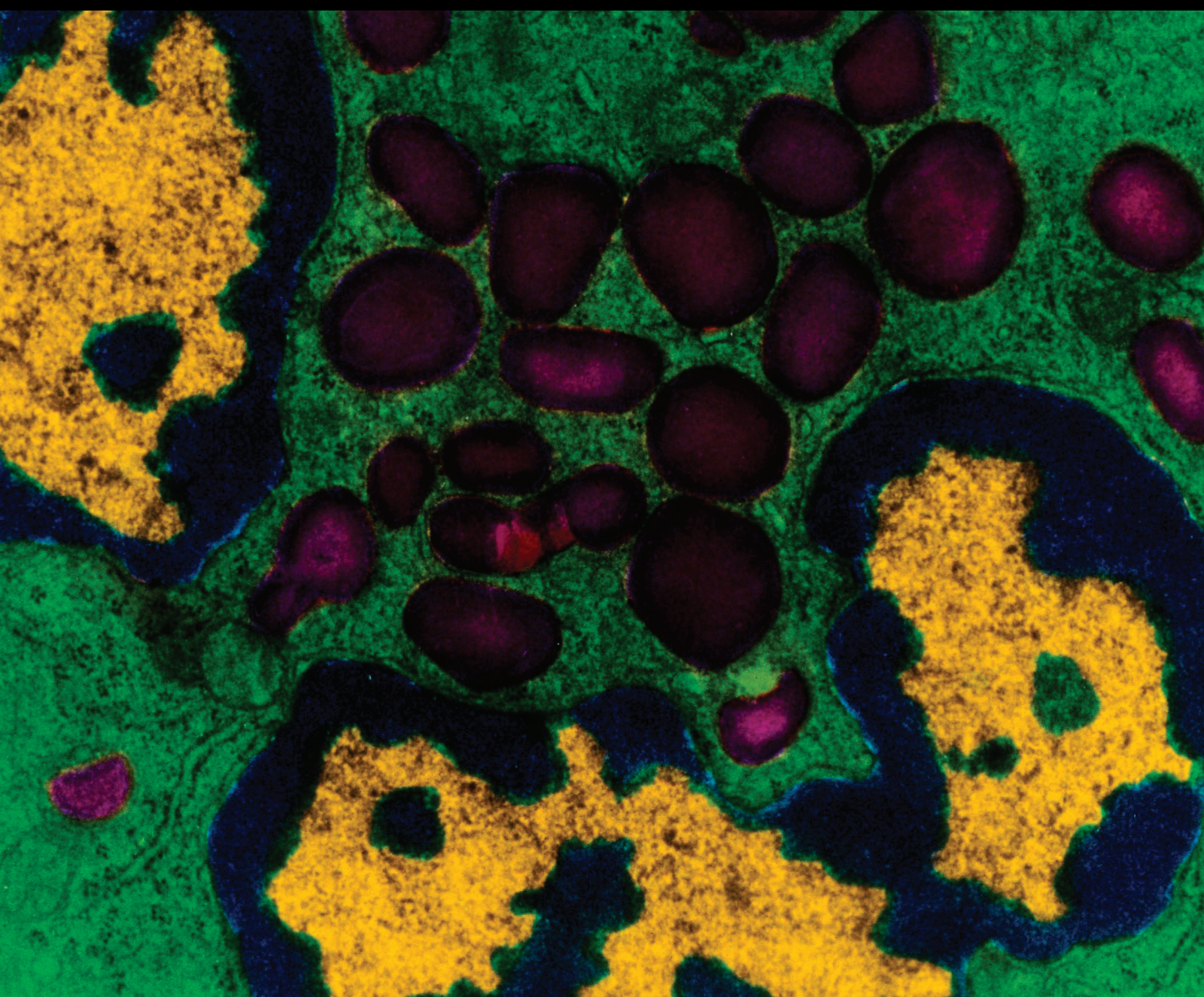

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Editorial Board

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Editorial

Microglia in Health and Disease: A Double-Edged Sword

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Microgial cells are the resident immune cells of the central nervous system, comprising 5–10% of the glial cells in the brain [1]. These cells orchestrate fundamental processes for the development and function of the CNS. Microglia participate in neuronal development, in adult neurogenesis, and also in the modulation of synaptic transmission [2, 3]. Microglia are constantly surveying the parenchyma, and they detect changes in their microenvironment, contributing to the pathophysiology of several neurodegenerative diseases. This special issue aimed to give an overview of the current knowledge on the role of microglial cells and processes mediated by microglia during health and disease.

F. I. Baptista et al. investigated how elevated concentration of glucose and interleukin-1β (IL-1β) negatively affects the progression of diabetic retinopathy, the most common complication of diabetes. In this paper, the exposure to elevated glucose concentration, to mimic hyperglycemic conditions, upregulates IL-1β expression in retinal neural cell cultures, affecting microglial and macroglial cells in the retina. The authors also observed that IL-1β has an important role in retinal microglial activation and proliferation under diabetic-like conditions, and limiting IL-1β-triggered inflammatory processes may provide a new therapeutic strategy to prevent the progression of diabetic retinopathy.

The proinflammatory cytokines, such as IL-1β and tumor necrosis factor (TNF), are important inflammatory mediators in the CNS. To date, the role of microglial-derived TNF following spinal cord injury (SCI) is poorly understood, since the contribution of soluble TNF (solTNF) versus membrane-anchored TNF (mTNF) to tissue damage and functional recovery remains to be elucidated. D. G. Ellman et al. investigated the effect of solTNF and mTNF on SCI using genetically modified mice that express only mTNF. They showed that the absence of solTNF in mice does not affect lesion size and functional outcome after SCI, but TNF levels are significantly decreased within the lesioned spinal cord. These findings suggested that genetic ablation of solTNF does not affect lesion size and functional outcome after SCI.

After spinal cord injury, inflammatory stimulation and/or modification greatly improve the regenerative outcome in rodents. I. Bollaerts et al. revised the current knowledge on how acute inflammation is intertwined with axonal regeneration, an important component of CNS repair.

Other severe motor neuron disease is amyotrophic lateral sclerosis (ALS), and C. Parisi et al. reviewed the M1/M2 functional imprinting of primary microglia as a paradigm of pro-/anti-inflammatory function and the role played by P2X7 and miR-125b in microglia activation in ALS. The authors concluded that a subtle equilibrium in the timing and power of proinflammatory versus anti-inflammatory agents can imprint microglia to tip the balance toward toxicity or protection, motor neuron survival, or cell death in ALS.

The balance between proinflammatory versus anti-inflammatory agents is crucial in several neurodegenerative disorders. Accordingly, D. Leonoudakis et al. explored the protective mechanisms of securinine, a major natural alkaloid product from the root of the plant *Securinega suffruticosa*, in glial cells. The authors demonstrated that this natural product inhibits glial activation and subsequent generation of proinflammatory factors.

Several agents have been reported to afford neuroprotection through the control of microglial reactivity. M. H. Madeira et al. revised the literature regarding the main effects of caffeine, the major component of coffee and the most consumed psychostimulant in the world, in the modulation of microglial reactivity and neuroinflammation in neurodegenerative diseases. Also, L. Carniglia et al. summarized the current literature on the way several neuropeptides modulate microglial activity and response to tissue damage and how this modulation may affect pain sensitivity.

It has been increasingly recognized that glial cells, such as microglia, and inflammatory signaling play a major role in the pathogenesis of chronic pain. T. Berta et al. revised the major signaling pathways involved in microglial cell activation and chronic pain with an emphasis on caspases. Overall, they suggested that caspase-6 released from axonal terminals regulates microglial TNF secretion, synaptic plasticity, and chronic pain. Because of this, they hypothesized that caspase-6 could be targeted by antibodies to treat chronic pain.

Together, the reviews and research articles that are included in this special issue help to understand the role of microglial cells in health and disease.

Ana Raquel Santiago
Liliana Bernardino
Marta Agudo-Barriuso
Joana Gonçalves

References

[1] J. A. Kabba, Y. Xu, H. Christian et al., "Microglia: housekeeper of the central nervous system," *Cellular and Molecular Neurobiology*, pp. 1–19, 2017.

[2] T. L. Tay, J. C. Savage, C. W. Hui, K. Bisht, and M. E. Tremblay, "Microglia across the lifespan: from origin to function in brain development, plasticity and cognition," *The Journal of Physiology*, vol. 595, pp. 1929–1945, 2017.

[3] L. Tian, C. W. Hui, K. Bisht et al., "Microglia under psychosocial stressors along the aging trajectory: consequences on neuronal circuits, behavior, and brain diseases," *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, vol. 79, Part A, pp. 27–39, 2017.

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Research Article

Elevated Glucose and Interleukin-1β Differentially Affect Retinal Microglial Cell Proliferation

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Diabetic retinopathy is considered a neurovascular disorder, hyperglycemia being considered the main risk factor for this pathology. Diabetic retinopathy also presents features of a low-grade chronic inflammatory disease, including increased levels of cytokines in the retina, such as interleukin-1 beta (IL-1β). However, how high glucose and IL-1β affect the different retinal cell types remains to be clarified. In retinal neural cell cultures, we found that IL-1β and IL-1RI are present in microglia, macroglia, and neurons. Exposure of retinal neural cell cultures to high glucose upregulated both mRNA and protein levels of IL-1β. High glucose decreased microglial and macroglial cell proliferation, whereas IL-1β increased their proliferation. Interestingly, under high glucose condition, although the number of microglial cells decreased, they showed a less ramified morphology, suggesting a more activated state, as supported by the upregulation of the levels of ED-1, a marker of microglia activation. In conclusion, IL-1β might play a key role in diabetic retinopathy, affecting microglial and macroglial cells and ultimately contributing to neural changes observed in diabetic patients. Particularly, since IL-1β has an important role in retinal microglia activation and proliferation under diabetes, limiting IL-1β-triggered inflammatory processes may provide a new therapeutic strategy to prevent the progression of diabetic retinopathy.

1. Introduction

Diabetic retinopathy is a leading cause of vision loss and blindness in the western countries and the most common complication of diabetes. Hyperglycemia is considered the primary pathogenic factor underlying diabetic retinopathy, being the breakdown of blood-retinal barrier (BRB), one of the first alterations clinically evident, and a hallmark of the disease [1].

In fact, early signs of neural dysfunction in the retina, namely, alterations in electroretinograms and loss of colour and contrast sensitivity, occur before the detection of microvascular changes in diabetic patients and animals [2–5]. Despite the progress in understanding the pathogenesis of diabetic retinopathy, the mechanisms underlying neural dysfunction are far from being completely understood.

Growing evidence indicates that diabetic retinopathy has features of a low-grade chronic inflammatory disease. Several genes involved in inflammatory processes are upregulated early in the diabetic rat retina [6, 7]. In the vitreous fluid of diabetic patients, the levels of interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor (TNF) are increased [8–10]. In addition, an increase in the production of cytokines, such as IL-1β and TNF, expression of adhesion molecules, leukocyte adhesion, and vascular permeability [11–13] have been observed in the retina of diabetic animals. Moreover, in the retinas of streptozotocin-induced diabetic rats, the levels of IL-1β are also increased [14–17], and this was correlated with an increase in BRB permeability [12, 14]. It has been shown that Müller glial cells isolated from diabetic rats acquire a reactive phenotype in response to diabetes, increasing the expression of inflammation-related genes [16]. In cultured retinal cells exposed to high glucose, an increase in $[Ca^{2+}]_i$ triggered by activation of purinergic receptors was observed in both retinal neurons and microglial cells [18]. This enhanced calcium response may also contribute to the

Table 1: List of primary antibodies.

Antibody	Western blot dilution	Immunocytochemistry dilution	Company
Mouse anti-TUJ-1	1 : 1000	1 : 1000	Covance
Mouse anti-GFAP	1 : 5000	1 : 500	Sigma
Mouse anti-CD11b	—	1 : 100	Serotec
Rabbit anti-Iba-1	—	1 : 200	Wako
Mouse anti-ED-1	1 : 1000	1 : 250	Serotec
Goat anti-IL-1β	—	1 : 50	RD
Mouse anti-IL-1RI	1 : 500	1 : 100	RD
Mouse anti-PCNA	1 : 500	—	Santa Cruz
Mouse anti-β-actin	1 : 5000	—	Sigma
Rabbit anti-Ki-67	—	1 : 100	Abcam

increase in the release of inflammatory mediators and neurotransmitters in diabetic retinas. Early retinal microglia activation is a common response in diabetic retinopathy and is associated with progressive neurodegeneration in the retina. Activation of microglia leads to an increase in their proliferation and migration, phagocytosis, and release of several proinflammatory mediators [19]. The retina has been viewed as an immune privileged tissue; however, strong evidence supports a role for microglia activation and local inflammation in the pathogenesis of diabetic retinopathy [17, 20, 21].

IL-1β is a proinflammatory cytokine known to upregulate a plethora of several inflammatory mediators, including IL-1β itself, TNF, inducible nitric oxide synthase, and chemokines [22–25]. IL-1β elicits responses in cells only through the activation of IL-1 type I receptor (IL-1RI) although it can also bind to IL-1 type II receptor (IL-1RII), a decoy receptor. Although an increase in retinal IL-1β levels has been described in diabetic conditions and correlated with the pathogenesis of diabetic retinopathy, it is still unclear which retinal cells express IL-1β and IL-1RI.

In order to better understand how high glucose and IL-1β impact retinal cells, we evaluated whether high glucose regulates IL-1β expression and investigated which retinal cell types produce IL-1β and express its receptor in primary retinal neural cell cultures. Importantly, we also evaluated the cell-specific effects of high glucose and IL-1β per se in retinal neural cell cultures to clarify which cell types are mainly affected.

2. Experimental Procedure

2.1. Ethics Statement. Procedures involving animals were conducted in accordance with the guidelines of the European Community directive for the use of animals in laboratory (2010/63/EU), translated to the Portuguese law in 2013 (Decreto-lei 113/2013), and in accordance with the Association for Research in Vision and Ophthalmology (ARVO) guidelines for the use of animals in vision research. The experiments were approved by our Institutional Ethics Committee (Comissão de Ética da Faculdade de Medicina da Universidade de Coimbra) (approval ID: FMUC/07/12). Moreover, people working with animals have received appropriate education (Federation of Laboratory Animal Science Associations (FELASA) course) as required by the Portuguese authorities, and all efforts were made to minimize animal suffering. Decapitation with surgical scissors was the method used to perform euthanasia of the Wistar rat pups (postnatal days 3–5).

2.2. Primary Cultures of Rat Retinal Neural Cells. Primary rat retinal neural cell cultures were obtained from the retinas of 3–5-day-old Wistar rats, as previously described [26, 27]. After 2 days in culture, cells were incubated with 25 mM D-glucose (final concentration 30 mM) or 25 mM D-mannitol (plus 5 mM glucose already present in cell culture media), which was used as an osmotic control, and maintained for further 7 days in culture. The concentration of glucose in control conditions was 5 mM. Cells were also exposed to IL-1β (10 ng/ml) or lipopolysaccharide (LPS; positive control for inflammation; 1 μg/ml) at day in vitro (DIV) 8 for 24 h.

2.3. Western Blot Analysis. Western blotting analysis of cellular lysates of retinal neural cell cultures was performed as previously described [27] with minor changes. Equal amounts of protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using 8%–12% gels. After electrophoretic transfer and blocking, the membranes were incubated with primary antibodies (listed in Table 1) overnight at 4°C. The secondary antibodies used were anti-mouse or anti-rabbit alkaline phosphatase-linked IgG secondary antibody (1 : 20,000; GE Healthcare, UK). Protein immunoreactive bands were visualized with the enhanced chemifluorescence substrate (ECF; GE Healthcare). Fluorescence was detected on an imaging system (Thyphoon FLA 9000, GE Healthcare), and the digital quantification of bands' immunoreactivity was performed using ImageQuant 5.0 software (Molecular Dynamics Inc., Sunnyvale. CA, USA). β-Actin was used as a protein loading control.

2.4. Immunocytochemistry. Immunocytohemistry was performed as previously described [27]. The primary antibodies used are listed in Table 1. The secondary antibodies used were Alexa Fluor 594-conjugated anti-mouse IgG (1 : 250)

and Alexa Fluor 488-conjugated anti-rabbit IgG (1 : 250). The nuclei were stained with DAPI (1 : 5000). Cells were visualized in a laser scanning confocal microscope LSM 710 META (Zeiss, Germany).

2.5. Assessment of Cell Viability. Cell viability of retinal neural cell cultures was evaluated as described previously [28].

2.6. Terminal Transferase dUTP Nick End Labeling (TUNEL) Staining. Cells undergoing apoptosis were identified by TUNEL assay using the DeadEnd Fluorimetric TUNEL system (Promega Corporation, USA) as previously described [29]. Cells were also stained with DAPI to label the nuclei. The images were acquired with an inverted fluorescence microscope (DM IRE2, Leica Microsystems, UK). At least a minimum of 10 random fields in each coverslip were counted.

2.7. Quantitative Real-Time PCR. Isolation of total RNA from retinal cells, cDNA synthesis, and qPCR was performed as previously described [30]. The primers for the target rat genes (IL-1β, NM_031512) and the reference gene (rat HPRT, NM_012583) were predesigned and validated by QIAGEN (QuantiTect Primers, QIAGEN).

2.8. ELISA. At DIV 9, the conditioned medium of each well containing primary retinal neural cells exposed to different experimental conditions (control, high glucose, mannitol, and LPS) was removed and stored at −80°C until performing the ELISA assay. Rat IL-1β ELISA development kit (PeproTech, UK) was used to measure the levels of IL-1β in retinal neural cell culture medium. Each sample was assayed in duplicate using 100 μl of culture medium per well.

2.9. Flow Cytometry. The analysis of cells undergoing apoptosis was performed using the annexin V-FITC assay kit (BD Biosciences), following the manufacturer's instructions and using PI staining. The stained cells were analyzed with a FACSCalibur (Becton Dickinson, USA) equipped with a 488 nm argon laser and a 635 nm red diode laser. The collected events per sample were 20,000. Flow cytometry data was analyzed with CellQuest software (Becton Dickinson) and plotted as a function of fluorescence intensity FL-1 (green) versus FL-3 (red) fluorescence. We used annexin V-FITC (emission 518 nm) versus PI (propidium iodide) (emission 617 nm) to identify viable cells (annexin V^-PI^-), early apoptotic cells (annexin V^+PI^-), necrotic cells (annexin V^-PI^+), and late apoptotic cells (annexin V^+PI^+).

2.10. Statistical Analysis. Results are presented as mean ± SEM. Statistical significance was determined by using Student's t test or ANOVA, followed by Dunnett's post hoc test. Differences were considered significant for $p < 0.05$.

3. Results

3.1. Rat Retinal Neurons and Glial and Microglial Cells Express IL-1β and IL-1RI. IL-1β is a proinflammatory cytokine that can be synthesized by several cell types, such as leukocytes, endothelial cells, neurons, and glial cells. Since the retina is composed by different cell types that potentially may produce IL-1β, we first analyzed whether retinal cells present in culture (neurons, macroglial and microglial cells) were able to synthesize IL-1β and identified those that can be directly affected by IL-1β, that is, cells that express IL-RI. The expression of IL-1β and the distribution of IL-1RI in primary rat retinal neural cell cultures were investigated by double-labeling immunocytochemistry. Specific cell markers were used to identify the different cell types present in the cell culture: TUJ-1 (neurons), GFAP (macroglial cells), and Iba-1 or CD11b (microglial cells).

As shown in Figure 1(a), IL-1β immunoreactivity is present in TUJ-1^+, $CD11b^+$, and $GFAP^+$ cells. Similarly, IL-$1R1^+$ cells were also immunoreactive to TUJ-1^+, Iba-1^+, and $GFAP^+$ cells (Figure 1(b)). These observations indicate that retinal neural cells express IL-1β and can be responsive to it, since they also express IL-1RI.

3.2. High Glucose Increases IL-1β Expression in Rat Retinal Neural Cells. Since hyperglycemia is considered the main risk factor for diabetic retinopathy [27, 28] and the levels of IL-1β are increased in the retinas of diabetic rats [14–17], we evaluated the effect of elevated glucose on IL-1β expression in retinal neural cultures in order to evaluate if high glucose per se is capable of upregulating IL-1β expression.

Firstly, we evaluated IL-1β mRNA expression in these cultures by qPCR. A significant increase in IL-1β mRNA content was observed in high glucose-treated cells (283.2 ± 32.8% of the control) (Figure 2(a)). No changes were detected in cells exposed to mannitol, demonstrating that the effect of glucose was not due to the increase in osmolarity. In addition, a significant increase in IL-1β mRNA was observed in cells treated with LPS (increase to 39,868.7 ± 4043.4% of the control) (Figure 2(a)), a positive control for IL-1β gene expression upregulation.

To determine whether the increase in IL-1β mRNA expression was concomitant with an increase in the production of IL-1β, we evaluated its protein levels in the culture media of retinal neural cells by ELISA assay. A significant increase in IL-1β levels was detected upon high glucose treatment (increase to 244.52 ± 52.2% of the control) (Figure 2(b)). Again, no changes were detected in cells exposed to mannitol, demonstrating that the effect was not due to the increase in osmolarity. As expected, a significant increase in IL-1β levels was also detected in LPS-treated cells (333.8 ± 98.3% of the control) (Figure 2(b)).

3.3. IL-1β Does Not Induce Retinal Neural Cell Death. Previous studies from our laboratory [29, 30] demonstrated that exposure of cultured retinal cells for 7 days to high glucose decreases cell viability, which is concomitant with an increase in the number of apoptotic nuclei detected by TUNEL assay. Since IL-1β is an important mediator of the inflammatory responses, and is involved in a variety of cellular processes, including cell proliferation, differentiation, and apoptosis [31, 32], we evaluated whether exposure to IL-1β per se could increase apoptosis in retinal neural cells.

Retinal cell cultures were exposed to IL-1β for 24 h, and retinal cell viability was evaluated by TUNEL assay and flow cytometry. IL-1β did not increase the number of $TUNEL^+$ cells as compared to control conditions (Figure 3(a)). To

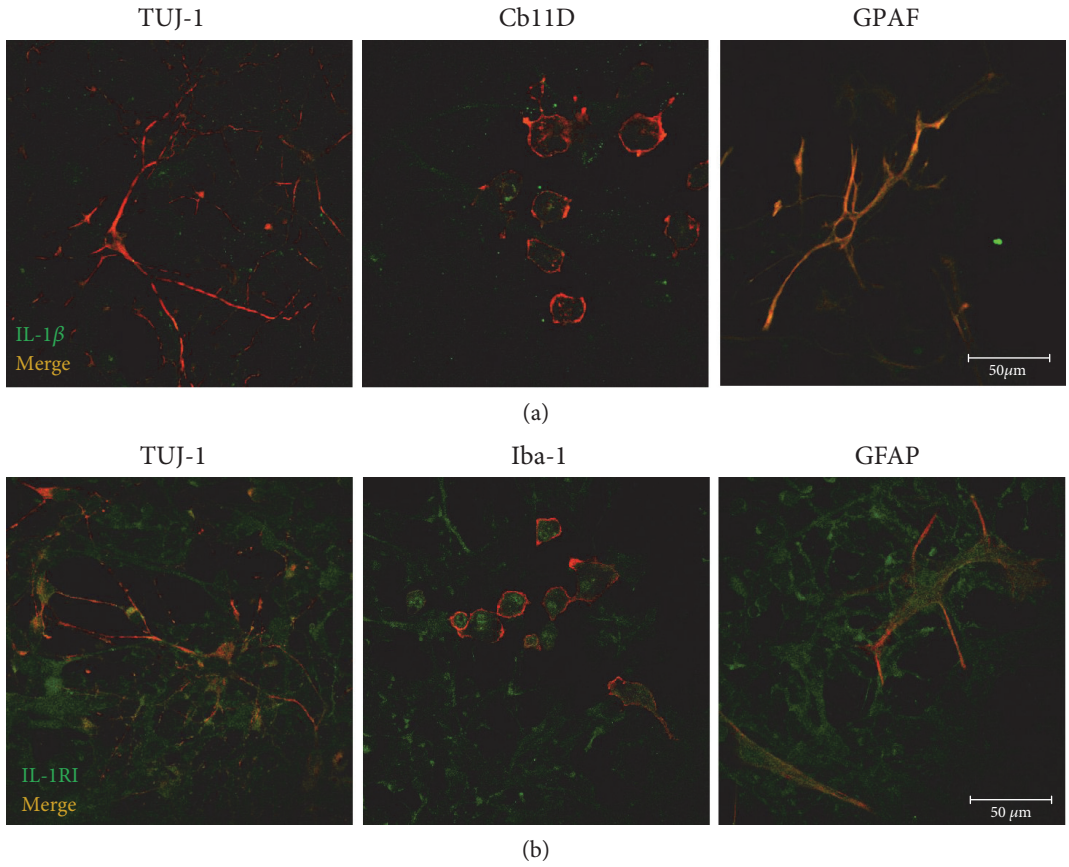

Figure 1: Rat retinal neurons and glial cells express IL-1β and IL-1RI. IL-1β (a) and IL-1RI (b) immunostaining (both in green) can be observed in rat retinal neurons (TUJ-1; red) and microglia (CD11b or iba-1; red) and macroglial cells (Müller cells and astrocytes; GFAP; red), indicating that retinal neural cells express IL-1β and can be responsive to it, since they express IL-1RI. Scale bar: 50 μm.

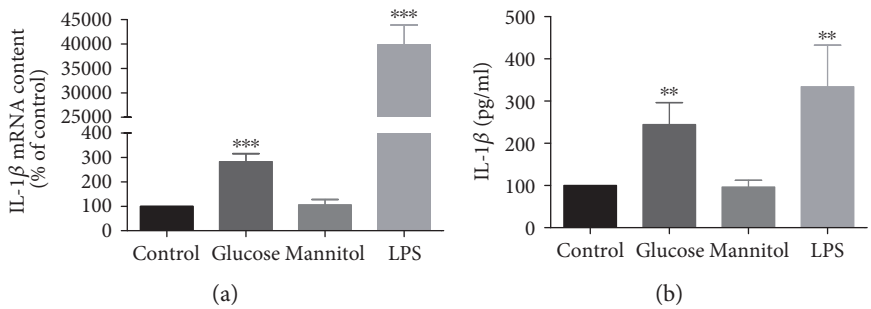

Figure 2: High glucose upregulates IL-1β expression in rat retinal cell cultures. Cells were exposed to 30 mM glucose or 24.5 mM mannitol (plus 5.5 mM glucose; osmotic control) for 7 days and IL-1β or LPS for 24 h. High glucose upregulates both IL-1β mRNA (a) and protein (b) levels. The results represent the mean ± SEM of at least 3 independent experiments and are presented as percentage of control. $^{**}p < 0.01$ and $^{***}p < 0.001$, significantly different from control, using Student's t-test.

confirm these results, we additionally performed flow cytometry with annexin V and PI, aiming to distinguish the features of apoptotic versus viable cells. A typical representative dot plot analysis of retinal cells after exposure to IL-1β is shown in Figure 3(b). Exposure to IL-1β for 24 h did not increase cell death in these cultures. Dot plot analysis showed that 94.8 ± 0.3% of cells exposed to IL-1β are viable, similar to control conditions (94.5 ± 0.5% of the cells were viable). Interestingly, the exposure of retinal neural cells to IL-1β for 24 h induced an increase in the reduction of MTT (120.5 ± 4.4% of the control; Figure 3(c)), which is frequently used as a viability or proliferative assay. Since IL-1β did not increase cell death, this observation suggests that IL-1β was enhancing cell proliferation. In order to verify whether the increase in the MTT reduction induced by IL-1β was mediated by the activation of IL-1RI, retinal cells were exposed

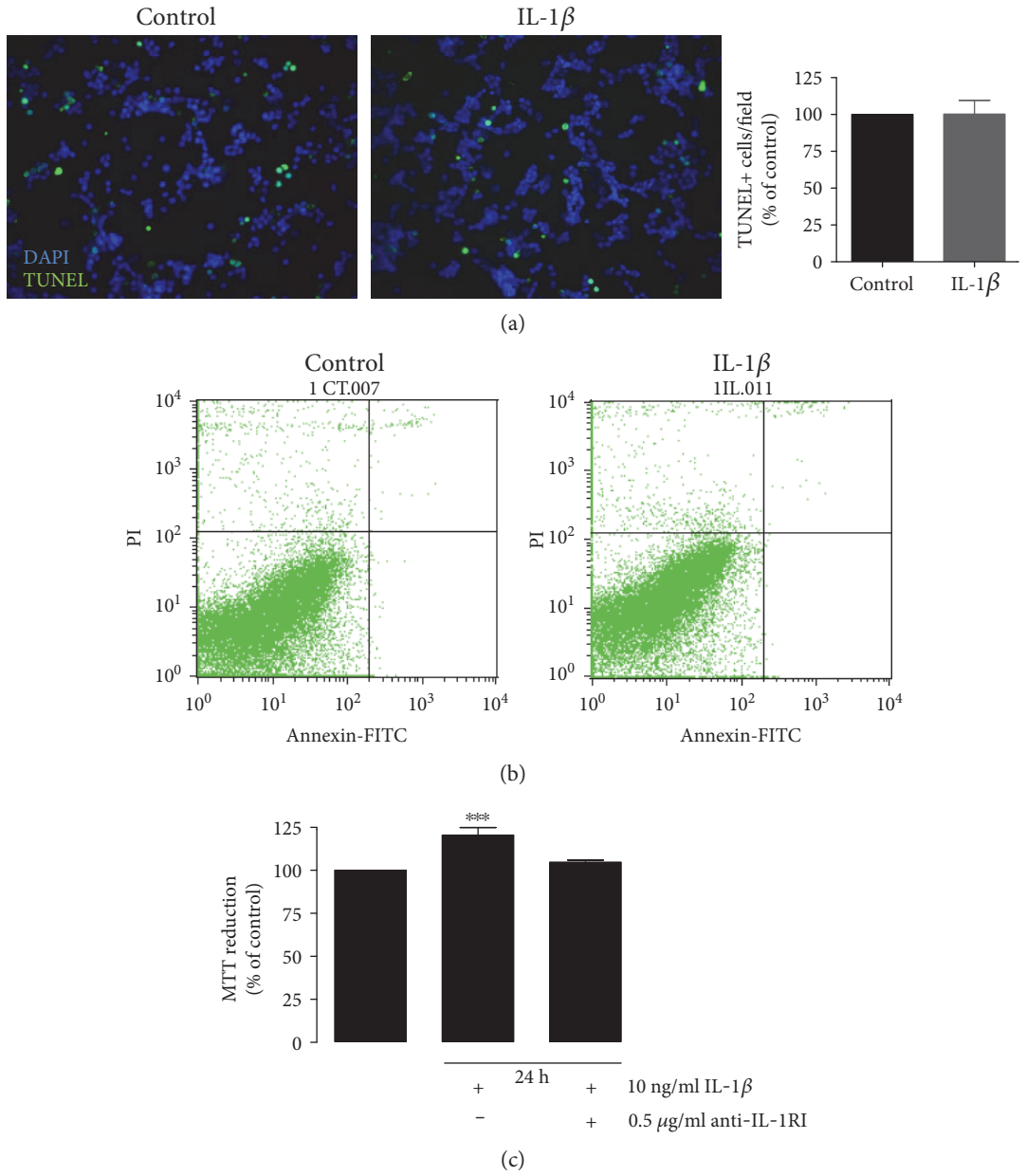

Figure 3: Exposure to IL-1β does not increase retinal neural cell death. Rat retinal neural cell cultures were exposed to IL-1β for 24 h, and cell death was evaluated by TUNEL assay. Representative images and quantification of the number of TUNEL$^+$ cells upon IL-1β treatment are shown. Scale bar: 50 μm (a). To further evaluate cell death, retinal cells were labeled with annexin V plus PI and were analyzed by flow cytometry (b). MTT was used as a cell viability assay (c). The results represent the mean ± SEM of, at least, 4 independent experiments. $^{***}p < 0.001$, significantly different from control as determined by ANOVA followed by Dunnett's post hoc test.

to IL-1β together with an antibody against IL-1RI. The presence of the antibody prevented the increase in the MTT reduction induced by IL-1β (Figure 3(c)).

3.4. High Glucose and IL-1β Differently Impact on Retinal Microglial Cell Proliferation. Since we have detected an increase in the MTT reduction and no changes in the number of TUNEL$^+$ cells in cultures exposed to IL-1β, we hypothesized that IL-1β could be affecting the proliferation of some cell types in these cultures. To evaluate this hypothesis, the proliferative capacity of retinal neural cells was assessed by Ki-67 or proliferating cell nuclear antigen (PCNA) immunoreactivity. Since elevated glucose increases the levels of IL-1β in retinal cultures, we also evaluated the effect of high glucose on retinal neural cell proliferation. As shown in Figure 4(a), a significant decrease in the number of Ki-67$^+$ cells was observed in cells exposed to elevated glucose for 7 days (decrease to 49.07 ± 6.5% of control). No changes were observed in mannitol-treated cells (data not shown). Conversely, a significant increase in the number of Ki-67$^+$ cells (increase to 149.8% ± 15.1% of control) was observed in cells incubated with IL-1β for 24 h

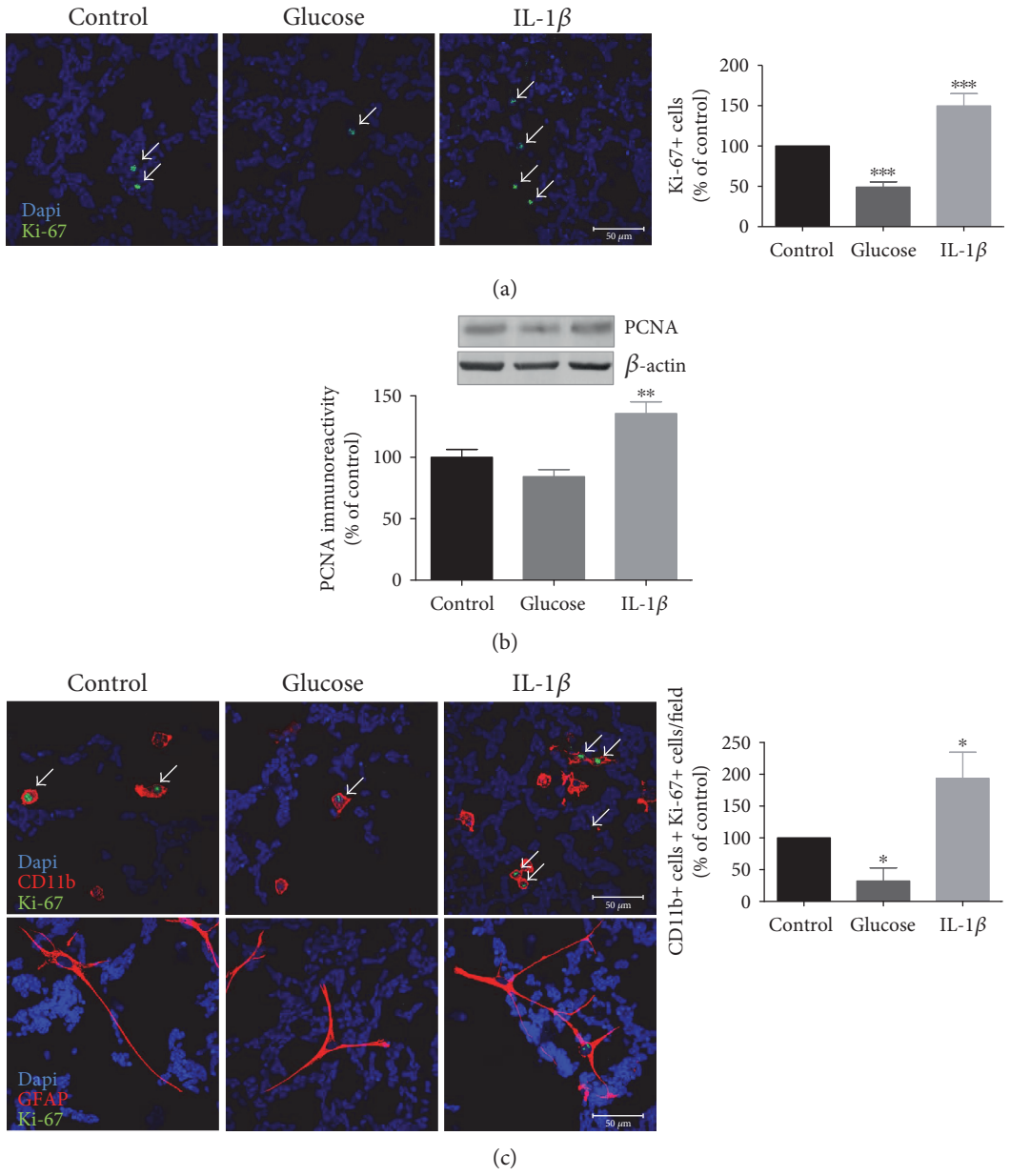

FIGURE 4: High glucose and IL-1β differently affect cell proliferation retinal neural cell cultures. Rat retinal neural cell cultures were exposed to high glucose for 7 days or IL-1β for 24 h and stained for Ki-67. Representative images and quantification of the number of Ki-67$^+$ cells upon high glucose and IL-1β treatment are shown. Scale bar: 50 μm (a). PCNA protein levels were evaluated by Western blotting. Representative Western blots are presented above the graphs, with the respective loading controls (β-actin) (b). To evaluate whether microglial and macroglial cells are proliferating, colocalization of CD11b$^+$ or GFAP$^+$ cells with Ki-67 was performed. Representative images and quantification of the number of CD11b$^+$Ki-67$^+$cells or GFAP$^+$Ki-67$^+$cells upon high glucose and IL-1β treatment are shown. Scale bar: 50 μm (c). Data represent means ± SEM of, at least, 7 independent experiments. $^*p < 0.05$, significantly different from control as determined by ANOVA followed by Dunnett's post hoc test.

(Figure 4(a)). Similar to what was observed with Ki-67 immunoreactivity, PCNA protein content decreased in high glucose-treated cells and increased in cells exposed to IL-1β (Figure 4(b)). These results suggest that prolonged exposure to high glucose and exposure to IL-1β induce opposite effects in cell proliferation.

To identify which cell types present in culture were proliferating, we performed double-labeling immunocytochemistry experiments using CD11b$^+$ (microglial) or GFAP$^+$ (macroglial) with Ki-67. Regarding microglial cells, there was a significant decrease in the number of Ki-67$^+$CD11b$^+$ cells (32.3% ± 20.5% of control) when retinal neural cells were exposed to elevated glucose, whereas in IL-1β-treated cells, a significant increase in the number of Ki-67$^+$CD11b$^+$ cells was observed (increase to 193.6% ± 41.1% of control) (Figure 4(c)). Concerning GFAP$^+$Ki-67$^+$ cells, very few or no positive cells were observed in control condition (average of 0.1 ± 0.1 Ki-67$^+$ colocalizing with GFAP$^+$ cells per field),

while in cells exposed to high glucose, we did not detect any $GFAP^+Ki\text{-}67^+$ cells. When cells were exposed to IL-1β, the average number of $Ki\text{-}67^+$ cells colocalizing with $GFAP^+$ cells per field was of 0.5 ± 0.2, demonstrating an increase in macroglial cell proliferation (Figure 4(c)).

These results suggest that while high glucose decreases retinal glial cell proliferation, particularly in microglial cells, IL-1β increases both microglial and macroglial cell proliferation.

To support this observation, we next evaluated the effect of high glucose and IL-1β in the total number of both $Iba\text{-}1^+$ and $GFAP^+$ cells. Microglial cells are very sensitive to alterations in their microenvironment, as well as to changes in cell culture conditions. They are considered to be major producers of IL-1β, which in turn can further activate microglial cells. High glucose induced a significant decrease in the number of $Iba\text{-}1^+$ cells ($49.2 \pm 5.1\%$ of control). However, IL-1β induced a significant increase in the number of $Iba\text{-}1^+$ cells ($144.9 \pm 10.9\%$ of control), as shown in Figures 5(a) and 5(b). The number of $GFAP^+$ cells was significantly decreased by high glucose ($77.3 \pm 1.6\%$ of control), but strongly increased by IL-1β ($153.9 \pm 8.6\%$ of control) (Figures 5(c) and 5(d)). Accordingly, GFAP protein content decreased in high glucose-treated cells ($74.7 \pm 5.4\%$ of control). Nevertheless, no alterations on GFAP protein content were observed when cells were exposed to IL-1β (Figure 5(e)).

However, we noticed that not only the number but also the morphology of micro- and macroglial cells was altered. Microglial cells presented a round-shaped morphology with less processes, suggesting a more activated state. On the other hand, in IL-1β-treated cell cultures, microglia cells presented both round-shaped and ramified morphology (Figure 5(d)). Regarding macroglial cells, we observed that cells exposed to high glucose were less ramified (Figure 5(c)). To check whether microglial cells were activated, we evaluated ED-1 immunoreactivity (marker for activated microglia) by immunocytochemistry and Western blotting. Interestingly, a significant increase in ED-1 protein levels was detected in cells exposed to high glucose for 7 days (increase to $127.6 \pm 9.0\%$) (Figure 5(g)). As expected, in IL-1β-treated cells, we also observed an increase in ED-1 protein levels ($131.8 \pm 10.9\%$ of control) (Figures 5(f) and 5(g)).

4. Discussion

Hyperglycemia is considered the major risk factor for the development of diabetic retinopathy. Nevertheless, in the last decade, growing evidence has shown that diabetic retinopathy has features of a low-grade chronic inflammatory disease [21]. In fact, it has been shown that microglial and macroglial cell activation is an important feature of neuroinflammation present in the diabetic retina [17, 31–34]. Moreover, increased production of inflammatory mediators, such as IL-1β [21, 35], TNF-α [35], and NO [12, 21] has been reported in the retinas of diabetic animals.

The results presented in this work show that a prolonged exposure to high glucose upregulates IL-1β expression in retinal neural cell cultures. In the central nervous system, the main cellular source of IL-1β under stress conditions is activated microglia, with a consequent upregulation of the cytokine in macroglia as well [25]. However, a previous study demonstrated that exposure to high glucose, for 4 days, is not sufficient to upregulate IL-1β expression in microglial cells [36]. Nevertheless, this study was performed using brain-derived microglial cells and with less time of exposure to high glucose (4 days) than in our study (7 days exposure) in retinal cell cultures.

Our group has previously shown that a long-term exposure (7 days) to high glucose decreases cell viability in retinal neural cell cultures [30]. Exposure to high glucose increases $[Ca^{2+}]_i$ responses in neurons and microglial cells in retinal neural cell cultures [18]. These alterations may lead to neurotransmitter and proinflammatory mediator release contributing to retinal cell death. Moreover, we demonstrated that as early as 3 days of exposure to high glucose also induces a small, but significant, decrease in cell viability, and a constant decline in cell viability was found for longer incubation periods, reaching a minimum at the last time point studied, 7 days [37]. The increase in the number of apoptotic nuclei detected by TUNEL assay after 7 days of incubation with high glucose was concomitant with a decrease in cell viability assessed with the MTT assay [28, 37]. Using several markers to identify the type of cells undergoing apoptosis in retinal cultures exposed to high glucose, we showed that very few $TUNEL^+$ cells were immunoreactive for neuronal (NeuN, TUJ-1), microglial (CD11b), or macroglial (GFAP, vimentin) cell markers. A high percentage of apoptotic cells in high glucose condition were ascribed to be photoreceptors and bipolar cells [37].

In the present study, exposure to IL-1β did not affect the number of apoptotic cells in retinal cell cultures. Interestingly, IL-1β increased the reduction of MTT. Despite being frequently used as a cell viability assay, it is also used as a proliferative assay. As high glucose decreases MTT reduction [28] and IL-1β increases it, these results lead us to hypothesize that exposure to high glucose and IL-1β have opposite effects on cell proliferation. In line with this, we further evaluated the effect of high glucose and IL-1β on cell proliferation by Ki-67 immunostaining. By cell counting, we detected a significant decrease in the number of $CD11b^+Ki\text{-}67^+$ cells and in the number of $CD11b^+$ cells in retinal cultures exposed to high glucose. Given that it has not detected an increase in $TUNEL^+CD11b^+$ cells in these cultures exposed to high glucose [29], this observation suggests that the decrease was not due to the apoptosis of $CD11b^+$ cells.

Since IL-1β increased the number of $CD11b^+$ cells, and high glucose increased IL-1β production in retinal neural cell cultures, we were expecting an increase in $CD11b^+$ cells in cultures exposed to high glucose. However, as mentioned above, high glucose decreased the number of $CD11b^+$ cells. A possible explanation is that high glucose might decrease the proliferation of $CD11b^+$ cells due to cell cycle arrest [38]. Nevertheless, there was an increase in ED1 levels in cells exposed to high glucose, indicating microglia activation. In fact, this possibility was supported by the increase in IL-1β levels when cells were exposed to high glucose, since activated microglia are prone to release increased levels of cytokines.

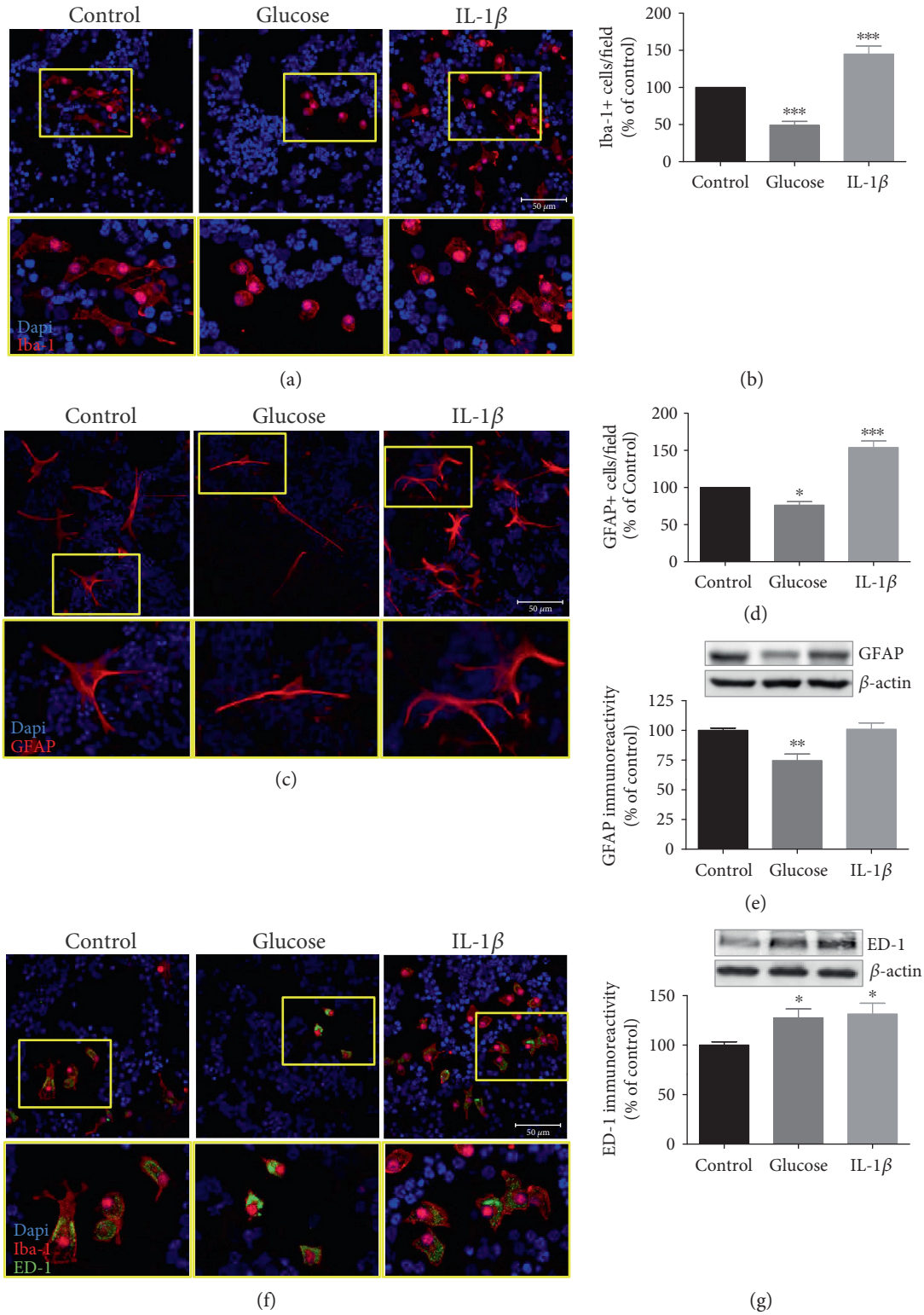

Figure 5: High glucose and IL-1β have opposite effects on retina microglial and macroglial cell proliferation. Rat retinal neural cell cultures were exposed to high glucose for 7 days or IL-1β for 24 h. Iba-1 immunoreactivity was assessed by immunocytochemistry (a, b). GFAP immunoreactivity was assessed by immunocytochemistry (c, d) or Western blotting (e). ED-1 immunoreactivity was assessed by immunocytochemistry (f) or Western blotting (g). Representative images (insets of higher magnifications images are shown below each panel) for Iba-1 (a), GFAP (c), or ED-1 (f) upon high glucose and IL-1β treatment are shown. Scale bar: 100 μm for (c). Scale bar: 50 μm for (a) and (f). Data represent means ± SEM of at least 4 independent experiments. $^{*}p < 0.05$, $^{***}p < 0.001$, significantly different from control as determined by ANOVA followed by Dunnett's post hoc test.

Similar to what was observed for microglial cells, IL-1β increased the number of GFAP$^+$ cells, as well as the number of GFAP$^+$ cells stained with Ki-67, indicating that glial cells are also proliferating. After injury, Müller cells become activated and undergo reactive gliosis, which is characterized by increased cell proliferation and gene expression alterations [39]. However, when retinal cells were exposed to high glucose, there was a decrease in GFAP levels and a decrease in the number of GFAP$^+$ cells, indicating that elevated glucose per se has an inhibitory effect on glial cell proliferation, despite upregulating the levels of IL-1β in these cultures. The reduction in glial cell proliferation is not due to glial cell death [40]. These observations suggest that the effect of IL-1β on glial cell proliferation is clearly dependent on its concentration in the cell culture media. With relatively low levels of IL-1β, this cytokine is not able to induce glial cell proliferation.

Continuous stimulation with stress signals can lead to chronic microglia overstimulation with a consequent loss of its autoregulatory mechanisms, amplifying inflammation [41] which may consequently be harmful for neurons. When retinal cultures were exposed to IL-1β for 24 h, we did not detect any changes in the levels of TUJ-1 (data not shown), a neuronal marker. Nevertheless, we cannot exclude the possibility that for longer periods of exposure to IL-1β, microglia cells may have detrimental effects in these cultures changing from a protective to a proinflammatory modus [42, 43].

It was previously demonstrated that exposure to high glucose increases TNF expression [37]. The blockade of TNF receptor 1, which is expressed in retinal neurons, prevents high glucose-induced cell death. Additionally, it was demonstrated that the secretion of TNF and monocyte chemotactic protein-1 by rat cortical microglia, triggered by exposure to high glucose, is mediated by reactive oxygen species production and NF-κB pathway activation, which may be mechanisms underlying neuronal injury and the pathogenesis of diabetic encephalopathy [44]. Moreover, IL-1RI-deficient mice are protected from diabetes-induced retinal pathology [45], indicating that IL-1β signalling may play a key role in the development of diabetic retinopathy. Taking into account that IL-1RI is a crucial locus of control of IL-1β activity, blocking IL-1RI activity should be considered as a possible therapeutic strategy for the treatment of diabetic retinopathy. In the retina, activated microglia and Müller cell responses are described not to be independent but involve bidirectional feedback signals that help initiate and propagate a coordinated adaptive response [46]. Therefore, in this study, changes in cell proliferation and expression of cell-specific markers suggest that there are adaptive responses that may help to limit neuronal cell death by directing and amplifying inflammatory processes in order to restore and maintain homeostasis in neuronal cell cultures.

5. Conclusions

In summary, our findings show that high glucose increases IL-1β production in retinal cell cultures. Moreover, we found that high glucose and IL-1β differently affect microglial and macroglial cells. High glucose decreased microglial and macroglial proliferation, whereas IL-1β increased their proliferation. These apparently opposing effects might be related with the levels of IL-1β. When cells are exposed to high glucose, the levels of IL-1β reached are significantly lower comparing to the condition when cells are exposed to IL-1β only. Other possible explanations might be the different exposure duration to IL-1β (7 days versus 24 h exposure) or the effect of high glucose on cell cycle arrest which may superimpose the potential proliferative effect of IL-1β.

Since overactivation of microglial cells may have deleterious effects in the retina, limiting IL-1β-mediated inflammatory processes could be a mechanism to prevent the progression of diabetic retinopathy.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Filipa I. Baptista and Célia A. Aveleira contributed equally to this work.

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References

[1] J. G. Cunha-Vaz, "The blood-retinal barriers system. Basic concepts and clinical evaluation," *Experimental eye Research*, vol. 78, no. 3, pp. 715–721, 2004.

[2] D. A. Antonetti, A. J. Barber, S. K. Bronson et al., "Diabetic retinopathy: seeing beyond glucose-induced microvascular disease," *Diabetes*, vol. 55, no. 9, pp. 2401–2411, 2006.

[3] M. S. Roy, R. D. Gunkel, and M. J. Podgor, "Color vision defects in early diabetic retinopathy," *Archives of Ophthalmology*, vol. 104, no. 2, pp. 225–228, 1986.

[4] M. L. Daley, R. C. Watzke, and M. C. Riddle, "Early loss of blue-sensitive color vision in patients with type I diabetes," *Diabetes Care*, vol. 10, no. 6, pp. 777–781, 1987.

[5] H. Sakai, Y. Tani, E. Shirasawa, Y. Shirao, and K. Kawasaki, "Development of electroretinographic alterations in streptozotocin-induced diabetes in rats," *Ophthalmic Research*, vol. 27, no. 1, pp. 57–63, 1995.

[6] A. M. Joussen, S. Huang, V. Poulaki et al., "In vivo retinal gene expression in early diabetes," *Investigative Ophthalmology & Visual Science*, vol. 42, no. 12, pp. 3047–3057, 2001.

[7] R. M. Brucklacher, K. M. Patel, H. D. VanGuilder et al., "Whole genome assessment of the retinal response to diabetes

reveals a progressive neurovascular inflammatory response," *BMC Medical Genomics*, vol. 1, no. 1, p. 26, 2008.

[8] A. M. Abu el Asrar, D. Maimone, P. H. Morse, S. Gregory, and A. T. Reder, "Cytokines in the vitreous of patients with proliferative diabetic retinopathy," *American Journal of Ophthalmology*, vol. 114, no. 6, pp. 731–736, 1992.

[9] T. Yuuki, T. Kanda, Y. Kimura et al., "Inflammatory cytokines in vitreous fluid and serum of patients with diabetic vitreoretinopathy," *Journal of Diabetes and Its Complications*, vol. 15, no. 5, pp. 257–259, 2001.

[10] J. I. Patel, G. M. Saleh, P. G. Hykin, Z. J. Gregor, and I. A. Cree, "Concentration of haemodynamic and inflammatory related cytokines in diabetic retinopathy," *Eye (London, England)*, vol. 22, no. 2, pp. 223–228, 2008.

[11] K. Miyamoto, S. Khosrof, S. E. Bursell et al., "Prevention of leukostasis and vascular leakage in streptozotocin-induced diabetic retinopathy via intercellular adhesion molecule-1 inhibition," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 19, pp. 10836–10841, 1999.

[12] A. Carmo, J. G. Cunha-Vaz, A. P. Carvalho, and M. C. Lopes, "Effect of cyclosporin-A on the blood—retinal barrier permeability in streptozotocin-induced diabetes," *Mediators of Inflammation*, vol. 9, no. 5, pp. 243–248, 2000.

[13] R. A. Kowluru, P. Koppolu, S. Chakrabarti, and S. Chen, "Diabetes-induced activation of nuclear transcriptional factor in the retina, and its inhibition by antioxidants," *Free Radical Research*, vol. 37, no. 11, pp. 1169–1180, 2003.

[14] A. Carmo, J. G. Cunha-Vaz, A. P. Carvalho, and M. C. Lopes, "L-arginine transport in retinas from streptozotocin diabetic rats: correlation with the level of IL-1 beta and NO synthase activity," *Vision Research*, vol. 39, no. 23, pp. 3817–3823, 1999.

[15] R. A. Kowluru and S. Odenbach, "Role of interleukin-1beta in the development of retinopathy in rats: effect of antioxidants," *Investigative Ophthalmology & Visual Science*, vol. 45, no. 11, pp. 4161–4166, 2004.

[16] C. Gerhardinger, M. B. Costa, M. C. Coulombe, I. Toth, T. Hoehn, and P. Grosu, "Expression of acute-phase response proteins in retinal Muller cells in diabetes," *Investigative Ophthalmology & Visual Science*, vol. 46, no. 1, pp. 349–357, 2005.

[17] J. K. Krady, A. Basu, C. M. Allen et al., "Minocycline reduces proinflammatory cytokine expression, microglial activation, and caspase-3 activation in a rodent model of diabetic retinopathy," *Diabetes*, vol. 54, no. 5, pp. 1559–1565, 2005.

[18] T. O. Pereira, G. N. da Costa, A. R. Santiago, A. F. Ambrosio, and P. F. dos Santos, "High glucose enhances intracellular Ca2+ responses triggered by purinergic stimulation in retinal neurons and microglia," *Brain Research*, vol. 1316, pp. 129–138, 2010.

[19] E. Schuetz and S. Thanos, "Microglia-targeted pharmacotherapy in retinal neurodegenerative diseases," *Current Drug Targets*, vol. 5, no. 7, pp. 619–627, 2004.

[20] A. M. Joussen, V. Poulaki, M. L. Le et al., "A central role for inflammation in the pathogenesis of diabetic retinopathy," *The FASEB Journal*, vol. 18, no. 12, pp. 1450–1452, 2004.

[21] T. S. Kern, "Contributions of inflammatory processes to the development of the early stages of diabetic retinopathy," *Experimental Diabetes Research*, vol. 2007, Article ID 95103, p. 14, 2007.

[22] I. Y. Chung and E. N. Benveniste, "Tumor necrosis factor-alpha production by astrocytes. Induction by lipopolysaccharide, IFN-gamma, and IL-1 beta," *Journal of Immunology*, vol. 144, no. 8, pp. 2999–3007, 1990.

[23] S. M. Sparacio, Y. Zhang, J. Vilcek, and E. N. Benveniste, "Cytokine regulation of interleukin-6 gene expression in astrocytes involves activation of an NF-kappa B-like nuclear protein," *Journal of Neuroimmunology*, vol. 39, no. 3, pp. 231–242, 1992.

[24] Z. Chai, S. Gatti, C. Toniatti, V. Poli, and T. Bartfai, "Interleukin (IL)-6 gene expression in the central nervous system is necessary for fever response to lipopolysaccharide or IL-1 beta: a study on IL-6-deficient mice," *The Journal of Experimental Medicine*, vol. 183, no. 1, pp. 311–316, 1996.

[25] N. J. Rothwell and G. N. Luheshi, "Interleukin 1 in the brain: biology, pathology and therapeutic target," *Trends in Neurosciences*, vol. 23, no. 12, pp. 618–625, 2000.

[26] A. R. Santiago, T. S. Pereira, M. J. Garrido, A. J. Cristovao, P. F. Santos, and A. F. Ambrosio, "High glucose and diabetes increase the release of [3H]-D-aspartate in retinal cell cultures and in rat retinas," *Neurochemistry International*, vol. 48, no. 6-7, pp. 453–458, 2006.

[27] F. I. Baptista, M. J. Pinto, F. Elvas, T. Martins, R. D. Almeida, and A. F. Ambrosio, "Diabetes induces changes in KIF1A, KIF5B and dynein distribution in the rat retina: Implications for axonal transport," *Experimental eye Research*, vol. 127C, pp. 91–103, 2014.

[28] A. R. Santiago, A. J. Cristovao, P. F. Santos, C. M. Carvalho, and A. F. Ambrosio, "High glucose induces caspase-independent cell death in retinal neural cells," *Neurobiology of Disease*, vol. 25, no. 3, pp. 464–472, 2007.

[29] Á. F. Castilho, C. A. Aveleira, E. C. Leal et al., "Heme oxygenase-1 protects retinal endothelial cells against high glucose- and oxidative/nitrosative stress-induced toxicity," *PloS One*, vol. 7, no. 8, article e42428, 2012.

[30] M. Ferreira-Marques, C. A. Aveleira, S. Carmo-Silva, M. Botelho, L. Pereira de Almeida, and C. Cavadas, "Caloric restriction stimulates autophagy in rat cortical neurons through neuropeptide Y and ghrelin receptors activation," *Aging (Albany NY)*, vol. 8, no. 7, pp. 1470–1484, 2016.

[31] M. Mizutani, C. Gerhardinger, and M. Lorenzi, "Muller cell changes in human diabetic retinopathy," *Diabetes*, vol. 47, no. 3, pp. 445–449, 1998.

[32] E. Rungger-Brandle, A. A. Dosso, and P. M. Leuenberger, "Glial reactivity, an early feature of diabetic retinopathy," *Investigative Ophthalmology & Visual Science*, vol. 41, no. 7, pp. 1971–1980, 2000.

[33] D. Gaucher, J. A. Chiappore, M. Pâques et al., "Microglial changes occur without neural cell death in diabetic retinopathy," *Vision Research*, vol. 47, no. 5, pp. 612–623, 2007.

[34] T. W. Gardner, D. A. Antonetti, A. J. Barber, K. F. LaNoue, and S. W. Levison, "Diabetic retinopathy: more than meets the eye," *Survey of Ophthalmology*, vol. 47, Supplement 2, pp. S253–S262, 2002.

[35] N. Demircan, B. G. Safran, M. Soylu, A. A. Ozcan, and S. Sizmaz, "Determination of vitreous interleukin-1 (IL-1) and tumour necrosis factor (TNF) levels in proliferative diabetic retinopathy," *Eye (London, England)*, vol. 20, no. 12, pp. 1366–1369, 2006.

[36] Y. Liu, M. Biarnes Costa, and C. Gerhardinger, "IL-1beta is upregulated in the diabetic retina and retinal vessels: cell-specific effect of high glucose and IL-1beta autostimulation," *PloS One*, vol. 7, no. 5, article e36949, 2012.

[37] G. N. Costa, J. Vindeirinho, C. Cavadas, A. F. Ambrosio, and P. F. Santos, "Contribution of TNF receptor 1 to retinal neural cell death induced by elevated glucose," *Molecular and Cellular Neurosciences*, vol. 50, no. 1, pp. 113–123, 2012.

[38] P. Rao, B. Lyn-Cook, and N. Littlefield, "High glucose concentration alters cell proliferation dynamics in human hepatoma cells," *International Journal of Toxicology*, vol. 18, no. 5, pp. 297–306, 1999.

[39] M. A. Dyer and C. L. Cepko, "Control of Muller glial cell proliferation and activation following retinal injury," *Nature Neuroscience*, vol. 3, no. 9, pp. 873–880, 2000.

[40] G. Costa, T. Pereira, A. M. Neto, A. J. Cristovao, A. F. Ambrosio, and P. F. Santos, "High glucose changes extracellular adenosine triphosphate levels in rat retinal cultures," *Journal of Neuroscience Research*, vol. 87, no. 6, pp. 1375–1380, 2009.

[41] Y. Toda, J. Tsukada, M. Misago, Y. Kominato, P. E. Auron, and Y. Tanaka, "Autocrine induction of the human pro-IL-1beta gene promoter by IL-1beta in monocytes," *Journal of Immunology*, vol. 168, no. 4, pp. 1984–1991, 2002.

[42] M. E. Lull and M. L. Block, "Microglial activation and chronic neurodegeneration," *Neurotherapeutics*, vol. 7, no. 4, pp. 354–365, 2010.

[43] M. Karlstetter, S. Ebert, and T. Langmann, "Microglia in the healthy and degenerating retina: insights from novel mouse models," *Immunobiology*, vol. 215, no. 9-10, pp. 685–691, 2010.

[44] Y. Quan, C. T. Jiang, B. Xue, S. G. Zhu, and X. Wang, "High glucose stimulates TNFalpha and MCP-1 expression in rat microglia via ROS and NF-kappaB pathways," *Acta Pharmacologica Sinica*, vol. 32, no. 2, pp. 188–193, 2011.

[45] J. A. Vincent and S. Mohr, "Inhibition of caspase-1/interleukin-1beta signaling prevents degeneration of retinal capillaries in diabetes and galactosemia," *Diabetes*, vol. 56, no. 1, pp. 224–230, 2007.

[46] M. Wang, W. Ma, L. Zhao, R. N. Fariss, and W. T. Wong, "Adaptive Muller cell responses to microglial activation mediate neuroprotection and coordinate inflammation in the retina," *Journal of Neuroinflammation*, vol. 8, no. 1, p. 173, 2011.

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Research Article

Anti-Inflammatory and Neuroprotective Role of Natural Product Securinine in Activated Glial Cells: Implications for Parkinson's Disease

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Glial activation and subsequent release of neurotoxic proinflammatory factors are believed to play an important role in the pathogenesis of several neurological disorders including Parkinson's disease (PD). Inhibition of glial activation and inflammatory processes may represent a therapeutic target to alleviate neurodegeneration. Securinine, a major natural alkaloid product from the root of the plant *Securinega suffruticosa*, has been reported to have potent biological activity and is used in the treatment of neurological conditions such as amyotrophic lateral sclerosis, poliomyelitis, and multiple sclerosis. In this study, we explored the underlying mechanisms of neuroprotection elicited by securinine, particularly its anti-inflammatory effects in glial cells. Our results demonstrate that securinine significantly and dose-dependently suppressed the nitric oxide production in microglia and astrocytic cultures. In addition, securinine inhibited the activation of the inflammatory mediator NF-κB, as well as mitogen-activated protein kinases in lipopolysaccharide- (LPS-) stimulated BV2 cells. Additionally, securinine also inhibited interferon-γ- (IFN-γ-) induced nitric oxide levels and iNOS mRNA expression. Furthermore, conditioned media (CM) from securinine pretreated BV2 cells significantly reduced mesencephalic dopaminergic neurotoxicity compared with CM from LPS stimulated microglia. These findings suggest that securinine may be a potential candidate for the treatment of neurodegenerative diseases related to neuroinflammation.

1. Introduction

Inflammation of the central nervous system (CNS) is a key contributing factor in several neurodegenerative diseases. Glial cells, including microglia and astrocytes, are known to be the major mediators of neuroinflammation. Activated microglial cells trigger an inflammatory response that is then maintained and often times amplified by astrocytes. This, in turn, exposes neurons to inflammatory mediators that can cause neuronal cell death [1]. Neurodegenerative CNS diseases including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and age-related macular degeneration (ARMD) have all been associated with chronic neuroinflammation and activation of both microglia and astrocytes [2]. This chronic inflammation can lead to the accumulation of neurotoxic molecules including proinflammatory cytokines, proteinases, and reactive oxygen species (ROS), which participate in the neurodegenerative process and can ultimately lead to neuronal cell death [3–5]. It is hypothesized that inhibiting microglial-mediated induction of the neuroinflammatory process may prove to be neuroprotective and therefore inhibitory compounds targeting this process are likely to have therapeutic value.

Medicinal plants and their active components are currently the subject of extensive biomedical research. Many of these plant-derived medications have been validated by traditional usage over the centuries, but the specific bioactive components have not been identified or are not fully characterized. Traditional herbal medicines with neurotrophic

and neuroprotective properties have been demonstrated to prevent neurodegeneration and neuroinflammatory diseases [6]. This suggests that these traditional medicinal herbs possess neuroprotective benefits through distinct and multiple mechanisms, including anti-inflammation [7–10]. Natural compounds specifically targeted to blocking microglial activation and therefore blocking the initiation of CNS inflammation may prove to be an effective therapy to prevent neurodegenerative and neuroinflammatory diseases.

Securinine, a major natural alkaloid product from the root of the plant *Securinega suffruticosa,* has been reported to have potent biological activity and has been used clinically in several countries [11]. Securinine has been found to be an antagonist of the γ-amino butyric acid (GABA) A receptor and as such is currently used in the treatment of neurological conditions such as ALS, poliomyelitis, and multiple sclerosis [12–14]. Additionally, securinine has been shown to be a macrophage agonist, enhancing bacterial clearance via a mechanism distinct from toll-like receptors (TLRs) [15]. Securinine appears to stimulate p38 MAPK activity through antagonism of the GABA A receptor in monocytes [16]. Securinine has also been found to stimulate apoptosis in p53-null colon cancer cells [1, 17]. However, the potential anti-inflammatory role of securinine on glial activation and the mechanisms involved have not been thoroughly explored.

In the present study, to determine whether securinine can suppress glial inflammatory activation and act as a mediator of neuroinflammation, we tested the effects of securinine on lipopolysaccharide- (LPS-) induced activation of microglia and astrocytes. The results demonstrated that securinine inhibits the activation of the inflammatory mediator NF-κB, as well as its upstream MAPK activators including ERK. Further, securinine also silences iNOS expression and NO production, both of which are activated by NF-κB. Importantly, we demonstrate that inhibition of LPS-induced microglial activation with securinine is neuroprotective in the context of primary mesencephalic dopaminergic neurons representing an in vitro model of PD.

2. Experimental Procedures

2.1. Cell Culture. The cell culture reagents used in this study were purchased from Mediatech, Inc. (Manassas, VA, USA). BV2 microglial cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture (10,000 u/mL) (Sigma Aldrich, St Louis, MO). RAW 264.7 macrophage cell lines (ATCC, TBI-71) were grown and maintained in DMEM supplemented with 10% heat inactivated FBS and 1% Penicillin-streptomycin mixture (10,000 u/mL) at 37°C, 5% CO_2.

Primary mouse astrocytes and microglia were isolated as described previously [18]. We used postnatal day 2 pups to generate primary glial cultures. Briefly, cortical tissues from C57/BL6 mice were dissected out and the tissue was digested with papain for 30 minutes at 37°C and plated in poly-L-lysine coated T75 flasks in DMEM supplemented with 10% FBS. For astrocytes culture isolation, after 1 week of culture, the flasks were shaken to remove microglia followed by trypsinization and plating onto poly-L-lysine coated plates or chambered slides at 1×10^5 cells/well. The cells were only used for a maximum of three passages; more than 95% of cells stained positively for the astrocyte marker GFAP in these cultures.

Primary microglial cultures were prepared from the cortex of C57/BL6 mice as described above. Briefly, mixed glial cells were cultured for 3-4 weeks in DMEM supplemented with 10% FBS. The astrocyte monolayer was removed by incubation for 30 min with 0.25% trypsin/2.12 mM EDTA (Mediatech, Inc., Manassas, VA) diluted 1 : 4 with DMEM. The remaining isolated microglial cells were plated into 96-well plates at a density of 5×10^4 cells/well and 6-well plates at 5×10^5 cells/well. The purity of microglia cultures was assessed using microglial marker CD11b or Iba1; 95% of cells stained positively for these microglial markers; 1–3% stained positively for GFAP marker. The microglial cells were cultured for 2 days before drug treatment.

Primary mesencephalic cultures were isolated as previously described from our laboratory [19]. Briefly, ventral mesencephalon was dissected from embryonic day 14 of C57/BL6 mice. The tissue was dissociated mechanically and then digested enzymatically with papain solution as per manufactures instructions (Worthington Biochemical, Lakewood, NJ). After 4 d in vitro, the neuronal cultures were taken for condition media exposure from activated microglial cells.

2.2. Cell Viability Assay. Cell viability was determined by calorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described previously from our laboratory [20]. BV2 microglial cells were plated into 96-well culture plates at a density of 1×10^4 cells/mL in 100 μL culture medium per well. The cells were allowed to settle for 24 h before the addition of different concentrations of securinine for 24 h (Sigma Aldrich, USA). After 24 h of treatment, MTT solution (5 mg/mL) was added to each well and plate was incubated at 37°C for 2–4 h. The medium was aspirated and the resulting formazan crystals were dissolved in 200 μL of dimethyl sulfoxide before measuring the absorbance at 570 nm using a spectrophotometer.

2.3. Measurement of Nitrite ($NO2^-$) Levels. The level of $NO2^-$, a stable downstream product of NO, secreted by activated glial cells in culture media was measured using the Griess Reagent assay method as previously described [21]. The microglia, astrocytes, and macrophages cultures were exposed to inflammation inducing agents in presence or absence of different concentration of securinine for 24 h. Following the treatments, NO levels in the culture media were measured by mixing with equal volume of Griess Reagent and OD was measured at 540 nm on a microplate reader (Molecular Devices, CA). The data are representative of results obtained from four independent experiments performed in triplicate (mean ± SD).

2.4. NFκB-Luciferase Activity Assay. BV2 cells stably expressing an NFκB-luciferase construct were plated into 96-well cell culture plates at a density of 10,000 cells/well. The NFκB

binding reporter plasmid contains three copies of the κB-binding sequence fused to the firefly luciferase gene which was purchased from Clontech (Mountain View, CA, USA). Cells were preexposed to securinine followed by cotreatment with LPS for 24 hr treatment. Luciferase activity was measured using the Dual-Luciferase® Reporter Assay System kit as per the manufacturer's instructions (Promega).

2.5. Western Blotting Analysis. Following various treatments, BV2 cells and astrocytes were collected from Petri dishes by trypsinization and lysed in cold lysis buffer containing protease inhibitors as previously described [21]. The whole cell lysates were sonicated and the protein concentration was measured using a Bradford protein assay kit (Bio-Rad, Hercules, CA). Twenty-five to 50 μg of total protein was subjected to 10% SDS-PAGE and protein was transferred to a polyvinylidene difluoride membrane. Immunodetection was carried out by standard procedures using the following dilutions of antibodies: iNOS (rabbit polyclonal antibody, 1 : 1000), phospho-NFkB (p65) (rabbit polyclonal antibody, 1 : 1000), PARP (rabbit polyclonal antibody, 1 : 1000), the phospho- or total forms of ERK1/2, p38 MAPK, JNK, and pSTAT1 (rabbit polyclonal antibodies, 1 : 1000, Cell Signaling Technology, USA), and beta-actin (mouse monoclonal antibody, 1 : 10,000, Sigma Aldrich, USA). Secondary antibodies were horseradish peroxidase conjugated to either goat anti-rabbit IgG or anti-mouse IgG (Cell Signaling Technology, USA). The membranes were developed using an enhanced chemiluminescence (ECL) detection system (Pierce, USA). Band intensities were determined using the Image-Pro Plus 6.0 software (Bio-Rad).

2.6. Quantitative Polymerase Chain Reaction (qPCR) Analysis. BV2 cells and primary astrocytes cultured were challenged with LPS (BV2, 500 ng/mL; primary astrocytes, 1 μg/mL + IFN-γ 40 U/mL) in the presence or absence of securinine (10 μM) for 6 h. Total RNA was isolated from the cells via TRIzol method and cDNA was synthesized using Promega GoScript™ Reverse Transcription System (Madison, WI, USA). Quantitative PCR was performed using SYBR Green PCR Master Mix reagent and gene-specific primers. Data were analyzed by using the comparative threshold cycle (Ct) method. Altered mRNA expression levels following treatment were calculated following normalization to GAPDH. The ratios obtained after normalization are expressed as fold change versus corresponding controls [20].

2.7. Neurotoxicity of BV2 Conditioned Media in Primary Mesencephalic Cultures. Primary mesencephalic cultures were isolated as described above. After 4 days in vitro, dopaminergic neurons were exposed to conditioned media (CM) from BV2 microglial cells treated with LPS ± securinine. The CM was prepared as follows: BV2 cells cultured in 6-well plates were stimulated with LPS (500 ng/mL) in the presence and absence of securinine (10 μM) for 24 h. CM was collected from all the groups and centrifuged at 2,000 g for 10 min to remove any cell debris. The CM from control, securinine, and LPS versus LPS + securinine-treated cells was diluted (1 : 4) in neuronal culture media before adding to mesencephalic cultures. After 72 h exposure, the cells were fixed in 4% paraformaldehyde and immunostained with anti-TH antibody and Alexa Fluor 488 secondary antibody. Total numbers of TH-positive neurons were counted in 3–5 separate wells for each condition. Experiments were repeated with cultures isolated from three independent dissections.

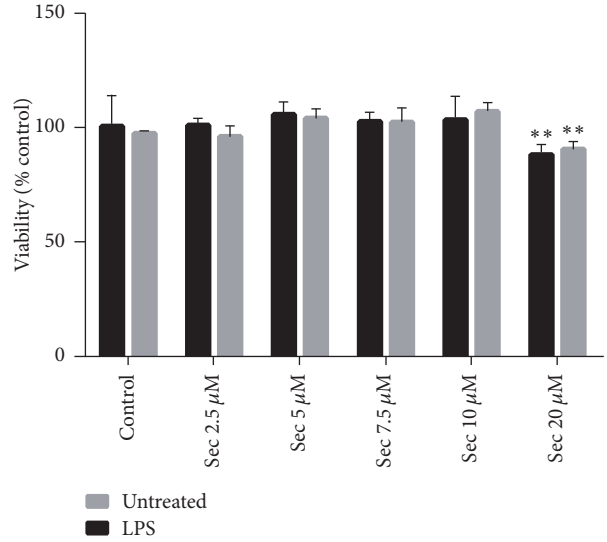

Figure 1: Effects of securinine on the cell viability of the BV2 microglia cell line. BV2 cells were treated with 2.5–20 μM of securinine without LPS treatment or in the presence of 500 ng/mL LPS treatment for 24 h. Cell viability was measured via the MTT assay and data was expressed as mean ± SEM for three independent experiments. $^{**}P < 0.05$, significantly different from the value in control cells.

2.8. Statistical Analysis. Unless otherwise stated, all experiments were performed in triplicate and repeated at least three times. The data are presented as mean ± SEM and statistical comparisons between groups were performed by one-way ANOVA followed by Student's t-test. Multiple comparisons of data from in vitro experiments were evaluated by two-way ANOVA followed by Bonferroni post hoc testing. Statistical significance was set at $P < 0.05$ for all analyses.

3. Results

3.1. Securinine Does Not Elicit Cellular Toxicity in Microglial BV2 Cells at Concentrations up to 15 μM. To determine whether securinine displays cytotoxic effects in glial cell types, we treated BV2 cells, an immortalized murine microglial cell line, with increasing concentrations of the compound for 24 h, followed by assessment of cell viability using the MTT assay. No significant cytotoxicity was observed up to 15 μM, whereas 15% toxicity was observed at 20 μM (Figure 1). This toxicity may be due to the induction of apoptosis as previously reported at 30 μM in cancer cell lines [17]. Based on this data, we chose to use 10 μM securinine for all subsequent assays. Additionally, the concentration of LPS (500 ng/mL) used to induce NO production also did not affect cell viability (data not shown).

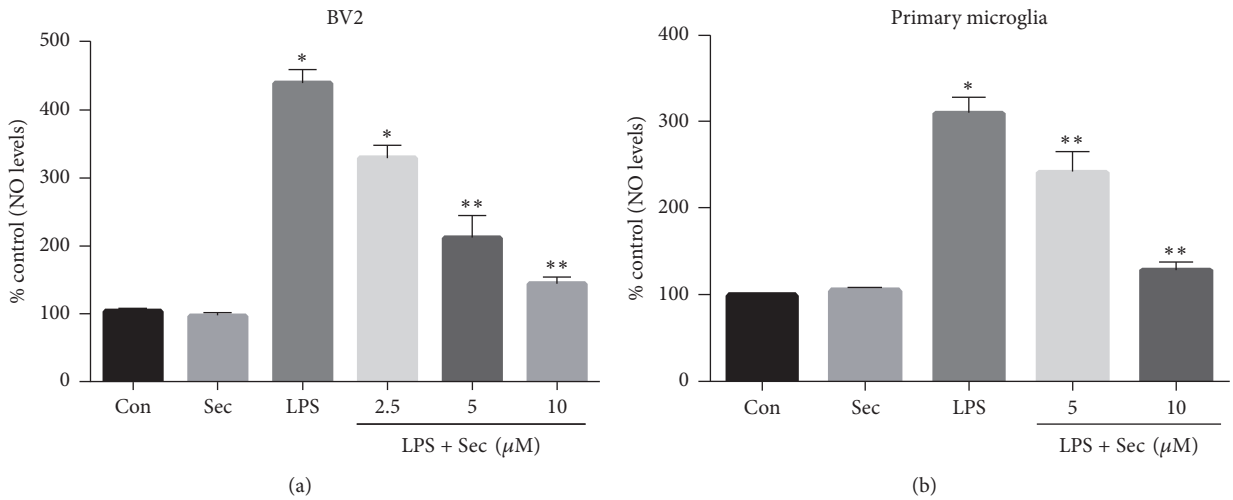

Figure 2: Securinine inhibits LPS-induced NO production in the BV2 cell line and in mouse primary microglia challenged with LPS exposure. BV2 microglial cells (a) and mouse primary microglia (b) were pretreated with securinine (2, 5, and 10 μM) for 1 h and then stimulated with LPS (500 ng/mL) for 24 h. NO levels in the medium were determined using the Griess Reagent system. The data are expressed as mean ± SD, $n = 4$. $^{*}P < 0.01$, significantly different from control samples. $^{**}P < 0.05$ significantly different from the LPS-treated group alone.

3.2. Securinine Inhibits LPS-Induced Inflammatory NO Production in Both BV2 Cells and Primary Mouse Microglia. To examine the potential anti-inflammatory activity of securinine in the context of microglial activation, we initially studied the effects of securinine on the production of inflammatory mediators in BV2 and mouse primary microglia, both challenged with LPS. As part of the inflammatory response, activated glial cells rapidly induce the expression of inducible nitric oxide synthase (iNOS), a key enzyme required for generating nitric oxide (NO), a reactive nitrogen species (RNS) causing protein and mitochondrial damage leading to apoptosis [22]. In BV2 cells, LPS markedly induced NO production as detected by the accumulation of nitrite in the culture medium after 24 h. In the presence of securinine however, there was a dose-dependent inhibition of NO production to almost control levels at the highest drug concentration. Securinine alone had no effect on NO production (Figure 2(a)). Likewise, we observed significant inhibition of LPS-induced NO production by securinine within primary microglia cell cultures (Figure 2(b)).

3.3. Securinine Inhibits LPS-Induced Expression of the NO-Synthesizing Enzyme, iNOS, and Proinflammatory Cytokines. To examine whether the reduction of NO production by securinine was due to reduced mRNA and protein expression of iNOS and also effect of securinine on the expression of various proinflammatory cytokines, real-time PCR and Western blot analyses were conducted in LPS-stimulated BV2 and primary microglial cells. Both primary and BV2 cells were preincubated 1 h with securinine (10 μM) and then stimulated for 6 h with LPS (500 ng/mL). As shown in Figures 3(A1) and 3(A2), securinine significantly reduced the expression of proinflammatory molecules such as TNF-α and IL-1β in both BV2 and primary microglial cells, while the compound alone did not induce any significant changes in gene expression. Furthermore, as shown by qPCR and Western blotting (Figures 3(B1) and 3(B2)), securinine dose-dependently inhibited LPS-induced iNOS expression at the mRNA and protein levels in both primary BV2 and microglial cells.

3.4. Securinine Attenuates LPS-Induced NFκB Activation. LPS and other inflammatory stimuli are known to induce iNOS expression in glia cells via the activation of the transcription factor NFκB. Therefore, we examined whether the anti-inflammatory effects of securinine were due to blockade of NF-κB activation within BV2 cells. NF-κB is normally activated by phosphorylation of IκB proteins, targeting them for rapid degradation through the ubiquitin-proteasome pathway and releasing NF-κB to enter the nucleus where it regulates gene expression. Western blot analysis demonstrated LPS-induced phosphorylation of p65 subunit which was significantly inhibited by pretreatment with securinine (Figure 4(a)). Furthermore, LPS-induced NFκB-dependent transcriptional activity was also significantly reduced by treatment with securinine (10 μM). These data suggest that securinine can prevent NFκB-mediated induction of inflammatory pathways.

3.5. Securinine Reduces LPS-Induced MAPK Phosphorylation. Inhibitors targeting MAPK signaling pathways have been known to exhibit anti-inflammatory activity [23]. To determine whether anti-inflammatory activity of securinine is due to modulation of MAPKs activity, BV2 cells were pretreated with securinine (10 μM) for 1 h and then stimulated with LPS for an additional 1 h incubation period. Based on preliminary time course studies (data not shown), 1 h treatment of LPS was determined to be optimal for MAPK phosphorylation. Western blot analysis was carried out using phosphoantibodies or total antibodies against the three MAPKs, p38 MAPK, JNK, and ERK1/2. Western blot analysis showed that the

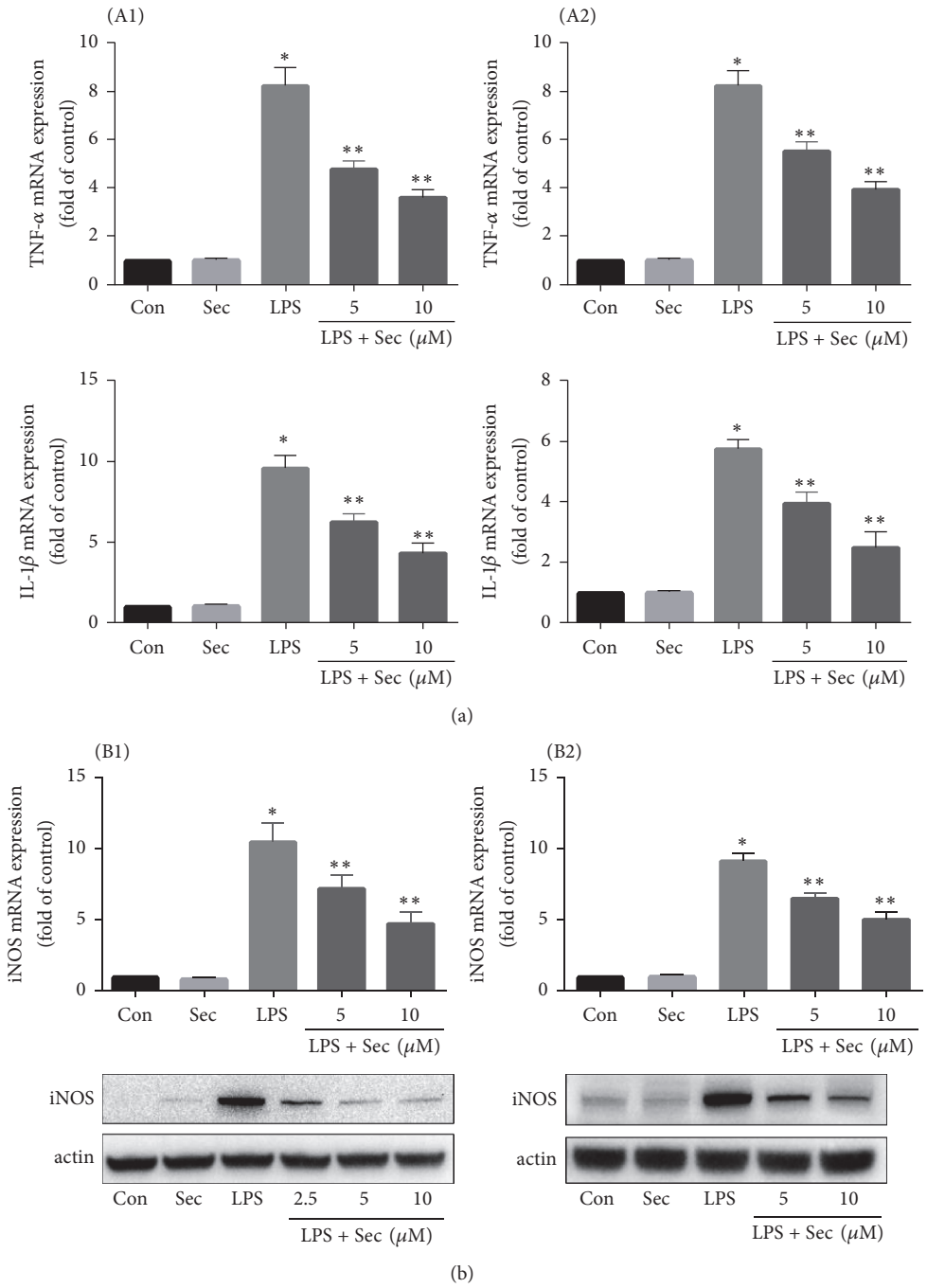

Figure 3: Securinine represses LPS-induced proinflammatory cytokines and iNOS expression in BV2 and mouse primary microglia. (a) Securinine inhibits proinflammatory cytokine expression following LPS exposure. BV2 and primary microglial cells were preincubated with securinine for 1 h before stimulation with LPS (500 ng/mL). After 6 h of stimulation, total RNA was isolated and levels of mRNA expression of proinflammatory cytokines TNF-α and IL-1β analyzed via quantitative real-time PCR ((A1) BV2 cells and (A2) mouse primary microglia). (b) Securinine represses LPS-induced iNOS mRNA and protein expression in BV2 (B1) and primary microglia cells. After 24 h of stimulation with LPS (500 ng/mL), the level of iNOS protein was monitored by Western blot analysis ((B1) BV2 cells and (B2) primary microglia cells). Data are mean ± SD of three different experiments. $^{*}P < 0.001$ versus control; $^{**}P < 0.05$ versus LPS-treated group alone.

securinine significantly repressed the phosphorylation of p38 MAPK, JNK, and ERK1/2, respectively, but did not affect the expression levels of ERK1/2, JNK, and p38 MAPK in LPS-stimulated BV2 microglia (Figure 5).

3.6. Securinine Reduces IFN-γ-Induced NO Production, iNOS mRNA Expression, and STAT1α Activation. Multiple transcription factors participate in the regulation of iNOS promotor activity. In addition to LPS, IFN-γ is a well-established

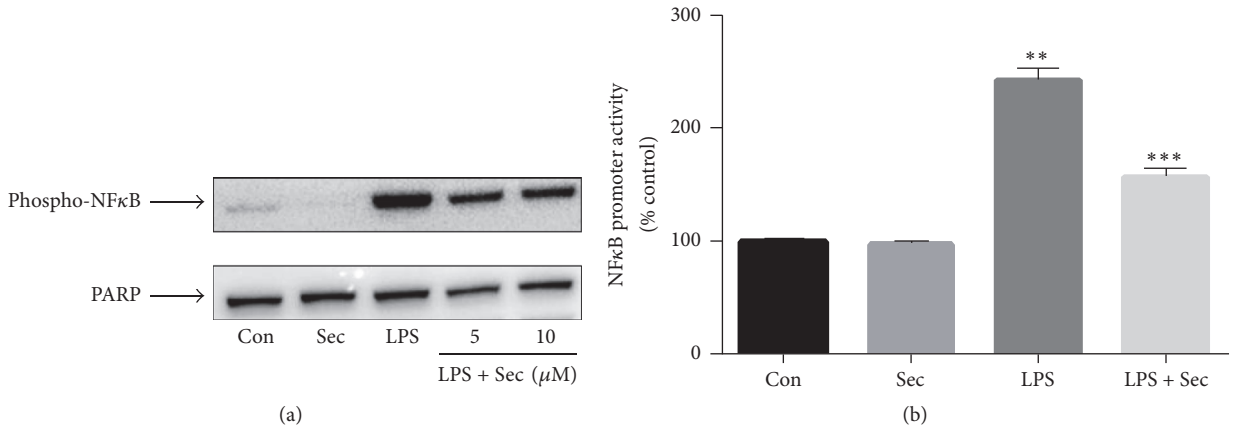

Figure 4: Securinine attenuates LPS-induced activation of NF-κB in BV2 microglia. (a) BV2 microglial cells were treated with securinine for 1 h followed by stimulation with 500 ng/mL LPS. After 30 min of LPS stimulation, isolated nuclear lysates were subjected to Western blotting to assess translocation of phosphor-65 protein to the nucleus. PARP was used as a nuclear loading control. (b) BV2 cells were transiently transfected with NF-κB-Luc plasmid for 24 h and then treated with 500 ng/mL LPS for 4 h ± securinine. Whole cell lysates were assayed via the luciferase activity (mean ± SE, $n = 4$); $^{*}P < 0.05$ versus control. $^{**}P < 0.05$ versus LPS. $^{***}P < 0.05$ versus LPS.

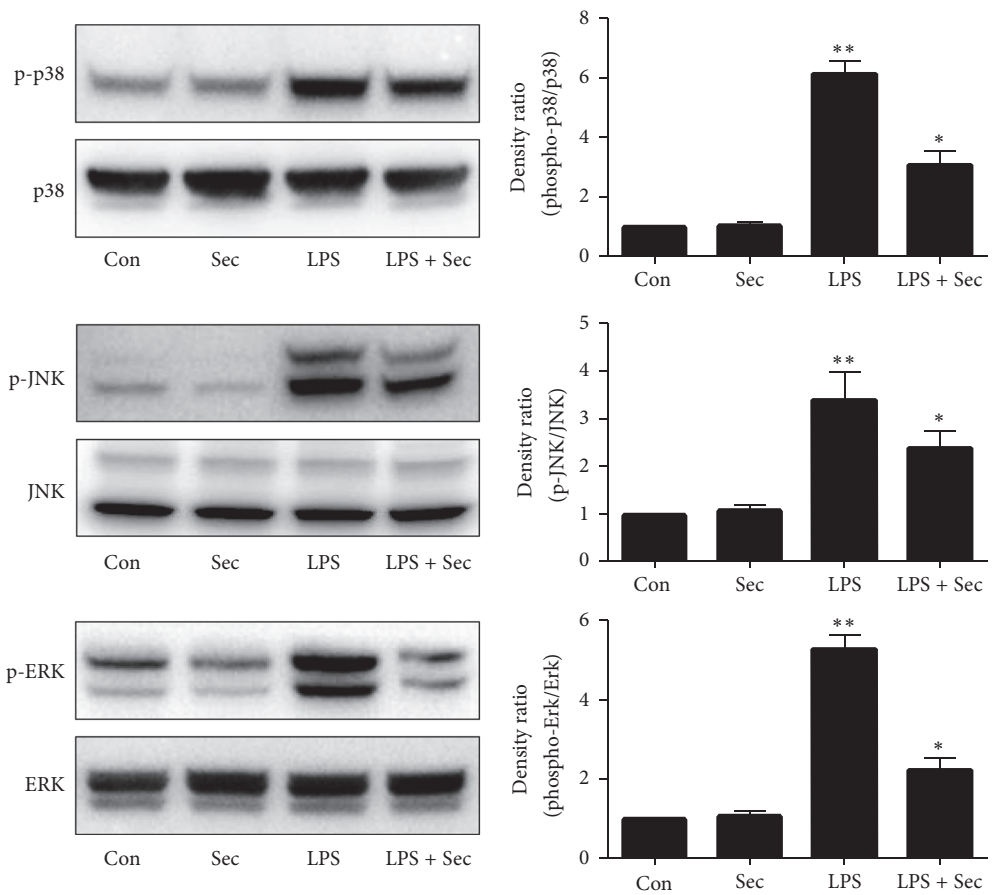

Figure 5: Securinine reduces LPS-induced MAPK phosphorylation in BV2 microglial cells. BV2 cells were pretreated with securinine (10 μM) for 1 h and then exposed to LPS (500 ng/mL) for 30 minutes. Whole cell lysates (50 μg of protein) were subjected to Western blot analysis using antibodies specific for phosphorylated forms of p38MAPK, ERK1/2, and JNK (mean ± SE, $n = 4$). Equal loadings of cell lysates were confirmed by reprobing the blots with total p38MAPK, ERK1/2, and JNK antibodies. The quantification data are shown in the right panel. $^{**}P < 0.01$ versus control; $^{*}P < 0.05$ versus LPS.

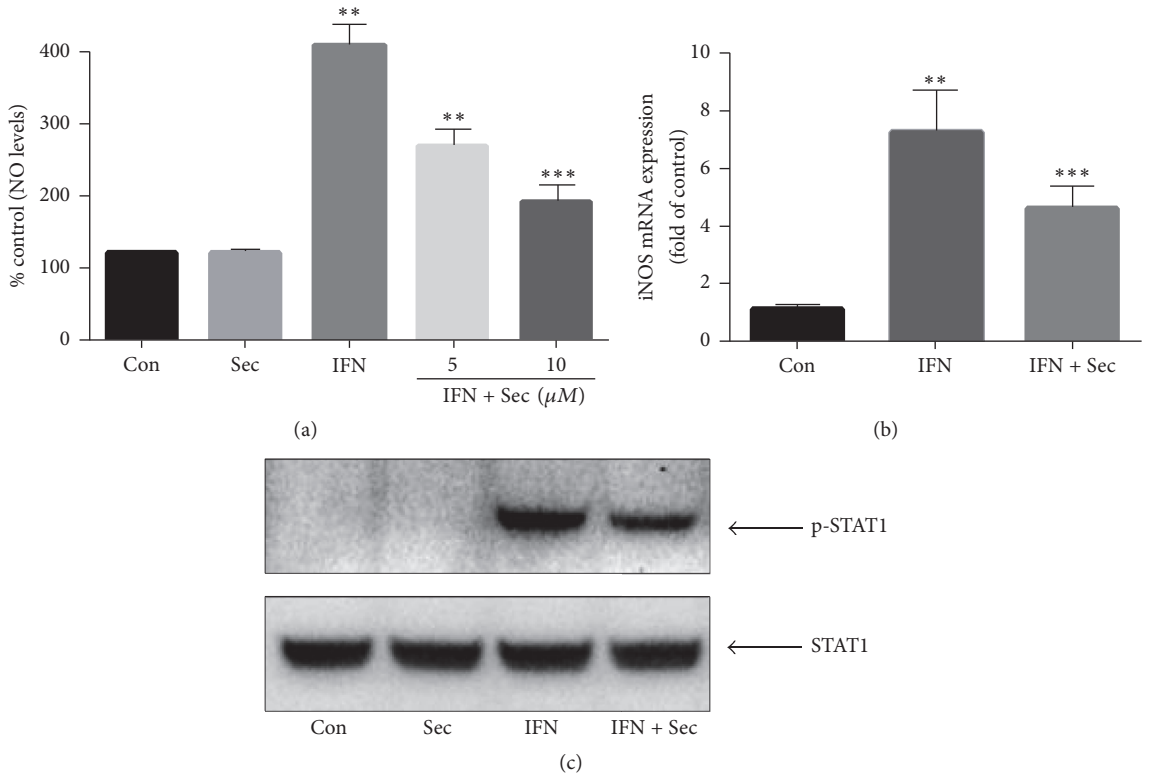

Figure 6: Securinine pretreatment reduces IFN-γ-induced NO production, iNOS mRNA expression, and phosphorylation of STAT1α in BV2 microglia. BV2 microglial cells were pretreated with securinine for 1 h and then challenged with IFN-γ (50 U/mL) for 24 h (a), 6 h (b), or 30 min (c). The expression levels of nitric oxide, iNOS mRNA, and tyrosine phosphorylation of STAT1α were determined by Griess Reagent assay (a), real-time PCR analysis (b), or Western blotting (c), respectively. The data are expressed as mean ± SD, $n = 4$. $^{**}P < 0.01$ significantly different from control samples. $^{***}P < 0.05$, significantly different from the LPS-treated group.

stimulus that promotes the expression of inflammatory molecules (e.g., iNOS) through STAT1α, but independent of NF-κB [23]. Thus, the effects of securinine on IFN-γ activation were also investigated. As shown in Figure 6(a), securinine inhibited IFN-γ-induced NO production in a concentration-dependent manner with maximum inhibition achieved at 10 μM. Securinine also significantly reduced IFN-γ-induced iNOS mRNA expression levels (Figure 6(b)) and phosphorylation of STAT1α (Figure 6(c)).

3.7. Securinine Protects Neurons through Inhibition of Microglial Activation. Previous studies from our own laboratory and others have demonstrated that activated microglia induce neural cell death and amplify the progression of neuronal degeneration [20, 24]. Since our data suggested that securinine can suppress microglial activation, we investigated whether this translated to noncell autonomous neuroprotective effects. Conditioned media (CM) from LPS-treated BV2 cells in the presence or absence of securinine pretreatment were added to primary mesencephalic cultures and cell viability monitored in the latter via tyrosine hydroxylase (TH) immunocytochemistry and TH-positive neuronal counting (Figures 7(a) and 7(b)). Results from these studies demonstrated that CM from LPS-stimulated microglia (LPS-CM) showed significant toxicity to TH-positive neurons, presumably as a consequence of toxic factors secreted by the activated microglia. In addition to cell loss, neuritic processes were also shortened in surviving TH-positive neurons (Figure 7(b)). Securinine alone, under normal conditions, did not influence TH-positive viability. However, pretreatment of microglial cells with securinine provided significant neuroprotection against toxicity associated with CM isolated from the activated microglia (LPS/Scu-CM versus LPS-CM), suggesting that securinine may exert its neuroprotective effects, at least partly, via reducing the production and secretion of inflammatory mediators from the microglia (Figure 7(b)).

3.8. Effects of the Securinine on the Inflammatory Activation of RAW 264.7 Macrophage Cells and Primary Astrocyte Cultures. Finally, in order to explore whether securinine can potentially also elicit anti-inflammatory effects in other cell types beyond microglia, we assessed NO production in RAW 264.7 macrophage cells and also in primary astrocyte cultures in the presence and absence of pretreatment with the compound. Securinine was found to dose-dependently decrease NO production in LPS-stimulated RAW264.7 macrophage cells (Figure 8(a)) and also in primary mouse astrocyte cultures (Figure 8(b)). When astrocytes were stimulated with LPS plus IFN-γ, greater levels of NO production were achieved, which were similarly inhibited by the securinine (Figure 8(c)). These

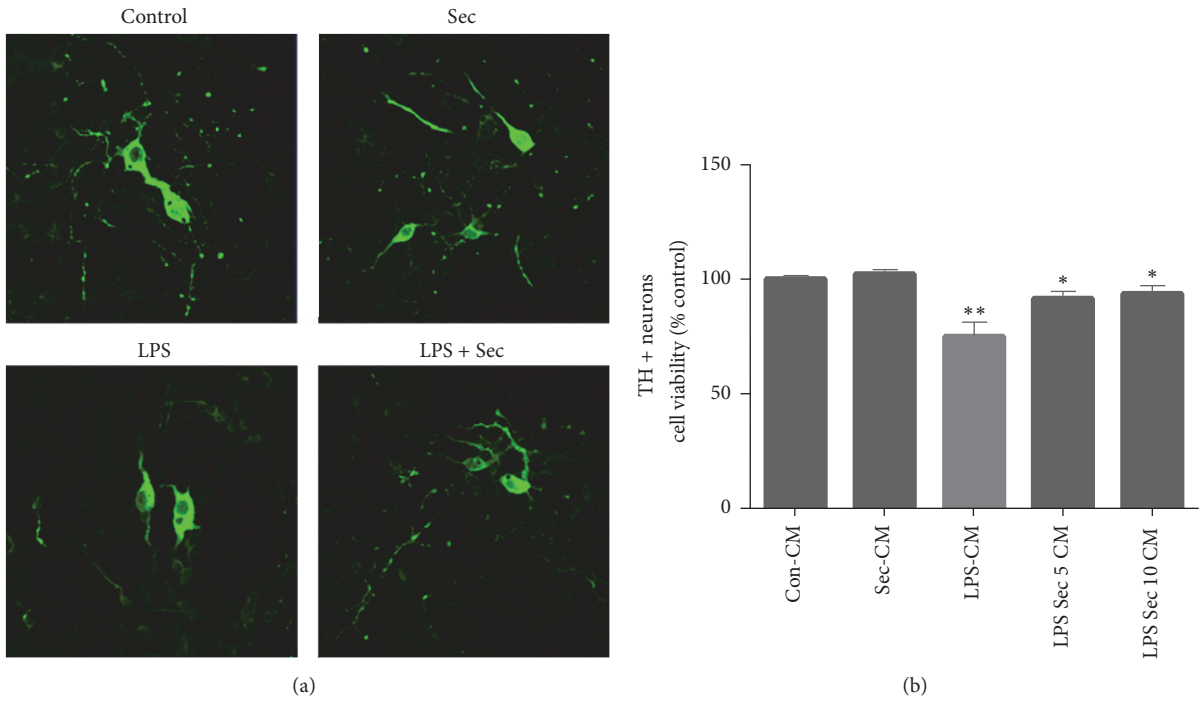

Figure 7: Securinine protects primary mesencephalic dopaminergic neurons through inhibition of BV2 cells activation. BV2 cells were stimulated with LPS (500 ng/mL) ± securinine (10 μM) for 24 h. CM from control (BV2-CM), securinine-treated (Sec-CM), LPS-treated (LPS-CM), and LPS/securinine-treated (5 and 10 μM; LPS + Sec 5 μM-CM, LPS + Sec 10 μM-CM) BV2 cells was added to primary mesencephalic cultures. After 72 h, cultures were immunostained for TH^+ neurons (a) and the number of TH^+ neurons was counted (b). Data are expressed as mean ± SD, $n = 4$; $^{**}P < 0.01$, compared with control-CM group, $^{*}P < 0.05$, compared with LPS-CM group.

results indicated that the small molecule securinine also has anti-inflammatory effects in peripheral macrophages and in astrocytes.

4. Discussion

In the present study, we examined the anti-inflammatory properties of the small molecule natural product securinine in glial populations and in macrophages and have delineated the potential signal transduction pathways involved in these processes. Our studies demonstrate that securinine strongly inhibits the inflammatory activation of microglia, astrocytes, and macrophages. Securinine significantly and dose-dependently reduced NO production in LPS-stimulated BV2 microglial cells and primary microglia and astrocyte cultures. NF-κB and MAPK pathways were at least partly involved in the anti-inflammatory mechanisms of securinine. Furthermore, securinine also inhibited IFN-γ-induced NO production and STAT1α activation. Finally, conditioned media from securinine pretreated BV2 microglial cells significantly reduced dopaminergic neurotoxicity compared to LPS-treated group alone. These results suggested that securinine might have therapeutic potential for various glial-mediated neuroinflammatory diseases.

Activation of microglial cells, the resident immune macrophage-like cells in the brain, is beneficial during acute infection or toxic insult by eliminating "sick" neurons that are no longer functional. However, in the presence of ongoing progressive brain damage, they can become chronically activated, resulting in sustained aberrant inflammatory response [25]. Glial cell activation and increased production of proinflammatory products derived from them have been implicated in the pathophysiology of several neurodegenerative diseases, such as AD, PD, and HD [26]. Recently, much attention has been paid to therapeutic strategies aimed at inhibiting neurotoxic microglial activation. Although nonsteroidal anti-inflammatory (NSAIDs) medications demonstrate neuroprotection in various disease models, such medications can have serious side effects following their long-term usage, leading to searches for better alternatives [27]. Recent studies have demonstrated that natural products and their components are good alternative candidates for therapeutic purposes due to their reputation for being safe, inexpensive, and readily available [28].

Activated microglia secretes proinflammatory mediators including cytokines such as IL-6 and TNF-α, reactive oxygen species, and reactive nitrative species such as NO. Nitric oxide (NO) is a unique biological messenger which mediates several physiological functions. However, under conditions of excessive production, NO appears to be neurotoxic suggesting that NO may play an important role in pathophysiology of neurodegenerative diseases [29]. In this current study, we have demonstrated that production of NO by LPS-activated microglia (both BV2 and primary microglia) is significantly inhibited in a dose-dependent manner by securinine at both the mRNA and protein levels.

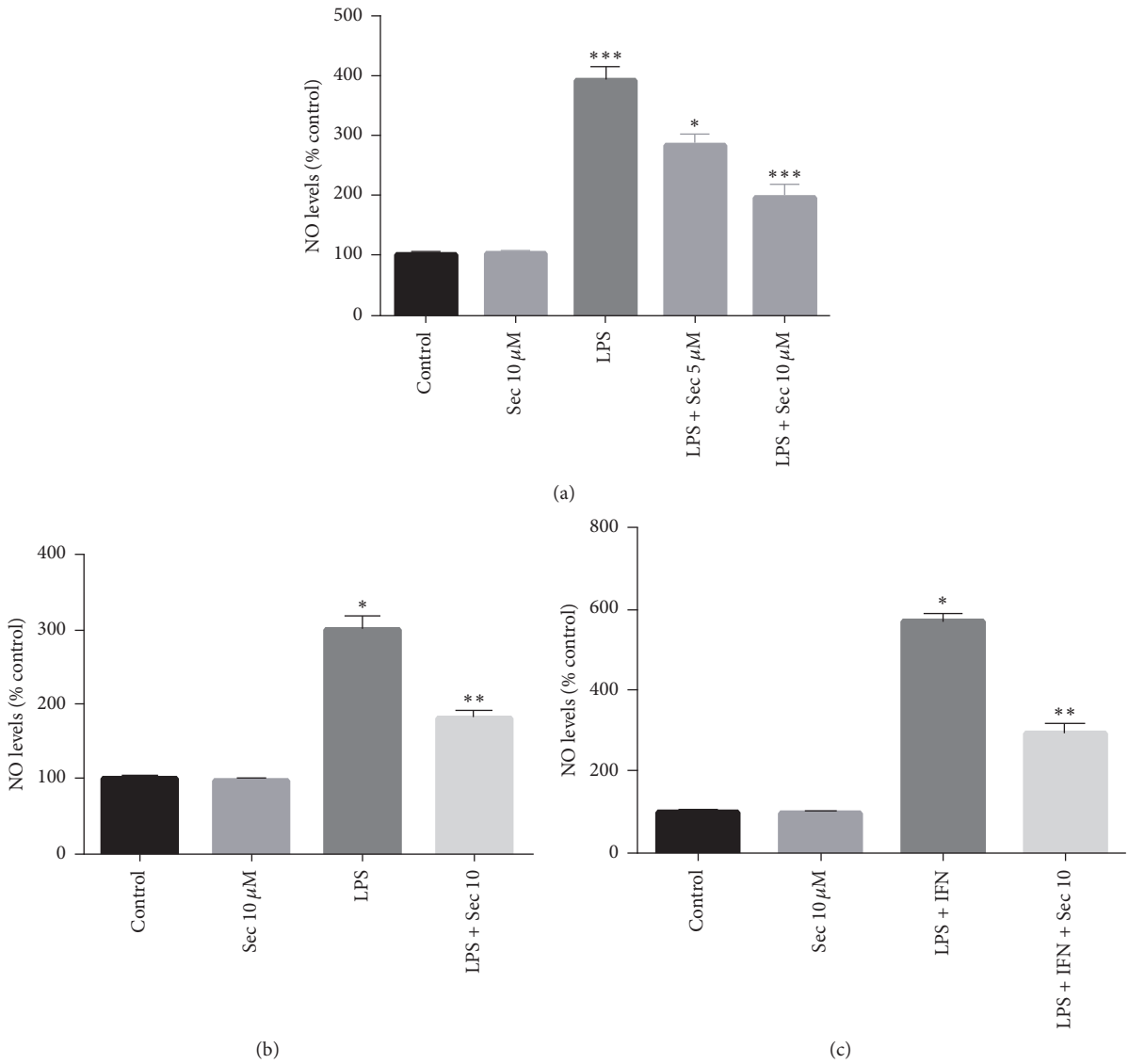

Figure 8: Securinine pretreatment inhibits LPS-induced NO production in RAW 264.7 macrophage cells and in primary astrocyte cultures. RAW 264.7 macrophage cells or primary astrocyte cultures (10×10^4 cells/well in 48-well plates) were incubated with 500 ng/mL of LPS or LPS/IFN-γ (50 unit/mL) in the presence or absence of securinine (10 μM) for 24 h. The amount of nitrite secreted into the supernatant was measured using the Griess Reagent assay ((a) RAW 264.7 cells, $^{***}P < 0.05$ versus control; (b and c) primary astrocyte cultures). The data were expressed as the mean ± SD ($n = 3$) and are representative of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different from cells treated with LPS only.

We next examined the effects of securinine on LPS-induced activation of various MAPKs as well as NF-κB-mediated pathways. It is well established that MAPKs and NF-κB transcription factors play an important role in regulation of expression of proinflammatory cytokines and enzymes including iNOS, TNF-α, IL-1β, IL-6, and COX-2 [30]. Therefore, for a compound to exhibit anti-inflammatory effects this should require an ability to attenuate the activation of these pathways. The signaling mechanisms involved in NF-κB activation have been well established and involve a cascade of cytoplasmic proteins leading ultimately to the translocation of the p65 subunit of NF-κB into the nucleus resulting in transcription of downstream proinflammatory genes [31, 32].

In this study, we show, as expected, phosphorylation of all three MAPKs following LPS treatment of BV2 microglia as well as phosphorylation of NF-κB p65 and increased NF-κB promotor activity. Securinine dose-dependently inhibited LPS-induced NF-κB phosphorylation and activation as well as LPS-induced p38, JNK, and ERK phosphorylation in these cells.

Earlier studies have demonstrated that the expression of iNOS is regulated coordinately by the action of several transcription factors such as NF-κB, AP-1, and STAT1 [33].

Interestingly, our results demonstrate that, in addition to attenuating NF-κB activation, securinine also inhibited IFN-γ-induced NO production, iNOS mRNA expression, and STAT1α activation (Figure 6). These results indicate that securinine regulates proinflammatory gene expression in both NF-κB dependent and independent manner.

Recent studies have emphasized the role of peripheral macrophages infiltration in reactive gliosis following traumatic brain injury (TBI) and also in the case of spinal cord injuries [34]. Furthermore, when the blood-brain barrier is compromised during neurological disorders such as multiple sclerosis and Alzheimer's and Parkinson's diseases, peripheral immune cells including monocytes, neutrophils, T cells, and B cells can enter the brain where they execute distinct cell-mediated effects [35]. Our studies with macrophages and astrocytes further demonstrate that securinine not only inhibits microglial activation but also inhibits peripheral macrophages and astrocytes indicating the broad anti-inflammatory action of securinine.

A recent study reported that securinine can reduce the glial inflammatory responses induced by beta-amyloid protein, improving both cognitive deficits and neurodegeneration in beta AP (25–35)-treated rats [36] Although the study suggested that securinine was neuroprotective in this model, the exact mechanisms involved in this process were not explored. In the present study, cultured media from LPS/securinine-treated microglia provided almost complete dopaminergic neuroprotection compared with CM from LPS-stimulated microglia. Taken in total, our results suggest that securinine offers neuroprotective effects via reducing abnormal production of proinflammatory mediators. Although CM experiments utilizing LPS-stimulated BV2 microglia with primary mesencephalic cultures may not completely mimic the in vivo situation, it likely, at least in part, reflects the pathological condition under which activated microglia can influence the survival of neighboring neurons in the living brain. Further studies are, however, required to validate mechanisms underlying the neuroprotective property of securinine in animal models of inflammation-mediated neurodegenerative diseases including PD.

5. Conclusion

In summary, our results demonstrate that the neuroprotective properties of securinine may be due to inhibition of glial activation and subsequent generation of proinflammatory factors. Mechanistically, this appears to involve inhibition of the p38 MAP kinase-NF-κB pathway resulting in reduced expression of proinflammatory genes and release of corresponding gene products.

Conflicts of Interest

All authors declare that there are no conflicts of interest.

Acknowledgments

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References

[1] V. H. Perry and C. Holmes, "Microglial priming in neurodegenerative disease," *Nature Reviews Neurology*, vol. 10, no. 4, pp. 217–224, 2014.

[2] L. Minghetti, "Role of inflammation in neurodegenerative diseases," *Current Opinion in Neurology*, vol. 18, no. 3, pp. 315–321, 2005.

[3] C. K. Glass, K. Saijo, B. Winner, M. C. Marchetto, and F. H. Gage, "Mechanisms underlying inflammation in neurodegeneration," *Cell*, vol. 140, no. 6, pp. 918–934, 2010.

[4] S. T. Dheen, C. Kaur, and E.-A. Ling, "Microglial activation and its implications in the brain diseases," *Current Medicinal Chemistry*, vol. 14, no. 11, pp. 1189–1197, 2007.

[5] M. E. Lull and M. L. Block, "Microglial activation and chronic neurodegeneration," *Neurotherapeutics*, vol. 7, no. 4, pp. 354–365, 2010.

[6] H. Li, F. Ma, M. Hu et al., "Polysaccharides from medicinal herbs as potential therapeutics for aging and age-related neurodegeneration," *Rejuvenation Research*, vol. 17, no. 2, pp. 201–204, 2014.

[7] A. P. Kulkarni, L. A. Kella Way, and G. J. Kotwal, "Herbal complement inhibitors in the treatment of neuroinflammation: future strategy for neuroprotection," *Annals of the New York Academy of Sciences*, vol. 1056, pp. 413–429, 2005.

[8] L.-W. Chen, Y.-Q. Wang, L.-C. Wei, M. Shi, and Y.-S. Chan, "Chinese herbs and herbal extracts for neuroprotection of dopaminergic neurons and potential therapeutic treatment of Parkinson's disease," *CNS and Neurological Disorders—Drug Targets*, vol. 6, no. 4, pp. 273–281, 2007.

[9] A. K. Jäger and L. Saaby, "Flavonoids and the CNS," *Molecules*, vol. 16, no. 2, pp. 1471–1485, 2011.

[10] M. Shimazawa, S. Chikamatsu, N. Morimoto, S. Mishima, H. Nagai, and H. Hara, "Neuroprotection by Brazilian green propolis against in vitro and in vivo ischemic neuronal damage," *Evidence-Based Complementary and Alternative Medicine*, vol. 2, no. 2, pp. 201–207, 2005.

[11] E. Chirkin, W. Atkatlian, and F.-H. Porée, "The securinega alkaloids," *Alkaloids: Chemistry and Biology*, vol. 74, pp. 1–120, 2015.

[12] J. A. Beutler, E. W. Karbon, A. N. Brubaker, R. Malik, D. R. Curtis, and S. J. Enna, "Securinine alkaloids: a new class of GABA receptor antagonist," *Brain Research*, vol. 330, no. 1, pp. 135–140, 1985.

[13] G. R. Buravtseva, "Result of application of securinine in acute poliomyelitis," *Farmakologiia I Toksikologiia*, vol. 21, no. 5, pp. 7–12, 1958.

[14] R. Copperman, G. Copperman, and A. Der Marderosian, "From Asia securinine—a central nervous stimulant is used in treatment of amytrophic lateral sclerosis," *Pennsylvania Medicine*, vol. 76, no. 1, pp. 36–41, 1973.

[15] K. Lubick, M. Radke, and M. Jutila, "Securinine, a GABAa receptor antagonist, enhances macrophage clearance of phase II C. burnetii: comparison with TLR agonists," *Journal of Leukocyte Biology*, vol. 82, no. 5, pp. 1062–1069, 2007.

[16] M. Shipman, K. Lubick, D. Fouchard et al., "Proteomic and systems biology analysis of monocytes exposed to securinine,

a $GABA_A$ receptor antagonist and immune adjuvant," *PLOS ONE*, vol. 7, no. 9, Article ID e41278, 2012.

[17] S. Rana, K. Gupta, J. Gomez et al., "Securinine induces p73-dependent apoptosis preferentially in p53-deficient colon cancer cells," *FASEB Journal*, vol. 24, no. 6, pp. 2126–2134, 2010.

[18] T. T. Tamashiro, C. L. Dalgard, and K. R. Byrnes, "Primary microglia isolation from mixed glial cell cultures of neonatal rat brain tissue," *Journal of Visualized Experiments*, no. 66, Article ID e3814, 2012.

[19] S. Rajagopalan, A. Rane, S. J. Chinta, and J. K. Andersen, "Regulation of ATP13A2 via PHD2-HIF1α signaling is critical for cellular iron homeostasis: implications for Parkinson's disease," *Journal of Neuroscience*, vol. 36, no. 4, pp. 1086–1095, 2016.

[20] S. J. Chinta, S. Rajagopalan, A. Ganesan, and J. K. Andersen, "A possible novel anti-inflammatory mechanism for the pharmacological prolyl hydroxylase inhibitor 3,4-dihydroxybenzoate: implications for use as a therapeutic for Parkinson's disease," *Parkinson's Disease*, vol. 2012, Article ID 364684, 12 pages, 2012.

[21] S. J. Chinta, A. Ganesan, P. Reis-Rodrigues, G. J. Lithgow, and J. K. Andersen, "Anti-inflammatory role of the isoflavone diadzein in lipopolysaccharide-stimulated microglia: implications for parkinson's disease," *Neurotoxicity Research*, vol. 23, no. 2, pp. 145–153, 2013.

[22] C.-Q. Li, L. J. Trudel, and G. N. Wogan, "Nitric oxide-induced genotoxicity, mitochondrial damage, and apoptosis in human lymphoblastoid cells expressing wild-type and mutant p53," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 16, pp. 10364–10369, 2002.

[23] J. Blanchette, M. Jaramillo, and M. Olivier, "Signalling events involved in interferon-γ-inducible macrophage nitric oxide generation," *Immunology*, vol. 108, no. 4, pp. 513–522, 2003.

[24] S. Wang, H. Wang, H. Guo, L. Kang, X. Gao, and L. Hu, "Neuroprotection of Scutellarin is mediated by inhibition of microglial inflammatory activation," *Neuroscience*, vol. 185, pp. 150–160, 2011.

[25] R. M. Ransohoff and V. H. Perry, "Microglial physiology: unique stimuli, specialized responses," *Annual Review of Immunology*, vol. 27, pp. 119–145, 2009.

[26] M. L. Block, L. Zecca, and J.-S. Hong, "Microglia-mediated neurotoxicity: uncovering the molecular mechanisms," *Nature Reviews Neuroscience*, vol. 8, no. 1, pp. 57–69, 2007.

[27] E. Auriel, K. Regev, and A. D. Korczyn, "Nonsteroidal anti-inflammatory drugs exposure and the central nervous system," *Handbook of Clinical Neurology*, vol. 119, pp. 577–584, 2014.

[28] D. K. Choi, S. Koppula, and K. Suk, "Inhibitors of microglial neurotoxicity: focus on natural products," *Molecules*, vol. 16, no. 2, pp. 1021–1043, 2011.

[29] J. E. Yuste, E. Tarragon, C. M. Campuzano, and F. Ros-Bernal, "Implications of glial nitric oxide in neurodegenerative diseases," *Frontiers in Cellular Neuroscience*, vol. 9, article no. 322, 2015.

[30] S. Akira, K. Takeda, and T. Kaisho, "Toll-like receptors: Critical proteins linking innate and acquired immunity," *Nature Immunology*, vol. 2, no. 8, pp. 675–680, 2001.

[31] P. Viatour, M.-P. Merville, V. Bours, and A. Chariot, "Phosphorylation of NF-κB and IκB proteins: implications in cancer and inflammation," *Trends in Biochemical Sciences*, vol. 30, no. 1, pp. 43–52, 2005.

[32] D. Liu, Z. Wang, S. Liu, F. Wang, S. Zhao, and A. Hao, "Anti-inflammatory effects of fluoxetine in lipopolysaccharide(LPS)-stimulated microglial cells," *Neuropharmacology*, vol. 61, no. 4, pp. 592–599, 2011.

[33] H. Kleinert, A. Pautz, K. Linker, and P. M. Schwarz, "Regulation of the expression of inducible nitric oxide synthase," *European Journal of Pharmacology*, vol. 500, no. 1-3, pp. 255–266, 2004.

[34] M. Das, S. Mohapatra, and S. S. Mohapatra, "New perspectives on central and peripheral immune responses to acute traumatic brain injury," *Journal of Neuroinflammation*, vol. 9, article 236, 2012.

[35] M. Prinz and J. Priller, "The role of peripheral immune cells in the CNS in steady state and disease," *Nature Neuroscience*, vol. 20, no. 2, pp. 136–144, 2017.

[36] X. Lin and Z. Jun-Tian, "Neuroprotection by D-securinine against neurotoxicity induced by beta-amyloid (25–35)," *Neurological Research*, vol. 26, no. 7, pp. 792–796, 2004.

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Review Article

Unconventional Role of Caspase-6 in Spinal Microglia Activation and Chronic Pain

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Chronic pain affects ~20% of the worldwide population. The clinical management of chronic pain is mostly palliative and results in limited success. Current treatments mostly target the symptoms or neuronal signaling of chronic pain. It has been increasingly recognized that glial cells, such as microglia, and inflammatory signaling play a major role in the pathogenesis of chronic pain. Caspases (CASPs) are a family of protease enzymes involved in apoptosis and inflammation. They are pivotal components in a variety of neurological diseases. However, little is known about the role of CASPs in microglial modulation as to chronic pain. In particular, our recent studies have shown that CASP6 regulates chronic pain via microglial inflammatory signaling. Inhibition of microglia and CASP signaling might provide a new strategy for the prevention and treatment of chronic pain.

1. Introduction

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. Acute pain is transient and serves as a warning of disease or a threat to the body. In contrast, chronic pain is a persistent and debilitating condition for which there are few treatment options. Chronic pain conditions include arthritis-induced pain, cancer pain, chemotherapy-induced pain, diabetic pain, migraine, fibromyalgia, and inflammatory and neuropathic pain [1]. In this review, we will mostly present studies involving animal models of inflammatory (e.g., injection of proinflammatory solutions such as carrageenan and complete Freund's adjuvant) and neuropathic pain (e.g., peripheral nerve injury such as spared nerve injury or chronic constriction injury). Previous reviews have been published with detailed descriptions and limitations of using these animal models to study chronic pain [2–5].

Inflammatory pain and neuropathic pain are characterized by spontaneous and evoked pain. Typical evoked pains include hyperalgesia (increased response to painful stimuli) and allodynia (painful response to normally innocuous stimuli). In particular, mechanical or tactile allodynia is probably the most commonly observed symptom in inflammatory and neuropathic pain animal models. Two major neuronal mechanisms underlie this symptom: central sensitization and disinhibition [6]. Central sensitization denotes a state of hyperexcitability of the neurons of the dorsal horn such that their responsiveness to synaptic inputs is increased and involves the modulation of NMDA and AMPA receptors in spinal neurons. The spinal injection of NMDA directly activates dorsal horn NMDA receptors and results in mechanical allodynia [7]. Disinhibition is characterized by a reduction in the effectiveness of the spinal inhibitory GABA and glycine neurons. Pharmacological blockade of GABA or glycine-mediated spinal inhibition also produces mechanical allodynia [8]. The balance between excitatory and inhibitory influences on spinal neuronal circuits plays a crucial role in maintaining physiological pain response. Inflammation or nerve injury leads to an increase in excitation and/or decrease in inhibition resulting in augmented neuronal excitability, which can manifest as chronic pain.

Current treatments of chronic pain include antidepressants, anticonvulsants, sodium channel blockers, NMDA antagonists, and opioids. However, these drugs only target neuronal pathways or symptoms and are limited by their

side effects. For instance, opioids are often accompanied by side effects such as respiratory depression, sedation, nausea, vomiting, constipation, dependence, tolerance, and addiction [9]. Therefore, there is an urgent need for new therapeutic targets. Recently, several studies have highlighted the role of nonneuronal mechanisms, such as immune and glial regulation, in chronic pain. Indeed, it is now widely accepted to consider chronic pain as a neuroimmune disease [10–13]. In particular, nerve injury induces significant activation of glial cells in the spinal cord, and the activated glial cells contribute to central sensitization and disinhibition via proinflammatory mediators [14]. Inhibitors of glial cells are able to attenuate chronic pain [15, 16] and may offer new therapeutic avenues.

Microglia are prominent glial cells in the spinal cord and contribute to chronic pain [17, 18]. As of this writing (29 July 2016), a PubMed search for "Microglia Chronic Pain" retrieves 509 articles, of which ~20% were published within the preceding 12 months. Clearly, microglial cells in chronic pain are a hot topic and fast growing area of research. Naturally, in such diverse and rapidly developing research, we cannot possibly cover all of the work that has been carried over the last two decades and we certainly expect additional progress will have been made by the time this review is published. We apologize to authors whose work we have not discussed.

In this review, we summarize the major signaling pathways involved in microglial activation and chronic pain with an emphasis on caspases (CASPs). In particular, potential microglial mechanisms and therapeutic approaches for the modulation of CASP6 in chronic pain are also described.

2. Microglia Signaling and Chronic Pain

Microglia are innate immune cells in the central nervous system constantly scavenging their environment using their ramified branches for the maintenance of hemostasis [18]. In animal models with nerve injury, microglia proliferate, change shapes (e.g., larger cell bodies and fewer ramifications), and increase the expression of microglial markers such as CD11b, ionized calcium-binding adapter molecule 1 (IBA1), and CX3C chemokine receptor 1 (CX3CR1) (Figure 1(a)). However, changes in the morphology and expression of these markers are not indicative of microglial activation and participation to pain symptoms. Notably, microglia proliferation and IBA1 expression increased are very limited after tissue inflammation or bone cancer, but pain in these animal models is still efficiently attenuated by the inhibition of microglial signaling [19, 20]. Several studies have reported the microglial phosphorylation of p38 (p-p38) in several animal models of pain involving nerve injury, spinal cord injury, formalin-induced acute inflammatory pain, postoperative pain, and chronic exposure to opioids [21–27]. Furthermore, these studies have also shown that microglial p-p38 leads to the production of proinflammatory cytokines that can alter pain pathways and p38 inhibition significantly attenuates pain. Therefore, the spinal phosphorylation of p38 might represent a better marker for microglial activation and participation in pain compared with classical microglial markers such as IBA1.

Considerable effort has been devoted to understanding the mechanisms by which microglial cells are activated and how they contribute to chronic pain. In recent years, many neuron-microglia pathways have emerged in chronic pain, including the chemokine receptor signaling (e.g., CX3CR1) [28], toll-like receptor signaling (e.g., TLR2 and TLR4) [29, 30], purinergic receptor signaling (e.g., P2Y12R and P2X4R) [31, 32], and tyrosine-protein kinase receptor signaling (e.g., CSF1R) [33] (Figure 1(b)).

Adenosine triphosphate (ATP), chemokines (CX3CL1 and INFγ), and proteases (MMP9 and CASP6) are released from the spinal projections of the primary sensory neurons following peripheral tissue or nerve injury. ATP, chemokines, and proteases induce signaling via ligand-gated ion channels and G protein-coupled receptors (GPCRs). These mediators are not unique to primary sensory neurons and may be secondary to microglial activation. For instance, CX3CL1 release requires the production of cathepsin S (CatS, a lysosomal protein), which is induced by the stimulation of the microglial receptors P2X7R or CSF1R [33–35]. Stimulation of the P2X7R also elicits the production of IL-1β and its maturation via CatS and CASP1 [36], which can further increase microglia activation via the interleukin receptor IL1R [37] and it is also a key contributor in the hyperexcitability of the nociceptive dorsal horn neurons [14]. IL-1β and IL-18 can also be processed by microglial CatB and CASP1/11 in inflammatory pain [38], suggesting a major role for the microglial Cat and CASP signaling in chronic pain.

Probably the most studied and well-characterized microglial signaling pathway is the TLR4 signaling pathway. Toll-like receptors are known to regulate innate immunity and respond to diverse invading pathogens and damage-associated molecular patterns. For example, TLR4 associates lipopolysaccharide (LPS) from the walls of Gram-negative bacteria. TLR4 is predominantly expressed in microglia and spinal injection of LPS-induced pain behaviors [39]. Increased TLR4 expression correlates with the development of pain after nerve injury and its inhibition significantly attenuates nerve injury-induced pain [40–42]. Notably, TLR4- and TLR2-deficient mice demonstrate decreased microglial reactivity and attenuated pain after nerve injury [30, 40]. TLRs have also been proposed to sense endogenous injury signals including fibronectin and heat shock proteins. After nerve injury, HSP90 is upregulated in the spinal cord and its inhibition attenuates TLR4 mediated pain [43].

TLR signaling, such as most of the aforementioned signaling, converges in the phosphorylation/activation of the mitogen-activated protein kinases (MAPK), ERK, and p38 [15]. In particular, p38 is persistently activated exclusively in microglia, whereas ERK is only activated in microglia in the first week after nerve injury [44]. Microglial MAPK phosphorylation usually results in the rapid activation of signal-dependent transcription factors, including members of the nuclear factor-kB (NF-kB) and interferon regulatory factor (IRF) families [23, 45, 46]. These factors can then work in combinatorial manner to rapidly express hundreds of

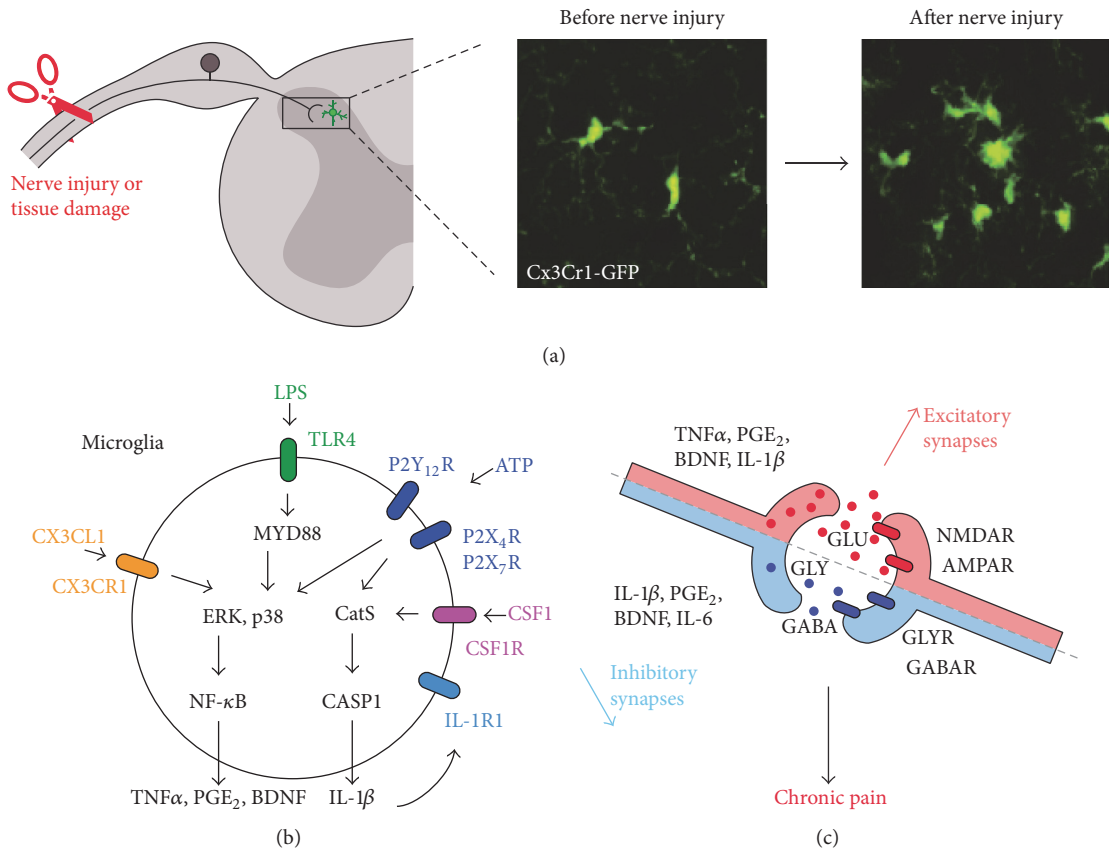

Figure 1: Microglial reactivity to nerve injury and signaling in chronic pain. (a) Nerve injury induces CX3CR1 expression in spinal microglia of mice expressing GFP under the control of CX3CR1 promoter. (b) Tissue and nerve injury results in the release of ATP, CX3CL1, and CSF1 leading to the activation of microglia shown by the phosphorylation of p38 and pERK and the production of prostaglandins, cytokines, and growth factors (e.g., TNFα and BDNF). Microglial cells also produce the cytokine IL-1b via the cathepsin/caspase-1 pathway, which can be further activated by binding to IL-1R1. (c) Microglial cytokines, prostaglandins, and growth factors modulate excitatory (glutamatergic synapses—GLU/NMDA and AMPA receptors) and inhibitory (GABAergic and glycinergic synapses—GABA/GABAR and GLY/GLYR) synaptic transmission. For instance, BDNF produces disinhibition of GABAergic lamina I neurons leading to chronic pain.

genes known to increase pain sensitivity, including the proinflammatory cytokines TNFα, inducible nitric oxide synthase (iNOS), and brain-derived neurotrophic factor (BDNF) as well as the purinergic receptors P2X4R and P2Y12R [19, 47, 48]. In particular, the microglial production of proinflammatory cytokines and neurotrophic factors can further recruit microglia, activate surrounding astrocytes, and promote the sensitization of central nervous system nociceptive circuits (Figure 1(c)).

Proinflammatory cytokines such as TNFα, prostaglandin PGE_2, and IL-1β can increase the excitatory synaptic transmission by both pre- and postmechanisms of the excitatory synapses by enhancing the release of glutamate and increasing the trafficking and modulating AMPA and NMDA receptors. In parallel these cytokines, including IL-1β, PGE_2, and IL-6, can also reduce or promote the loss of the inhibitory synaptic transmission (i.e., disinhibition). Furthermore, inhibitory synaptic transmission can also be reversed. Microglial release of growth factor BDNF downregulates the potassium-chloride cotransporter KCC2 in lamina I GABA positive neurons leading to the accumulation of the intracellular chloride, such as these inhibitory neurons changing phenotype and becoming excitatory [49]. Previous reviews have been published and are available with further details about these mechanisms [15, 18, 50].

3. Caspase Signaling and Chronic Pain

CASPs are cysteinyl-aspartate-specific proteases and best known for triggering apoptotic cell death [51]. CASPs are generally present in cells as inactive precursor enzymes with little or no proteases activity. Two major pathways regulate the activation of CASPs. The extrinsic pathway is elicited by the biding of extracellular death ligands (such as TNFα) to transmembrane death receptors, whereas the intrinsic pathways are induced by cell stress (such as oxidative stress) or damage. Both pathways can lead to apoptosis by the activation of the initiator CASP2, 8, 9, and 10 and the executioner CASP3, 6, and 7, or neuroinflammation via CASP1, 4, 5, and 11 [52].

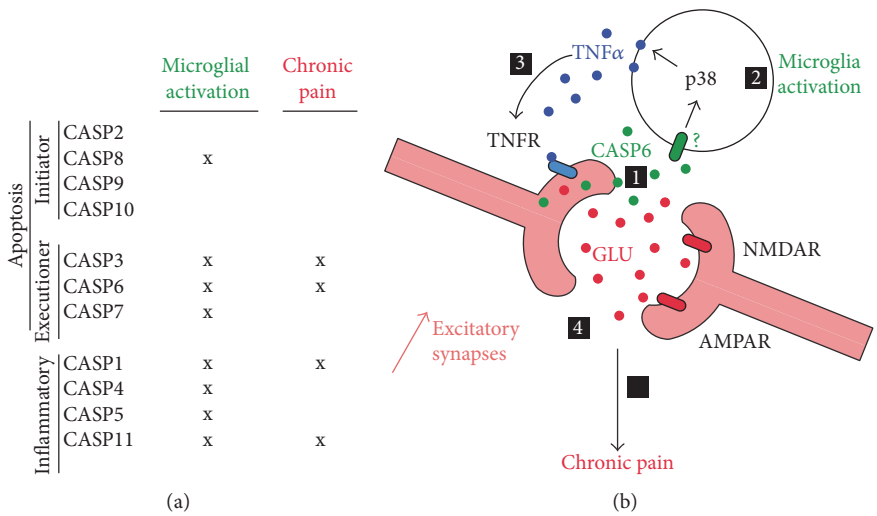

FIGURE 2: Caspases in microglial activation and chronic pain and schematic representation of the CASP6 neuroinflammatory mechanism. (a) Regulation of apoptotic and inflammatory caspases in microglia activation and chronic pain. (b) Tissue and nerve injury leads to the release of CASP6 from the central afferents of primary sensory neurons [1]; this leads to the microglial activation shown by the phosphorylation of p38 and production of TNFα [2]; consequently TNFα binds to the presynaptic TNFR increasing the release of glutamate and the excitatory synaptic transmission [3], which ultimately results in chronic pain [4].

Several chronic pain syndromes are associated with increase in TNFα and oxidative stress in the both peripheral and central nervous systems, which may lead to CASP activation and neuroinflammation [11]. Indeed several CASPs that regulate microglial activation also participate in chronic pain [19, 36, 38, 53–57] (Figure 2(a)). Although peripheral inhibition of CASP1, 2, 3, 8, and 9 significantly attenuated inflammatory and neuropathic pain behaviors [56], the peripheral mechanisms of CASPs remain elusive. Here, we focus on the central mechanisms of CASP signaling in chronic pain and microglial activation for which we have a better understanding. In chronic pain conditions, CASP activation in the spinal cord leads to both apoptosis and neuroinflammatory responses (e.g., microglia activation).

After peripheral nerve injury, apoptotic cells are observed in the dorsal horn of the spinal cord [57]. This apoptosis is driven by the activation of CASP3 in the inhibitory GABAergic interneurons of the superficial dorsal horn and causes the loss of these neurons, the decrease of the spinal inhibition, and the appearance of neuropathic pain. Spinal injection of pan caspase inhibitor Z-VAD-FMK to block the CASP3 activation prevents the number of apoptotic cells and decrease of spinal inhibition and alleviates neuropathic pain. Interestingly, the alleviation of neuropathic pain by Z-VAD-FMK outlasts its discontinuation, suggesting that degeneration of inhibitory interneurons contributes to the chronicity of pain. However, the neuronal activation of CASP3 and the apoptosis of GABAergic interneurons in animal models of neuropathic pain are controversial [58]. In particular, CASP3 and apoptotic signaling have been shown to occur also in glial cells after the same peripheral injury [58].

Little is known about the potential contribution of CASPs to glial cell functions and chronic pain. However, it has been recently reported that intracranial injection of LPS induces microglial activation of CASP3/7 without leading to apoptosis but instead to the release of proinflammatory mediators and neuroinflammation [55]. As further proof of the central role of CASP3/7 in microglial activation, the use of Z-DEVD-FMK (a specific CASP3 inhibitor) significantly reduced the LPS-induced release of proinflammatory mediators by microglial cells. Interestingly, the spinal delivery of same inhibitor Z-DEVD-FMK or CASP3 siRNA attenuates neuropathic pain after peripheral nerve injury [59]. Whether these treatments attenuate neuropathic pain via the inhibition of apoptosis and/or neuroinflammation remains to be investigated.

CASP1 and CASP11 certainly play a critical role in regulating neuroinflammation and are increased in chronic pain conditions [4, 38]. As briefly mentioned above, the activation of CASP1 and CASP11 plays a role in the maturation of proinflammatory cytokines, such as IL-1β and IL-18. The best known activator of CASP1 is the inflammasome, a complex of proteins (such as nucleotide-binding domain leucine-rich repeat containing proteins NLRP1 and NPLR3, NLR family CARD domain-containing protein NLRC4, and the apoptosis-associated speck-like protein containing a CASP recruitment domain or in short ASC) that are aggregated by various inflammatory conditions in immune and glial cells and lead to the CASP1 cleavage [60]. Although several mechanisms can contribute to allodynia after spinal injection of LPS, it has been observed that this treatment enhances CASP1 and ASC secretion providing evidence of the involvement of the inflammasome complex [36]. Furthermore, inhibition of CASP1 by Z-VAD-FMK both prevents IL-1β release and attenuates LPS-induced allodynia. However, how and to which extent the inflammasome complex is activated in microglia and chronic pain is still unclear.

There are several variants of inflammasome complexes, but the best know complex involves the activation of NLRP3 [60]. However, a recent study using NLRP3-deficient mice show no defects in regulating the transcriptional expression of ASC, CASP1, and IL-1β after spinal injections of LPS or after intraplantar formalin injection or after nerve injury [61]. In a peripheral inflammatory pain model, NLRC4 inflammasome complex but not NLRP3 was implicated in IL-1β increase in the skin and behavioral responses [62]. Similarly, NLRC4 and not NLRP3 may be involved in processing spinal IL-1β after LPS injection or nerve injury. In contrast, microglial NLRP3/CASP1 seems to be involved in chronic pain arising from the combination of nerve injury and prolonged exposure to opioids [63], suggesting the different pathways and contributions of CASP1 and inflammasome signaling to various neuroinflammatory and chronic pain conditions.

4. Caspase-6 in Neuron-Microglia Signaling and Chronic Pain

CASP6 is widely expressed in the brain and in the peripheral nervous system. CASP6 is well known as an executioner CASP and can cleave nuclear structural proteins (e.g., lamin) leading to apoptosis and neurodegeneration. CASP6 is involved in neurodegenerative diseases such as Huntington and Alzheimer diseases [64]. Our recent research suggests that CASP6 has an important and nonapoptotic role in the development of chronic pain in both inflammatory and neuropathic animal models (Berta CASP6).

Several lines of evidence suggest a unique role for this CASP in microglia activation and pain control: (1) CASP6 is highly expressed in the neuronal axons of primary sensory neurons that terminate in the superficial dorsal horn (laminae I-II) of the spinal cord [19, 65], (2) CASP6 is coexpressed with the calcitonin gene related peptide (CGRP), a well-known peptide involved in inflammation and pain [66], (3) CASP6 surrounds microglial cell bodies and processes, and (4) CASP6 levels in the cerebrospinal fluid significantly increase after inflammation [19]. To define the specific role of CASP6 in pain, we employed in vivo and in vitro approaches.

In vivo results in various animal models are very consistent, showing that the CASP6 inhibition by Z-VEID-FMK (a specific CASP6 inhibitor) or deletion can attenuate inflammatory and neuropathic pain (Berta 2014, 2016). Notably, the intrathecal injection of a specific antibody against the active form of CASP6 was effective in blocking formalin-induced pain. Because antibodies with few exception do not penetrate cells [67], this result further proved the presence and role of extracellular CASP6 in pain. In line with an extracellular action of CASP6, intrathecal injection of recombinant CASP6 (rCASP6) is sufficient to induce pain symptoms, such as mechanical allodynia. Importantly, spinal rCASP6 treatment did not produce signs of axonal degeneration, as we found no loss of peptidergic axons (CGRP+) or nonpeptidergic axons labeled with IB4 (an isolectin glycoprotein) in the dorsal horn. However pretreatment with minocycline, a microglial inhibitor that has been shown to attenuate pain [68], significantly reduced the rCASP6-evoked mechanical allodynia, which suggested an action of CASP6 on microglia.

In vitro experiments have demonstrated that stimulation of primary microglial culture with rCASP6 elicited a significant and dose-dependent release of TNF-α, but minimal or no release of other proinflammatory cytokines, including IL-6 and IL-1β [19]. The treatment of microglia with an inhibitor of p38 suppressed the rCASP6-induced TNF-α release, suggesting an important role of p38 in CASP6-triggered TNF-α release. Impairment of spinal TNF-α was also observed in mice with CASP6 deficiency compared to their wild-type controls after tissue injury and damage [19, 69].

Mechanistically, rCASP6 was also sufficient to enhance spontaneous excitatory postsynaptic currents (sEPSCs frequency) in spinal cord slices via microglial and TNF-α signaling [19]. Finally, rCASP6-activated microglial culture medium increased sEPSC frequency in spinal cord slices via TNF-α. Together, these data suggest that CASP6 released from axonal terminals regulates microglial TNF-α secretion, synaptic plasticity, and chronic pain (Figure 2(b)).

5. Conclusions and Future Directions

Millions of people suffer from chronic pain, which is now widely recognized as a neuroimmune disease. However, current treatments are limited to symptomatic palliation mostly focusing on blocking neurotransmission. Targeting neuroinflammation and, in particular, microglial signaling may offer new therapeutic strategies for a better treatment of chronic pain [11].

Spinal microglia contribute to the generation of inflammatory and neuropathic pain, postoperative pain, and opioid-induced tolerance in rodents. Although human studies have established glial activation in chronic pain states using functional magnetic resonance imaging [70] and in postmortem spinal cords [71], clinical trial with inhibitors targeting microglia, such as minocycline (a tetracycline antibiotic) and propentofylline (a CNS glial modulator), has shown no or limited promise in chronic pain [15]. These generic microglia inhibitors are undesirable, since it is well documented that microglia in neuroinflammatory diseases can have multiple phenotypes with pathological and protective functions [18]. Furthermore, nonpathological microglia have an important role within the immune system and are pivotal in maintaining and restoring physiological homeostasis [72]. Therefore, we should target specific pathological microglial signaling.

Among several microglial signaling, CASPs are promising targets to reduce neuroinflammation and chronic pain. Following tissue and nerve injury, CASPs play an important role not only in apoptosis, but also in mounting neuroinflammatory responses including microglia activation. There is increasing evidence that CASP1, CASP6, and CASP11 activation is involved in microglia activation and the maturation of proinflammatory cytokines in inflammatory and neuropathic pain conditions [19, 38, 53, 63]. Inhibition of these caspases has shown extraordinary promise in various disease models including painful conditions. For example, a blockade of

CASP6 by the peptide Z-VEID-FMK reduces inflammation and neuropathic-induced mechanical allodynia in mice models [19]. Unfortunately, the vast majority of CASP inhibitors are peptides that often lack selectivity [73].

Our data suggest that CASP6 released from axonal terminals regulates microglial TNF-α secretion, synaptic plasticity, and chronic pain (Figure 2(b)). Because of this unique presence of CASP6 in the extracellular milieu, we hypothesized that CASP6 could be targeted by antibodies. Indeed, formalin-induced second-phase pain was suppressed by spinal injection of a neutralizing antibody against the activated form of CASP6 [19]. The use of this antibody can confer many therapeutic advantages over the peptide inhibitor, including selectivity and accessibility. Furthermore, new technologies are available to enable antibodies to cross the blood-brain barrier [74].

It is worth noting that CASP6 inhibition attenuates mechanical allodynia in male, but not in female, mice [69]. Although microglial proliferation occurs similarly in male and female rodents, it has been reported that ablation of microglia and inhibition of microglial signaling attenuate inflammatory and neuropathic pain only in males, but not females [75]. These findings highlight the importance of including both sexes in basic research and they should be considered for future human trials and clinical practice. However, microglia may play different roles in different phases of chronic pain development as well as in different chronic pain conditions. Notably, it has been shown that inhibition of microglial signaling in animal models of bone cancer pain and spinal cord injury effectively attenuated chronic pain also in females [30, 76].

In conclusion, given the pace of recent advances in our appreciation of chronic pain as a neuroimmune disease and in our understanding of the reciprocal signaling between neurons and microglia (i.e. CASP6 signaling), it is at last realistic to expect that new and improved treatments will become available for a more successful management of clinical chronic pain.

Disclosure

Temugin Berta and Chul-Kyu Park are cosenior authors.

Competing Interests

The authors declare no conflict of interests.

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References

[1] R.-D. Treede, W. Rief, A. Barke et al., "A classification of chronic pain for ICD-11," *Pain*, vol. 156, no. 6, pp. 1003–1007, 2015.

[2] J. S. Mogil, "Animal models of pain: progress and challenges," *Nature Reviews Neuroscience*, vol. 10, no. 4, pp. 283–294, 2009.

[3] A. S. Jaggi, V. Jain, and N. Singh, "Animal models of neuropathic pain," *Fundamental and Clinical Pharmacology*, vol. 25, no. 1, pp. 1–28, 2011.

[4] J. M. Boyce-Rustay, P. Honore, and M. F. Jarvis, "Animal models of acute and chronic inflammatory and nociceptive pain," *Methods in Molecular Biology*, vol. 617, pp. 41–55, 2010.

[5] D. Le Bars, M. Gozariu, and S. W. Cadden, "Animal models of nociception," *Pharmacological Reviews*, vol. 53, no. 4, pp. 597–652, 2001.

[6] C. A. von Hehn, R. Baron, and C. J. Woolf, "Deconstructing the neuropathic pain phenotype to reveal neural mechanisms," *Neuron*, vol. 73, no. 4, pp. 638–652, 2012.

[7] D. W. Gil, C. V. Cheevers, and J. E. Donello, "Transient allodynia pain models in mice for early assessment of analgesic activity," *British Journal of Pharmacology*, vol. 153, no. 4, pp. 769–774, 2008.

[8] L. Sivilotti and C. J. Woolf, "The contribution of GABAA and glycine receptors to central sensitization: disinhibition and touch-evoked allodynia in the spinal cord," *Journal of Neurophysiology*, vol. 72, no. 1, pp. 169–179, 1994.

[9] F. Porreca and M. H. Ossipov, "Nausea and vomiting side effects with opioid analgesics during treatment of chronic pain: mechanisms, implications, and management options," *Pain Medicine*, vol. 10, no. 4, pp. 654–662, 2009.

[10] M. Calvo, J. M. Dawes, and D. L. H. Bennett, "The role of the immune system in the generation of neuropathic pain," *The Lancet Neurology*, vol. 11, no. 7, pp. 629–642, 2012.

[11] R.-R. Ji, Z.-Z. Xu, and Y.-J. Gao, "Emerging targets in neuroinflammation-driven chronic pain," *Nature Reviews Drug Discovery*, vol. 13, no. 7, pp. 533–548, 2014.

[12] R.-D. Gosselin, M. R. Suter, R.-R. Ji, and I. Decosterd, "Glial cells and chronic pain," *Neuroscientist*, vol. 16, no. 5, pp. 519–531, 2010.

[13] P. M. Grace, M. R. Hutchinson, S. F. Maier, and L. R. Watkins, "Pathological pain and the neuroimmune interface," *Nature Reviews Immunology*, vol. 14, no. 4, pp. 217–231, 2014.

[14] Y. Kawasaki, L. Zhang, J.-K. Cheng, and R.-R. Ji, "Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1β, interleukin-6, and tumor necrosis factor-α in regulating synaptic and neuronal activity in the superficial spinal cord," *Journal of Neuroscience*, vol. 28, no. 20, pp. 5189–5194, 2008.

[15] R.-R. Ji, T. Berta, and M. Nedergaard, "Glia and pain: is chronic pain a gliopathy?" *Pain*, vol. 154, supplement 1, pp. S10–S28, 2013.

[16] E. D. Milligan and L. R. Watkins, "Pathological and protective roles of glia in chronic pain," *Nature Reviews Neuroscience*, vol. 10, no. 1, pp. 23–36, 2009.

[17] M. Tsuda, S. Beggs, M. W. Salter, and K. Inoue, "Microglia and intractable chronic pain," *Glia*, vol. 61, no. 1, pp. 55–61, 2013.

[18] S. Taves, T. Berta, G. Chen, and R.-R. Ji, "Microglia and spinal cord synaptic plasticity in persistent pain," *Neural Plasticity*, vol. 2013, Article ID 753656, 10 pages, 2013.

[19] T. Berta, C.-K. Park, Z.-Z. Xu et al., "Extracellular caspase-6 drives murine inflammatory pain via microglial TNF-α secretion," *Journal of Clinical Investigation*, vol. 124, no. 3, pp. 1173–1186, 2014.

[20] P. Honore, S. D. Rogers, M. J. Schwei et al., "Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of neurochemical changes in the spinal cord and

sensory neurons," *Neuroscience*, vol. 98, no. 3, pp. 585–598, 2000.

[21] Y. Cui, Y. Chen, J.-L. Zhi, R.-X. Guo, J.-Q. Feng, and P.-X. Chen, "Activation of p38 mitogen-activated protein kinase in spinal microglia mediates morphine antinociceptive tolerance," *Brain Research*, vol. 1069, no. 1, pp. 235–243, 2006.

[22] B. C. Hains and S. G. Waxman, "Activated microglia contribute to the maintenance of chronic pain after spinal cord injury," *Journal of Neuroscience*, vol. 26, no. 16, pp. 4308–4317, 2006.

[23] R.-R. Ji and M. R. Suter, "p38 MAPK, microglial signaling, and neuropathic pain," *Molecular Pain*, vol. 3, no. 1, article 33, 2007.

[24] Y.-R. Wen, M. R. Suter, R.-R. Ji et al., "Activation of p38 mitogen-activated protein kinase in spinal microglia contributes to incision-induced mechanical allodynia," *Anesthesiology*, vol. 110, no. 1, pp. 155–165, 2009.

[25] C. I. Svensson, M. Marsala, A. Westerlund et al., "Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing," *Journal of Neurochemistry*, vol. 86, no. 6, pp. 1534–1544, 2003.

[26] M. Tsuda, A. Mizokoshi, Y. Shigemoto-Mogami, S. Koizumi, and K. Inoue, "Activation of p38 Mitogen-Activated Protein Kinase in Spinal Hyperactive Microglia Contributes to Pain Hypersensitivity Following Peripheral Nerve Injury," *GLIA*, vol. 45, no. 1, pp. 89–95, 2004.

[27] S.-X. Jin, Z.-Y. Zhuang, C. J. Woolf, and R.-R. Ji, "p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain," *Journal of Neuroscience*, vol. 23, no. 10, pp. 4017–4022, 2003.

[28] A. K. Clark, A. A. Staniland, and M. Malcangio, "Fractalkine/CX3CR1 signalling in chronic pain and inflammation," *Current Pharmaceutical Biotechnology*, vol. 12, no. 10, pp. 1707–1714, 2011.

[29] L. Nicotra, L. C. Loram, L. R. Watkins, and M. R. Hutchinson, "Toll-like receptors in chronic pain," *Experimental Neurology*, vol. 234, no. 2, pp. 316–329, 2012.

[30] D. Kim, A. K. Myung, I.-H. Cho et al., "A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity," *Journal of Biological Chemistry*, vol. 282, no. 20, pp. 14975–14983, 2007.

[31] S. Beggs, T. Trang, and M. W. Salter, "P2X4R + microglia drive neuropathic pain," *Nature Neuroscience*, vol. 15, no. 8, pp. 1068–1073, 2012.

[32] H. Tozaki-Saitoh, M. Tsuda, H. Miyata, K. Ueda, S. Kohsaka, and K. Inoue, "P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury," *Journal of Neuroscience*, vol. 28, no. 19, pp. 4949–4956, 2008.

[33] Z. Guan, J. A. Kuhn, X. Wang et al., "Injured sensory neuron-derived CSF1 induces microglial proliferation and DAP12-dependent pain," *Nature Neuroscience*, vol. 19, no. 1, pp. 94–101, 2015.

[34] P. M. Grace, P. E. Rolan, and M. R. Hutchinson, "Peripheral immune contributions to the maintenance of central glial activation underlying neuropathic pain," *Brain, Behavior, and Immunity*, vol. 25, no. 7, pp. 1322–1332, 2011.

[35] A. K. Clark, R. Wodarski, F. Guida, O. Sasso, and M. Malcangio, "Cathepsin S release from primary cultured microglia is regulated by the P2X7 receptor," *GLIA*, vol. 58, no. 14, pp. 1710–1726, 2010.

[36] A. K. Clark, F. D'Aquisto, C. Gentry, F. Marchand, S. B. McMahon, and M. Malcangio, "Rapid co-release of interleukin 1β and caspase 1 in spinal cord inflammation," *Journal of Neurochemistry*, vol. 99, no. 3, pp. 868–880, 2006.

[37] E. Pinteaux, L. C. Parker, N. J. Rothwell, and G. N. Luheshi, "Expression of interleukin-1 receptors and their role in interleukin-1 actions in murine microglial cells," *Journal of Neurochemistry*, vol. 83, no. 4, pp. 754–763, 2002.

[38] L. Sun, Z. Wu, Y. Hayashi et al., "Microglial cathepsin B contributes to the initiation of peripheral inflammation-induced chronic pain," *Journal of Neuroscience*, vol. 32, no. 33, pp. 11330–11342, 2012.

[39] R. E. Sorge, M. L. LaCroix-Fralish, A. H. Tuttle et al., "Spinal cord toll-like receptor 4 mediates inflammatory and neuropathic hypersensitivity in male but not female mice," *Journal of Neuroscience*, vol. 31, no. 43, pp. 15450–15454, 2011.

[40] F. Y. Tanga, N. Nutile-McMenemy, and J. A. DeLeo, "The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 16, pp. 5856–5861, 2005.

[41] M. R. Hutchinson, Y. Zhang, K. Brown et al., "Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4)," *European Journal of Neuroscience*, vol. 28, no. 1, pp. 20–29, 2008.

[42] I. Bettoni, F. Comelli, C. Rossini et al., "Glial TLR4 receptor as new target to treat neuropathic pain: efficacy of a new receptor antagonist in a model of peripheral nerve injury in mice," *GLIA*, vol. 56, no. 12, pp. 1312–1319, 2008.

[43] M. R. Hutchinson, K. M. Ramos, L. C. Loram et al., "Evidence for a role of heat shock protein-90 in toll like receptor 4 mediated pain enhancement in rats," *Neuroscience*, vol. 164, no. 4, pp. 1821–1832, 2009.

[44] Z.-Y. Zhuang, P. Gerner, C. J. Woolf, and R.-R. Ji, "ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model," *Pain*, vol. 114, no. 1-2, pp. 149–159, 2005.

[45] T. Masuda, S. Iwamoto, R. Yoshinaga et al., "Transcription factor IRF5 drives P2X4R$^+$-reactive microglia gating neuropathic pain," *Nature Communications*, vol. 5, article 3771, 2014.

[46] T. Masuda, M. Tsuda, R. Yoshinaga et al., "IRF8 is a critical transcription factor for transforming microglia into a reactive phenotype," *Cell Reports*, vol. 1, no. 4, pp. 334–340, 2012.

[47] K. Kobayashi, H. Yamanaka, T. Fukuoka, Y. Dai, K. Obata, and K. Noguchi, "P2Y12 receptor upregulation in activated microglia is a gateway of p38 signaling and neuropathic pain," *The Journal of Neuroscience*, vol. 28, no. 11, pp. 2892–2902, 2008.

[48] T. Trang, S. Beggs, X. Wan, and M. W. Salter, "P2X4-receptor-mediated synthesis and release of brain-derived neurotrophic factor in microglia is dependent on calcium and p38-mitogen-activated protein kinase activation," *Journal of Neuroscience*, vol. 29, no. 11, pp. 3518–3528, 2009.

[49] J. A. M. Coull, S. Beggs, D. Boudreau et al., "BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain," *Nature*, vol. 438, no. 7070, pp. 1017–1021, 2005.

[50] T. Trang, S. Beggs, and M. W. Salter, "Brain-derived neurotrophic factor from microglia: a molecular substrate for neuropathic pain," *Neuron Glia Biology*, vol. 7, no. 1, pp. 99–108, 2012.

[51] R. C. Taylor, S. P. Cullen, and S. J. Martin, "Apoptosis: controlled demolition at the cellular level," *Nature Reviews Molecular Cell Biology*, vol. 9, no. 3, pp. 231–241, 2008.

[52] S. M. Man and T.-D. Kanneganti, "Converging roles of caspases in inflammasome activation, cell death and innate immunity," *Nature Reviews Immunology*, vol. 16, no. 1, pp. 7–21, 2016.

[53] D.-Y. Liang, X. Q. Li, W.-W. Li et al., "Caspase-1 modulates incisional sensitization and inflammation," *Anesthesiology*, vol. 113, no. 4, pp. 945–956, 2010.

[54] S. M. Burm, E. A. Zuiderwijk-Sick, A. E. J. T Jong et al., "Inflammasome-induced IL-1β secretion in microglia is characterized by delayed kinetics and is only partially dependent on inflammatory caspases," *Journal of Neuroscience*, vol. 35, no. 2, pp. 678–687, 2015.

[55] M. A. Burguillos, T. Deierborg, E. Kavanagh et al., "Caspase signalling controls microglia activation and neurotoxicity," *Nature*, vol. 472, no. 7343, pp. 319–324, 2011.

[56] E. K. Joseph and J. D. Levine, "Caspase signalling in neuropathic and inflammatory pain in the rat," *European Journal of Neuroscience*, vol. 20, no. 11, pp. 2896–2902, 2004.

[57] J. Scholz, D. C. Broom, D.-H. Youn et al., "Blocking caspase activity prevents transsynaptic neuronal apoptosis and the loss of inhibition in lamina II of the dorsal horn after peripheral nerve injury," *The Journal of Neuroscience*, vol. 25, no. 32, pp. 7317–7323, 2005.

[58] E. Polgár, D. I. Hughes, A. Z. Arham, and A. J. Todd, "Loss of neurons from laminas I-III of the spinal dorsal horn is not required for development of tactile allodynia in the spared nerve injury model of neuropathic pain," *The Journal of Neuroscience*, vol. 25, no. 28, pp. 6658–6666, 2005.

[59] F. Wu, X. Miao, J. Chen et al., "Down-regulation of GAP-43 by inhibition of caspases-3 in a rat model of neuropathic pain," *International Journal of Clinical and Experimental Pathology*, vol. 5, no. 9, pp. 948–955, 2012.

[60] H. Guo, J. B. Callaway, and J. P.-Y. Ting, "Inflammasomes: mechanism of action, role in disease, and therapeutics," *Nature Medicine*, vol. 21, no. 7, pp. 677–687, 2015.

[61] V. Curto-Reyes, G. Kirschmann, M. Pertin, S. K. Drexler, I. Decosterd, and M. R. Suter, "Neuropathic pain phenotype does not involve the NLRP3 inflammasome and its end product interleukin-1β in the mice spared nerve injury model," *PLoS ONE*, vol. 10, no. 7, Article ID e0133707, 2015.

[62] A. H. Lopes, J. Talbot, R. L. Silva et al., "Peripheral NLCR4 inflammasome participates in the genesis of acute inflammatory pain," *Pain*, vol. 156, no. 3, pp. 451–459, 2015.

[63] P. M. Grace, K. A. Strand, E. L. Galer et al., "Morphine paradoxically prolongs neuropathic pain in rats by amplifying spinal NLRP3 inflammasome activation," *Proceedings of the National Academy of Sciences*, vol. 113, no. 24, pp. E3441–E3450, 2016.

[64] R. K. Graham, D. E. Ehrnhoefer, and M. R. Hayden, "Caspase-6 and neurodegeneration," *Trends in Neurosciences*, vol. 34, no. 12, pp. 646–656, 2011.

[65] A. Nikolaev, T. McLaughlin, D. D. M. O'Leary, and M. Tessier-Lavigne, "APP binds DR6 to trigger axon pruning and neuron death via distinct caspases," *Nature*, vol. 457, no. 7232, pp. 981–989, 2009.

[66] A. Recober and A. F. Russo, "Calcitonin gene-related peptide: an update on the biology," *Current Opinion in Neurology*, vol. 22, no. 3, pp. 241–246, 2009.

[67] A. D. Sali, I. Karakasiliotis, M. Evangelidou, S. Avrameas, and P. Lymberi, "Immunological evidence and regulatory potential for cell-penetrating antibodies in intravenous immunoglobulin," *Clinical & Translational Immunology*, vol. 4, no. 10, article e42, 2015.

[68] S. Beggs, G. Currie, M. W. Salter, M. Fitzgerald, and S. M. Walker, "Priming of adult pain responses by neonatal pain experience: maintenance by central neuroimmune activity," *Brain*, vol. 135, no. 2, pp. 404–417, 2011.

[69] T. Berta, Y. Qadri, G. Chen, and R. Ji, "Microglial signaling in chronic pain with a special focus on caspase 6, p38 map kinase, and sex dependence," *Journal of Dental Research*, vol. 95, no. 10, pp. 1124–1131, 2016.

[70] M. L. Loggia, D. B. Chonde, O. Akeju et al., "Evidence for brain glial activation in chronic pain patients," *Brain*, vol. 138, no. 3, pp. 604–615, 2015.

[71] Y. Shi, B. B. Gelman, J. G. Lisinicchia, and S.-J. Tang, "Chronic-pain-associated astrocytic reaction in the spinal cord dorsal horn of human immunodeficiency virus-infected patients," *Journal of Neuroscience*, vol. 32, no. 32, pp. 10833–10840, 2012.

[72] D. Gomez-Nicola and V. H. Perry, "Microglial dynamics and role in the healthy and diseased brain: a paradigm of functional plasticity," *Neuroscientist*, vol. 21, no. 2, pp. 169–184, 2015.

[73] G. P. McStay, G. S. Salvesen, and D. R. Green, "Overlapping cleavage motif selectivity of caspases: implications for analysis of apoptotic pathways," *Cell Death and Differentiation*, vol. 15, no. 2, pp. 322–331, 2008.

[74] W. M. Pardridge and R. J. Boado, "Reengineering biopharmaceuticals for targeted delivery across the blood-brain barrier," *Methods in Enzymology*, vol. 503, pp. 269–292, 2012.

[75] R. E. Sorge, J. C. S. Mapplebeck, S. Rosen et al., "Different immune cells mediate mechanical pain hypersensitivity in male and female mice," *Nature Neuroscience*, vol. 18, no. 8, pp. 1081–1083, 2015.

[76] M. J. Chen, B. Kress, X. Han et al., "Astrocytic CX43 hemichannels and gap junctions play a crucial role in development of chronic neuropathic pain following spinal cord injury," *Glia*, vol. 60, no. 11, pp. 1660–1670, 2012.

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Review Article

Having a Coffee Break: The Impact of Caffeine Consumption on Microglia-Mediated Inflammation in Neurodegenerative Diseases

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Caffeine is the major component of coffee and the most consumed psychostimulant in the world and at nontoxic doses acts as a nonselective adenosine receptor antagonist. Epidemiological evidence suggests that caffeine consumption reduces the risk of several neurological and neurodegenerative diseases. However, despite the beneficial effects of caffeine consumption in human health and behaviour, the mechanisms by which it impacts the pathophysiology of neurodegenerative diseases still remain to be clarified. A promising hypothesis is that caffeine controls microglia-mediated neuroinflammatory response associated with the majority of neurodegenerative conditions. Accordingly, it has been already described that the modulation of adenosine receptors, namely, the A_{2A} receptor, affords neuroprotection through the control of microglia reactivity and neuroinflammation. In this review, we will summarize the main effects of caffeine in the modulation of neuroinflammation in neurodegenerative diseases.

1. Microglial Cells Play Crucial Roles in Neurodegenerative Diseases

The multitaskers microglial cells are active effectors and regulators of homeostasis in the central nervous system (CNS). Microglial cells constantly survey the surrounding environment, and as primary resident immune cells in the CNS, they respond to the presence of pathogens, stress, or injury [1]. In fact, for decades, it was believed that in homeostatic conditions microglial cells were in a nonreactive *resting* state, which could be transformed into a *reactive* state under pathological conditions. Nevertheless, the crucial role of microglial cells in the noninjured CNS has become more evident in recent years, and these cells not only are involved in immune pathological response but are essential during CNS development, participating in crucial processes such as in synaptic pruning [2, 3] and synaptic plasticity [4, 5]. Thus, the so-called *resting phenotype* should also reflect an active state and should be replaced by *surveillance state* [6].

Contrasting to the highly ramified organization presented by microglia in the *surveillance state*, reactive microglial cells are characterized by amoeboid morphology, which favours phagocytosis [7, 8]. This shift to a more activated phenotype results in increased release of proinflammatory and cytotoxic factors, such as tumour necrosis factor (TNF), interleukin-1β (IL-1β), nitric oxide (NO), and reactive oxygen species (ROS) [9], as well as in increased expression of surface molecules related to the innate immune response, as major histocompatibility complex (MHC) proteins and antigen-presenting receptors [10].

When studying microglial cell reactivity in the context of pathology, one major point is the dichotomy between their contribution to neuroprotection and neurodegeneration. Microglia activation and production of inflammatory mediators are known to be a response to neuronal dysfunction and death to control the damage and to promote recovery (reviewed in [11, 12]). Nevertheless, sustained reactivity of microglial cells has a detrimental role and contributes to

neurodegeneration, in which neuronal loss is accompanied by increased neuroinflammatory conditions [13–16].

In the two last decades, significant advances have been made in the understanding of the contribution of microglial cells to CNS diseases. The activation of these cells is recognized as a hallmark of a wide variety of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS), and in retinal diseases, such glaucoma, diabetic retinopathy, and age-related macular degeneration (reviewed in [9, 17]). Hence, suppression of microglial-associated deleterious effects has emerged as a potential therapeutic strategy to prevent neurodegeneration [18, 19].

2. The Modulation of the Adenosinergic System for Therapeutic Intervention in Neurodegenerative Diseases

Adenosine is a ubiquitously expressed purine nucleoside that acts as a homeostatic factor and a crucial neuromodulator in the CNS. In physiological conditions, the concentration of adenosine in the extracellular fluids is low (30–300 nM), but its levels increase to 10 μM or higher during enhanced nerve activity, hypoxia, ischemia, or CNS damage [20]. At high concentrations, adenosine is able to modulate the release of excitotoxic mediators, limit calcium influx, hyperpolarize neurons, and exert modulatory effects on glial cells [21].

Four types of G-coupled receptors coordinate cellular responses to extracellular adenosine: the inhibitory A_1 and A_3 receptors and the facilitatory A_{2A} and A_{2B} receptors [22]. These receptors are expressed on astrocytes, microglia, and infiltrating immune cells and regulate the immune response of these cells in the CNS [23–31]. The actions mediated by adenosine in the immune cells may be towards neuronal protection, but adenosine may also promote proinflammatory response, leading to neuronal damage (reviewed [32]).

In the last decades, the neuroprotective properties of adenosine in the CNS have been extensively documented [33–41]. The neuromodulatory effects mediated by adenosine rely on a balanced activation of the inhibitory A_1 receptor (A_1R) and excitatory A_{2A} receptor (A_{2A}R) [41]. A large body of evidence points to a neuroprotective role of A_1R activation, but this receptor is prone to rapid desensitization, limiting the time-lapse of action of possible neuroprotective therapies [41].

Concerning A_{2A}R, there is an apparent paradox on the role mediated by this receptor in inflammation. In the periphery, activation of A_{2A}R signaling suppresses inflammation [42], attenuates pulmonary ischemic injury [43, 44], and improves cardiac dysfunction [45]. In the CNS, A_{2A}R controls the release of BDNF from activated microglia [46], and its blockade prevents hippocampal LPS-induced neuroinflammation [47] and prevents IL-1β-induced exacerbation of neuronal toxicity [48]. Antagonists A_{2A}R prevent retinal microglia reactivity, affording protection to retinal neuronal cells [26, 27]. Importantly, blockade of A_{2A}R has been shown to confer neuroprotection against a broad spectrum of CNS insults [49, 50].

While in the periphery, the activation of A_{2A}R halts a rapid immune response (acute), in the CNS the activation of A_{2A}R aggravates the inflammatory response (chronic conditions) (reviewed in [51]). This dual role of A_{2A}R might reflect the complexity of actions in distinct cell types present in the CNS, which may lead to distinct effects upon CNS injury [41, 52]. The mechanisms by which the blockade of A_{2A}R is able to impact neuroprotection remains to be clarified, but two leading hypotheses have been explored: the control of glutamate excitotoxicity and the control of microglia-mediated neuroinflammation [40, 41].

The blockade of A_{2A}R has emerged as a potential therapeutic strategy, based on its ability to regulate proliferation, chemotaxis, and reactivity of glial cells, affording protection in several brain diseases (reviewed in [40, 50]).

Although less expressed, A_{2B}R and A_3R may also mediate protective effects in the CNS. By acting on A_{2B}R, adenosine has been shown to augment the production of IL-10 by microglial and macrophages cells, while preventing the release of proinflammatory cytokines [53]. The activation of A_3R has been shown to afford protective effects, namely, after brain ischemia [54] and in retinal neurodegeneration [55].

3. Caffeine: An Antagonist of Adenosine Receptors with Protective Functions in the CNS

Caffeine (1,3,7-trimethylxanthine) is the most widely consumed psychostimulant substance in the world, mainly found in dietary sources, such coffee, tea, and energy drinks [56]. Caffeine has been described as a CNS stimulant that promotes wakefulness, enhances mood and cognition, and produces stimulatory effects [57, 58]. In fact, caffeine exerts beneficial effects on human behaviour, which were not mimicked by the consumption of decaffeinated drinks [59].

Worldwide, it is estimated that caffeine consumption, from all sources, is around 70 to 76 mg/person/day. A single cup of coffee provides a dose of 0.4 to 2.5 mg/kg of caffeine, leading to a peak serum concentration of 0.25 to 2 mg/L or approximately 1 to 10 μM. In humans, 99% of caffeine is absorbed from the gastrointestinal tract in about 45 min after ingestion [56]. The first metabolic step, which represents on average 80% of the total process, is via N-3 demethylation to paraxanthine (1,7-dimethylxanthine) by the cytochrome P450 1A2 enzyme [60] and was recently found to be associated with the variability of caffeine consumption between individuals [61]. Other two important products of caffeine metabolism are theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine), which represent about 16% of the total metabolites [62]. After long-term consumption of high doses of caffeine, these metabolites can also contribute to its pharmacological actions, since it can result in an accumulation of methylxanthines in the body, due to end-product inhibition of demethylation, and thereby should be also considered when investigating the pharmacological actions of caffeine [63].

Most of the studies about the beneficial effects of coffee have been focused largely on caffeine, but coffee contains over 1,000 components that may have neuroprotective effects [64–67]. Interestingly, decaffeinated coffee is protective in *Drosophila* models of PD [68], suggesting that other coffee constituents may provide neuroprotection. Eicosanoyl-5-hydroxytryptamide, a constituent of coffee, has been demonstrated to ameliorate the phenotype of a PD model associated with decreased protein aggregation and phosphorylation, improved neuronal integrity, and reduced neuroinflammation [69]. Also, chlorogenic acid, trigonelline, and melanoidins are also able to impact gene transcription and regulation of body fat percentage [70, 71].

The biochemical mechanisms that underlie the actions of caffeine are dependent on the dose. In the brain, the molecular targets of caffeine at nontoxic doses are the adenosine receptors A_1 and A_{2A} [56].

One of the most recognized actions of caffeine is its ability to reduce sleep and sleepiness. Caffeine, acting on $A_{2A}R$, promotes wakefulness, as demonstrated by genetic manipulation of the $A_{2A}R$ in the nucleus accumbens [72].

Ethanol and caffeinated beverages are frequently consumed in combination, a fact that might be due to the popular belief that caffeine can offset the acute intoxicating actions of ethanol. In fact, it has been shown that caffeine is able to attenuate ethanol-induced motor incoordination in rats [73], an effect that was also observed with A_1R antagonists, but not with antagonists of $A_{2A}R$. Interestingly, caffeine administration also prevents the hypnotic effects induced by ethanol, an effect suggested to be mediated by $A_{2A}R$ antagonism, since knockout (KO) mice for this receptor display similar behaviour [74].

Caffeine is also associated with alterations in neurotransmitter release and increase neuronal firing (via A_1R), as well as enhancing dopaminergic transmission (via $A_{2A}R$), globally affecting neuronal processes associated with mood and cognition (reviewed in [56]). Caffeine has been shown to control synaptic plasticity [75], to revert memory impairments [76, 77], and to prevent mood alteration triggered by chronic stress [78]. Importantly, these effects were also observed in the presence of selective $A_{2A}R$ antagonists prompting the critical role of this receptor to the actions of caffeine. Indeed, using $A_{2A}R$-KO mice, it was recently shown that the neuroprotective effects of caffeine in a PD model rely on the presence of $A_{2A}R$ [79].

Several studies have been demonstrating protective effects of caffeine in patients and animal models of neurodegenerative diseases, mainly by reducing excitotoxicity, apoptosis, and neuroinflammation (reviewed in [80]).

4. Modulation of Microglia Reactivity and Neuroinflammation with Caffeine

Since the late 1990s, several studies have shown that caffeine reduces neuroinflammation in models of AD and PD (reviewed in [80]). Additionally, epidemiological studies have shown that caffeine might exert neuroprotective effects in humans [56, 81, 82].

Several studies have also focused their attention on the ability of caffeine to reduce microglia reactivity. In an *in vitro* system, using the murine BV-2 microglia cell line, it was demonstrated that 2 mM caffeine attenuates the expression of proinflammatory mediators, such as NO and TNF, and their regulatory genes, elicited by lipopolysaccharide (LPS) [83], widely known to induce potent neuroinflammatory responses in the brain [84]. The same study suggests the modulation of extracellular signal-regulated kinase (ERK) signaling cascade and consequent NF-κB activation as a main pathway for caffeine actions [83], which has been also related to the $A_{2A}R$ activation-induced macroglial cell reactivity [85]. In an animal model of inflammation, in which LPS was infused over a period of two or four weeks in the brain, caffeine administration (daily intraperitoneal injection) reduces LPS-induced microglia activation in three regions of the hippocampus, in a dose-dependent manner [86].

Importantly, caffeine alters neuronal functioning in physiological brain conditions, increasing the spontaneous firing (reviewed in [56]). The effects of caffeine in nonneuronal cells in nonpathological conditions have not been extensively studied. It was already described that brain sections of animals administered with caffeine *ad libitum* in the drinking water have altered microglia density and morphology, as observed by process retraction and enlargement of the cell soma, indicating a more reactive phenotype [87]. The authors suggested that caffeine might prime microglial cells, impacting the transition from the surveillance to the reactive state [88]. Notably, in the retina, caffeine intake does not change microglia reactivity and expression of proinflammatory markers [87].

5. Beneficial Effects of Caffeine in Alzheimer's Disease: Neuroinflammation and Neuroprotection

Alzheimer's disease is the most common type of dementia worldwide, clinically characterized by a progressive decline of cognitive functions and memory deficits [89]. The main neuropathological hallmarks of AD are extraneuronal deposition of amyloid-beta (Aβ) protein in the form of plaques and intraneuronal aggregation of the hyperphosphorylated microtubule-associated protein tau in the form of filaments, mainly in the cortex, hippocampus, and amygdala [90]. Furthermore, a strong neuroinflammatory component has been associated with AD pathology, with increased glial cell reactivity (microgliosis and astrogliosis), activation of both classic and alternate pathways of the complement system, upregulation of inflammatory markers, and increased phagocytic activity [91, 92].

The presence of Aβ oligomers has been described to lead to microglia-mediated neuroinflammatory response, with alterations in the phagocytic efficiency and sustained overproduction of inflammatory mediators, which may contribute to neurotoxicity and neuronal loss [93]. Indeed, microglia reactivity has been described not only in the brain, but also in the retinas of AD animal models [94] and

patients [95]. It remains to be elucidated whether microglia activation is a cause or a consequence of AD, but the role of microglia reactivity in the progression of the disease is unquestionable. Hence, interventions targeted to control microglial cell reactivity might delay the progression of AD.

Consumption of caffeine has been associated with reduction in the cognitive decline in healthy subjects (with advanced age) and also AD patients [96–99]. In AD animal models the beneficial effects of caffeine intake include amelioration of cognitive impairments [100, 101] and dementia [102].

It has been described that increased caffeine levels in the plasma are associated with reduced inflammatory cytokine levels in the hippocampus [103]. Remarkably, chronic administration of caffeine to a transgenic mouse model of progressive AD-like tau pathology mitigates several proinflammatory and oxidative stress markers in the hippocampus and prevents the development of spatial memory deficits [104].

Disruption of the blood-brain barrier (BBB) is an early pathological event in AD [105, 106] and may potentiate the accumulation of Aβ in the brain by allowing the transport of Aβ produced in the periphery [107]. Caffeine administration protects against AD-associated BBB dysfunction [106, 108] and reduces glial cell reactivity at sites of BBB leakage [106]. The effects of caffeine in the control of BBB integrity have been associated with its antagonistic actions on adenosine receptors and consequent inhibition of cyclic adenosine monophosphate (cAMP) activity and control of calcium intracellular stores [108]. Caffeine might control AD-associated increase on inflammatory mediators by reducing glial cell reactivity on the BBB leakage site [106] through a reduction in infiltration of immune cells from the periphery [109].

Moreover, using an animal model showing age-related CNS alterations that includes cognitive impairment, increased neuroinflammatory markers, and neurodegeneration, chronic administration of caffeine improves memory deficits and reduces the expression of ROS and proinflammatory cytokines TNF and IL-1β, further conferring antiapoptotic effects [110]. Similarly, the effects of caffeine are mimicked by selective antagonists of $A_{2A}R$ [111], suggesting that the actions of caffeine are mediated by the blockade of $A_{2A}R$. In accordance, both pharmacological blockade and genetic inactivation of $A_{2A}R$ afford neuroprotection against Aβ toxicity [112].

These reports reinforce the crucial importance of $A_{2A}R$ in Aβ toxicity and in the associated microglia reactivity and neuroinflammatory response in the context of AD, demonstrating also prophylactic properties of caffeine and the therapeutic potential of $A_{2A}R$ antagonists for the treatment of AD [113].

6. Caffeine Modulates Neuroinflammation in Parkinson's Disease: Possible Strategy for Neuroprotection?

Parkinson's disease (PD) is the second most common progressive neurodegenerative disorder. It is characterized by a progressive loss of dopaminergic neurons of the nigrostriatal pathway with the occurrence of Lewy bodies (abnormal deposits of α-synuclein), which clinically translates in muscular rigidity, resting tremor, bradykinesia, and postural instability [114]. The pathogenesis of PD has been also associated with chronic neuroinflammation [115] and oxidative stress [116], both contributing to BBB disruption [116, 117].

The brain is particularly susceptible to oxidative stress due to the high consumption of oxygen [116]. Oxidative stress has been associated with several neurodegenerative diseases, including PD. Indeed, there is evidence from postmortem human samples that oxidative stress might be a primary insult that leads to neuronal damage in PD [118, 119]. In substantia nigra, microglial cells have been proposed to be the main cells producing oxidative stress products [120], suggesting the involvement of these cells in the pathophysiology of PD. The involvement of neuroinflammation in PD was suggested after observation of increased number of reactive microglial cells and an upregulation of major histocompatibility complex class II (MHC-II) in PD patients [121]. Indeed, reactive microglia and neuroinflammatory response have been strongly associated with dopaminergic cell loss in PD (reviewed in [122–124]). Furthermore, elevated levels of proinflammatory cytokines such as TNF [125], IL-1β, and IL-6 [126] have been described in the striatum of PD patients. In the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) animal model of PD there is evidence demonstrating the neurotoxic contribution of microglia-produced TNF [127], IL-1β [128], IL-6, and NO [129] to the loss of dopaminergic neurons. These proinflammatory cytokines, along with factors released from the dying dopaminergic cells, seem to increase and sustain neuroinflammation, leading to an irreversible loss of dopaminergic neurons (reviewed in [130]). Hence, future therapeutic strategies should consider inhibition of microglia-mediated neuroinflammation, possibly in combination with neurotropic factors, aiming to delay the progression of PD.

Epidemiological studies have been associating the consumption of caffeine with reduced risk of developing PD [131–134]. Using the MPTP mouse model, it was shown that daily intraperitoneal administration of caffeine attenuates microglia reactivity and prevents BBB dysregulation, leading to decreased dopaminergic neuronal loss [135, 136]. Accordingly, even when introduced in the later phases of the neurodegenerative process, caffeine is also able to attenuate the inflammatory process and microglial cell expression of CD68 (a marker of reactive microglia), which suggests its ability to arrest or delay neuroinflammation and neurodegeneration [135]. Likewise, caffeine, even in low doses, is able to reverse functional motor deficits in PD animal models [137, 138].

Although the mechanisms underlying neuroprotection by caffeine remain a matter of debate, it has been widely suggested that the neuroprotective effects of caffeine involve the antagonism of $A_{2A}R$ [79, 139, 140]. Notably, pharmacological blockade of $A_{2A}R$ presents similar protective effects to the ones observed with caffeine in several experimental models of PD [35, 140–142]. Indeed, the critical contribution of $A_{2A}R$ to caffeine-mediated neuroprotection was recently demonstrated in mice lacking the $A_{2A}R$ gene (KO mice) and exposed to MPTP. In these animals, caffeine had no effect on

MPTP toxicity, namely, in striatal neuronal loss and motor activity impairment [79].

The selective $A_{2A}R$ antagonists istradefylline (KW 6002) [143] and preladenant (SCH 420814) [144] have been investigated in the past years in clinical trials for PD. The $A_{2A}R$ antagonists significant ameliorate the motor symptoms, but more studies are required to establish the clinical utility of these drugs [143, 144].

Taking into account the contribution of microglia-mediated neuroinflammation in the pathophysiology of PD and the beneficial effects of caffeine and $A_{2A}R$ antagonists, one can hypothesize that pharmacologic blockade of $A_{2A}R$ might offer potential therapeutic benefit in PD at the level of motor alterations, neuroinflammatory response, and neuroprotection.

7. The Effects of Caffeine in Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune, inflammatory disease of the CNS and the most common cause of chronic neurologic disability beginning in early to middle adult life [145]. The major pathological hallmarks of MS include dysregulation of BBB, which promotes macrophage and lymphocyte infiltration, and the presence of sclerotic plaques in the CNS [146]. In more advanced stages, the degenerative phase is characterized by demyelination and axonal damage that results in neuronal functional impairment in the brain and in the spinal cord. The demyelination process is associated with inflammation, which can occur through activation of resident astrocytes and microglia and by the inflammatory cytokine products of infiltrating immune cells (lymphocytes or macrophages) [147].

Although the exact role of microglial cells in MS is not completely elucidated, it is recognized that these cells are able to sustain and propagate the inflammatory response during autoimmune inflammation [148]. Reactive microglia, expressing MHC-II, exert functions of antigen-presenting cells during MS, therefore promoting the propagation of the inflammatory process and secretion of cytokine or chemokine [149]. Indeed, the detrimental role of microglia activation in MS models has been demonstrated, with the inhibition of these cells leading to a reduction in the myelin and axonal damage, and also in neurodegeneration [148, 150–152].

Nonetheless, microglial cells not only contribute to the neurodegenerative process, but also play an important role in the promotion of neuroprotection, downregulation of inflammatory process, and stimulation of tissue repair. This complex and dual role might be due to the high heterogeneity of myeloid populations (microglia, monocytes, and infiltrating T-cells), with distinct subtypes and distinct states of microglia reactivity (M1 and M2) associated with different pathologic or protective roles [153–155].

As previously mentioned, several reports have implicated the modulation of adenosine receptors in immune cells to a suppression of the inflammatory response (reviewed by [156]). Indeed, the levels of adenosine are reduced in the plasma of MS patients and the expression of $A_{2A}R$ and A_1R is up- and downregulated, respectively [157, 158]. Studies in animal models of MS confirmed the decreased expression of A_1R in microglial cells and an increase in both pro- and anti-inflammatory mediators [159, 160]. The administration of caffeine to these animals restores the levels of A_1R and attenuates the neuroinflammatory process and demyelination [160]. In accordance with studies in animal models of MS, high consumption of coffee may decrease the risk of developing MS [65, 161]. The authors suggest the suppression of the neuroinflammatory process and consequent production of proinflammatory cytokines as the mechanism underlying the observed association [65].

Similar to other brain conditions, the levels of $A_{2A}R$ have been shown to be upregulated in the brain [162] and in lymphocytes of MS patients [163]. Activation of $A_{2A}R$ has been associated with a strong anti-inflammatory response by immune cells [156, 164]. Correspondingly, genetic inactivation of $A_{2A}R$ has been reported to enhance the inflammatory cell infiltration and microglial cell activation in cortex, brainstem, and spinal cord in a MS animal model, also increasing demyelination and axonal damage [165]. These results suggest that adenosine acting on $A_{2A}R$ triggers neuroprotective effects. Intriguingly, the use of antagonists of $A_{2A}R$ also affords neuroprotection in a model of MS by reducing lymphocyte infiltration [166]. Indeed, a dual role for $A_{2A}R$ in autoimmune inflammation has been already described, with activation of $A_{2A}R$ leading to prevention of the disease in the early stages, whereas $A_{2A}R$ blockade affords protection in later stages by reducing neuroinflammation [167]. These results suggest that $A_{2A}R$ activity can impact the progression of the disease in multiple cellular and molecular targets, but we must keep in mind the possibility that the genetic deletion and pharmacological inactivation of the receptor produce opposite effects in the pathology. Global genetic deletion of $A_{2A}R$ occurs in all cellular elements, whereas the pharmacological blockade is suggested to target preferentially neutrophils and lymphocytes [168], reducing their infiltration and therefore exerting neuroprotective effects, as well as reducing microglia activation [40, 165]. Therefore, the $A_{2A}R$ has been considered a potential target for therapeutic approaches in MS. Still, chronic treatment with caffeine during the degenerative phase of MS animal model provides neuroprotection regardless of the $A_{2A}R$ genotype, implying that, in this disease, caffeine acts in a non-$A_{2A}R$-dependent manner [169].

8. Beneficial Properties of Caffeine beyond Brain Neurodegenerative Diseases: A Look into the Retina

Despite the extensive evidence regarding the effects of caffeine consumption in the brain, very little is known about the effects of caffeine consumption in retinal degeneration [170]. We have shown that caffeine administration reduces retinal neuroinflammation and microglial reactivity in an animal model of retinal degeneration induced by ischemia reperfusion (I-R). Notably, caffeine treatment is also able to prevent retinal neuronal cell apoptosis in these animals [171]. Accordingly, in animals subjected to I-R, pharmacological

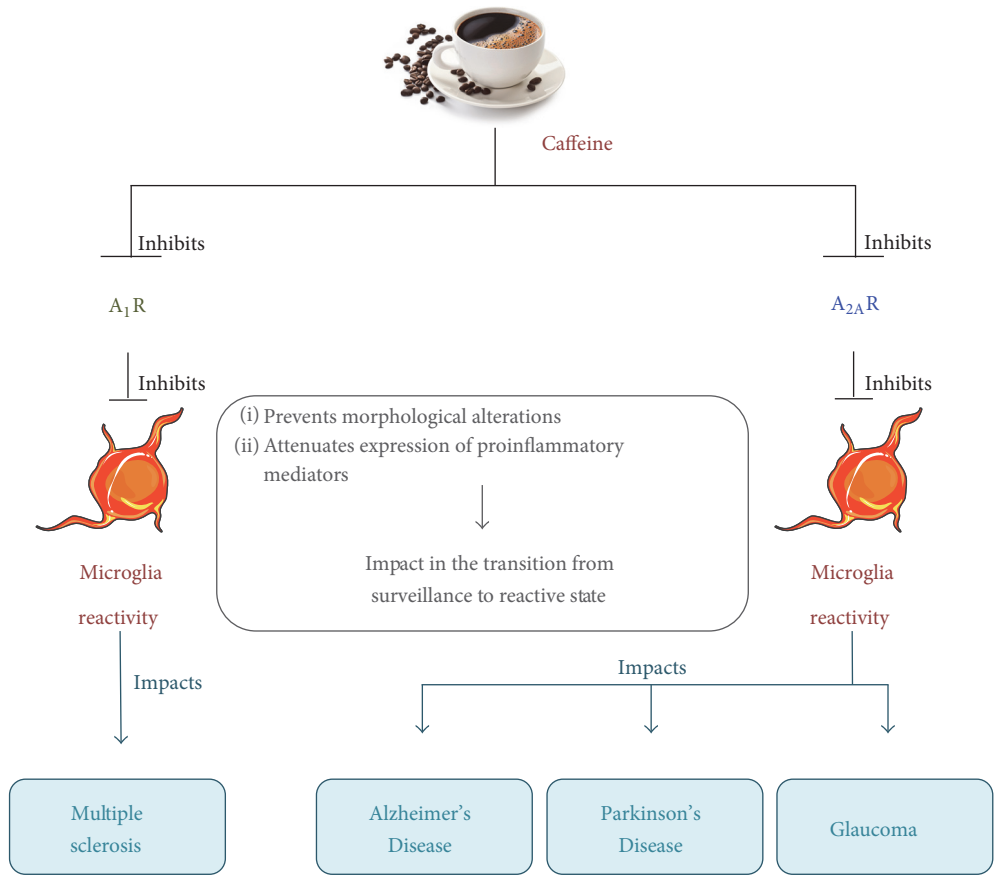

Figure 1: Caffeine reduces microglia-mediated inflammatory environment on CNS degenerative diseases. Schematic summary of the effects of caffeine intake on microglia reactivity and the associated CNS degenerative diseases.

blockade of $A_{2A}R$ prevents microglia reactivity and neuroinflammatory response [26]. Using retinal organotypic cultures and an I-R animal model we demonstrated that blockade of $A_{2A}R$ confers neuroprotection in the retinal through the control of microglia-mediated neuroinflammation [26, 87]. Hence, taking into account the antagonistic effects of caffeine in the $A_{2A}R$, one can hypothesize that protection against neuronal apoptosis in the retina afforded by caffeine might also be due to a reduction in microglia reactivity and neuroinflammatory response.

In addition, very recently, we demonstrated that caffeine intake prevents microglia-mediated neuroinflammation and increases the survival of retinal ganglion cells in an animal model of glaucoma [87], suggesting that caffeine may have a prophylactic effect in glaucoma. Still, the understanding of the effects of caffeine and $A_{2A}R$ antagonists in retinal neuroinflammation and neurodegeneration is still in a very early stage, but it appears as a promising therapeutic strategy for retinal neurodegenerative diseases [170].

9. Conclusions

Coffee is one of the most consumed beverages worldwide and its consumption has been demonstrated to impact human health. Taking in account the beneficial properties of caffeine in neurological and neurodegenerative diseases and the molecular targets of caffeine in the CNS, it is very important to elucidate the effects of caffeine to neuroinflammation.

Antagonists of adenosine receptors, namely, of $A_{2A}R$, have been vastly studied in neurodegenerative diseases. One hypothesis that has been gaining attention to explain the protective properties of caffeine and $A_{2A}R$ antagonists is the control of microglia-mediated neuroinflammation (Figure 1). Caffeine may block $A_{2A}R$ in microglial cells thus controlling exacerbated microglia reactivity and noxious inflammation, providing neuroprotection. Nevertheless, more studies are required to elucidate the cellular and molecular mechanisms of caffeine and its metabolites in the modulation of microglia-mediated neuroinflammation in neurodegenerative disorders.

Competing Interests

The authors declare that they have no competing interests.

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References

[1] H. Kettenmann, U.-K. Hanisch, M. Noda, and A. Verkhratsky, "Physiology of microglia," *Physiological Reviews*, vol. 91, no. 2, pp. 461–553, 2011.

[2] D. P. Schafer and B. Stevens, "Phagocytic glial cells: sculpting synaptic circuits in the developing nervous system," *Current Opinion in Neurobiology*, vol. 23, no. 6, pp. 1034–1040, 2013.

[3] S. Hong, L. Dissing-Olesen, and B. Stevens, "New insights on the role of microglia in synaptic pruning in health and disease," *Current Opinion in Neurobiology*, vol. 36, pp. 128–134, 2016.

[4] D. P. Schafer, E. K. Lehrman, A. G. Kautzman et al., "Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner," *Neuron*, vol. 74, no. 4, pp. 691–705, 2012.

[5] Y. Wu, L. Dissing-Olesen, B. A. MacVicar, and B. Stevens, "Microglia: dynamic mediators of synapse development and plasticity," *Trends in Immunology*, vol. 36, no. 10, pp. 605–613, 2015.

[6] U.-K. Hanisch and H. Kettenmann, "Microglia: active sensor and versatile effector cells in the normal and pathologic brain," *Nature Neuroscience*, vol. 10, no. 11, pp. 1387–1394, 2007.

[7] M. A. Petersen and M. E. Dailey, "Diverse microglial motility behaviors during clearance of dead cells in hippocampal slices," *GLIA*, vol. 46, no. 2, pp. 195–206, 2004.

[8] N. Stence, M. Waite, and M. E. Dailey, "Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices," *GLIA*, vol. 33, no. 3, pp. 256–266, 2001.

[9] M. E. Lull and M. L. Block, "Microglial activation and chronic neurodegeneration," *Neurotherapeutics*, vol. 7, no. 4, pp. 354–365, 2010.

[10] F. Aloisi, "Immune function of microglia," *GLIA*, vol. 36, no. 2, pp. 165–179, 2001.

[11] H. Zhang, F.-W. Wang, L.-L. Yao, and A.-J. Hao, "Microglia—friend or foe," *Frontiers in Bioscience—Scholar*, vol. 3, no. 3, pp. 869–883, 2011.

[12] Z. Chen and B. D. Trapp, "Microglia and neuroprotection," *Journal of Neurochemistry*, vol. 136, supplement 1, pp. 10–17, 2016.

[13] R. E. Mrak and W. S. T. Griffin, "Glia and their cytokines in progression of neurodegeneration," *Neurobiology of Aging*, vol. 26, no. 3, pp. 349–354, 2005.

[14] S. A. Sargsyan, P. N. Monk, and P. J. Shaw, "Microglia as potential contributors to motor neuron injury in amyotrophic lateral sclerosis," *GLIA*, vol. 51, no. 4, pp. 241–253, 2005.

[15] K. Biber, T. Owens, and E. Boddeke, "What is microglia neurotoxicity (Not)?" *GLIA*, vol. 62, no. 6, pp. 841–854, 2014.

[16] M. L. Block, L. Zecca, and J.-S. Hong, "Microglia-mediated neurotoxicity: uncovering the molecular mechanisms," *Nature Reviews Neuroscience*, vol. 8, no. 1, pp. 57–69, 2007.

[17] M. H. Madeira, R. Boia, P. F. Santos, A. F. Ambrósio, and A. R. Santiago, "Contribution of microglia-mediated neuroinflammation to retinal degenerative diseases," *Mediators of Inflammation*, vol. 2015, Article ID 673090, 15 pages, 2015.

[18] T. Möller and H. W. G. M. Boddeke, "Glial cells as drug targets: what does it take?" *GLIA*, vol. 64, no. 10, pp. 1742–1754, 2016.

[19] E. E. Spangenberg and K. N. Green, "Inflammation in Alzheimer's disease: lessons learned from microglia-depletion models," *Brain, Behavior, and Immunity*, 2016.

[20] R. A. Cunha, "Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors," *Neurochemistry International*, vol. 38, no. 2, pp. 107–125, 2001.

[21] N. Rebola, R. J. Rodrigues, C. R. Oliveira, and R. A. Cunha, "Different roles of adenosine A1, A2A and A 3 receptors in controlling kainate-induced toxicity in cortical cultured neurons," *Neurochemistry International*, vol. 47, no. 5, pp. 317–325, 2005.

[22] J.-F. Chen, C.-F. Lee, and Y. Chern, "Adenosine receptor neurobiology: overview," *International Review of Neurobiology*, vol. 119, pp. 1–49, 2014.

[23] L. Antonioli, C. Blandizzi, P. Pacher, and G. Haskó, "Immunity, inflammation and cancer: a leading role for adenosine," *Nature Reviews Cancer*, vol. 13, no. 12, pp. 842–857, 2013.

[24] D. Boison, J.-F. Chen, and B. B. Fredholm, "Adenosine signaling and function in glial cells," *Cell Death and Differentiation*, vol. 17, no. 7, pp. 1071–1082, 2010.

[25] O. Björklund, M. Shang, I. Tonazzini, E. Daré, and B. B. Fredholm, "Adenosine A1 and A3 receptors protect astrocytes from hypoxic damage," *European Journal of Pharmacology*, vol. 596, no. 1–3, pp. 6–13, 2008.

[26] M. H. Madeira, R. Boia, F. Elvas et al., "Selective A2A receptor antagonist prevents microglia-mediated neuroinflammation and protects retinal ganglion cells from high intraocular pressure-induced transient ischemic injury," *Translational Research*, vol. 169, pp. 112–128, 2016.

[27] M. H. Madeira, F. Elvas, R. Boia et al., "Adenosine A_{2A}R blockade prevents neuroinflammation-induced death of retinal ganglion cells caused by elevated pressure," *Journal of Neuroinflammation*, vol. 12, no. 1, article 115, 2015.

[28] C. Hammarberg, G. Schulte, and B. B. Fredholm, "Evidence for functional adenosine A_3 receptors in microglia cells," *Journal of Neurochemistry*, vol. 86, no. 4, pp. 1051–1054, 2003.

[29] E. Daré, G. Schulte, O. Karovic, C. Hammarberg, and B. B. Fredholm, "Modulation of glial cell functions by adenosine receptors," *Physiology and Behavior*, vol. 92, no. 1-2, pp. 15–20, 2007.

[30] L. Luongo, F. Guida, R. Imperatore et al., "The A1 adenosine receptor as a new player in microglia physiology," *GLIA*, vol. 62, no. 1, pp. 122–132, 2014.

[31] M.-C. Peakman and S. J. Hill, "Adenosine A_{2B}-receptor-mediated cyclic AMP accumulation in primary rat astrocytes," *British Journal of Pharmacology*, vol. 111, no. 1, pp. 191–198, 1994.

[32] M. R. Blackburn, C. O. Vance, E. Morschl, and C. N. Wilson, "Adenosine receptors and inflammation," *Handbook of Experimental Pharmacology*, vol. 193, pp. 215–269, 2009.

[33] M. P. Abbracchio and F. Cattabeni, "Brain adenosine receptors as targets for therapeutic intervention in neurodegenerative diseases," *Annals of the New York Academy of Sciences*, vol. 890, pp. 79–92, 1999.

[34] J. Wardas, "Neuroprotective role of adenosine in the CNS," *Polish Journal of Pharmacology*, vol. 54, no. 4, pp. 313–326, 2002.

[35] K. Ikeda, M. Kurokawa, S. Aoyama, and Y. Kuwana, "Neuroprotection by adenosine A2A receptor blockade in experimental models of Parkinson's disease," *Journal of Neurochemistry*, vol. 80, no. 2, pp. 262–270, 2002.

[36] T. W. Stone, S. Ceruti, and M. P. Abbracchio, "Adenosine receptors and neurological disease: neuroprotection and neurodegeneration," *Handbook of Experimental Pharmacology*, vol. 193, pp. 535–587, 2009.

[37] E. Ongini, M. Adami, C. Ferri, and R. Bertorelli, "Adenosine A_{2A} receptors and neuroprotection," *Annals of the New York Academy of Sciences*, vol. 825, pp. 30–48, 1997.

[38] J.-F. Chen, P. K. Sonsalla, F. Pedata et al., "Adenosine A_{2A} receptors and brain injury: broad spectrum of neuroprotection, multifaceted actions and 'fine tuning' modulation," *Progress in Neurobiology*, vol. 83, no. 5, pp. 310–331, 2007.

[39] R. A. Cunha, "How does adenosine control neuronal dysfunction and neurodegeneration?" *Journal of Neurochemistry*, 2016.

[40] A. R. Santiago, F. I. Baptista, P. F. Santos et al., "Role of microglia adenosine A_{2A} receptors in retinal and brain neurodegenerative diseases," *Mediators of Inflammation*, vol. 2014, Article ID 465694, 13 pages, 2014.

[41] R. A. Cunha, "Neuroprotection by adenosine in the brain: from A_1 receptor activation to A_{2A} receptor blockade," *Purinergic Signalling*, vol. 1, no. 2, pp. 111–134, 2005.

[42] M. V. Sitkovsky, "Use of the A_{2A} adenosine receptor as a physiological immunosuppressor and to engineer inflammation *in vivo*," *Biochemical Pharmacology*, vol. 65, no. 4, pp. 493–501, 2003.

[43] D. J. Lapar, V. E. Laubach, A. Emaminia et al., "Pretreatment strategy with adenosine A2A receptor agonist attenuates reperfusion injury in a preclinical porcine lung transplantation model," *Journal of Thoracic and Cardiovascular Surgery*, vol. 142, no. 4, pp. 887–894, 2011.

[44] T. B. Reece, P. I. Ellman, T. S. Maxey et al., "Adenosine A_{2A} receptor activation reduces inflammation and preserves pulmonary function in an in vivo model of lung transplantation," *Journal of Thoracic and Cardiovascular Surgery*, vol. 129, no. 5, pp. 1137–1143, 2005.

[45] T. B. Reece, V. E. Laubach, C. G. Tribble et al., "Adenosine A_{2A} receptor agonist improves cardiac dysfunction from pulmonary ischemia-reperfusion injury," *Annals of Thoracic Surgery*, vol. 79, no. 4, pp. 1189–1195, 2005.

[46] C. Gomes, R. Ferreira, J. George et al., "Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A_{2A} receptor-dependent manner: A_{2A} receptor blockade prevents BDNF release and proliferation of microglia," *Journal of Neuroinflammation*, vol. 10, article 16, 2013.

[47] N. Rebola, A. P. Simões, P. M. Canas et al., "Adenosine A_{2A} receptors control neuroinflammation and consequent hippocampal neuronal dysfunction," *Journal of Neurochemistry*, vol. 117, no. 1, pp. 100–111, 2011.

[48] A. P. Simões, J. A. Duarte, F. Agasse et al., "Blockade of adenosine A_{2A} receptors prevents interleukin-1β-induced exacerbation of neuronal toxicity through a p38 mitogen-activated protein kinase pathway," *Journal of Neuroinflammation*, vol. 9, article 204, 2012.

[49] S.-S. Dai and Y.-G. Zhou, "Adenosine 2A receptor: a crucial neuromodulator with bidirectional effect in neuroinflammation and brain injury," *Reviews in the Neurosciences*, vol. 22, no. 2, pp. 231–239, 2011.

[50] C. V. Gomes, M. P. Kaster, A. R. Tomé, P. M. Agostinho, and R. A. Cunha, "Adenosine receptors and brain diseases: neuroprotection and neurodegeneration," *Biochimica et Biophysica Acta—Biomembranes*, vol. 1808, no. 5, pp. 1380–1399, 2011.

[51] R. A. Cunha, J. F. Chen, and M. V. Sitkovsky, "Opposite modulation of peripheral inflammation and neuroinflammation by adenosine A2A receptors," in *Interaction between Neurons and Glia in Aging and Disease*, pp. 53–79, Springer, New York, NY, USA, 2007.

[52] J.-F. Chen, H. K. Eltzschig, and B. B. Fredholm, "Adenosine receptors as drug targets—what are the challenges?" *Nature Reviews Drug Discovery*, vol. 12, no. 4, pp. 265–286, 2013.

[53] W. Wei, C. Du, J. Lv et al., "Blocking A_{2B} adenosine receptor alleviates pathogenesis of experimental autoimmune encephalomyelitis via inhibition of IL-6 production and Th17 differentiation," *Journal of Immunology*, vol. 190, no. 1, pp. 138–146, 2013.

[54] G.-J. Chen, B. K. Harvey, H. Shen, J. Chou, A. Victor, and Y. Wang, "Activation of adenosine A3 receptors reduces ischemic brain injury in rodents," *Journal of Neuroscience Research*, vol. 84, no. 8, pp. 1848–1855, 2006.

[55] J. Galvao, F. Elvas, T. Martins, M. F. Cordeiro, A. F. Ambrósio, and A. R. Santiago, "Adenosine A3 receptor activation is neuroprotective against retinal neurodegeneration," *Experimental Eye Research*, vol. 140, pp. 65–74, 2015.

[56] B. B. Fredholm, K. Bättig, J. Holmén, A. Nehlig, and E. E. Zvartau, "Actions of caffeine in the brain with special reference to factors that contribute to its widespread use," *Pharmacological Reviews*, vol. 51, no. 1, pp. 83–133, 1999.

[57] A. Nehlig, J.-L. Daval, and G. Debry, "Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects," *Brain Research Reviews*, vol. 17, no. 2, pp. 139–170, 1992.

[58] C. F. Haskell, D. O. Kennedy, K. A. Wesnes, and A. B. Scholey, "Cognitive and mood improvements of caffeine in habitual consumers and habitual non-consumers of caffeine," *Psychopharmacology*, vol. 179, no. 4, pp. 813–825, 2005.

[59] A. Smith, "Effects of caffeine on human behavior," *Food and Chemical Toxicology*, vol. 40, no. 9, pp. 1243–1255, 2002.

[60] L. Gu, F. J. Gonzalez, W. Kalow, and B. K. Tang, "Biotransformation of caffeine, paraxanthine, theobromine and theophylline by cDNA-expressed human CYP1A2 and CYP2E1," *Pharmacogenetics*, vol. 2, no. 2, pp. 73–77, 1992.

[61] S. Denden, B. Bouden, A. Haj Khelil, J. Ben Chibani, and M. H. Hamdaoui, "Gender and ethnicity modify the association between the CYP1A2 rs762551 polymorphism and habitual coffee intake: evidence from a meta-analysis," *Genetics and Molecular Research*, vol. 15, no. 2, 2016.

[62] N. L. Benowitz, P. Jacob III, H. Mayan, and C. Denaro, "Sympathomimetic effects of paraxanthine and caffeine in humans," *Clinical Pharmacology and Therapeutics*, vol. 58, no. 6, pp. 684–691, 1995.

[63] C. P. Denaro, C. R. Brown, M. Wilson, P. Jacob III, and N. L. Benowitz, "Dose-dependency of caffeine metabolism with repeated dosing," *Clinical Pharmacology and Therapeutics*, vol. 48, no. 3, pp. 277–285, 1990.

[64] M. Lee, E. G. McGeer, and P. L. McGeer, "Quercetin, not caffeine, is a major neuroprotective component in coffee," *Neurobiology of Aging*, vol. 46, pp. 113–123, 2016.

[65] A. K. Hedström, E. M. Mowry, M. A. Gianfrancesco et al., "High consumption of coffee is associated with decreased multiple sclerosis risk; results from two independent studies," *Journal of Neurology, Neurosurgery and Psychiatry*, vol. 87, no. 5, pp. 454–460, 2016.

[66] Y. Mikami and T. Yamazawa, "Chlorogenic acid, a polyphenol in coffee, protects neurons against glutamate neurotoxicity," *Life Sciences*, vol. 139, Article ID 14470, pp. 69–74, 2015.

[67] G. Basurto-Islas, J. Blanchard, Y. C. Tung et al., "Therapeutic benefits of a component of coffee in a rat model of Alzheimer's disease," *Neurobiology of Aging*, vol. 35, no. 12, pp. 2701–2712, 2014.

[68] K. Trinh, L. Andrews, J. Krause et al., "Decaffeinated coffee and nicotine-free tobacco provide neuroprotection in Drosophila models of Parkinson's disease through an NRF2-dependent mechanism," *Journal of Neuroscience*, vol. 30, no. 16, pp. 5525–5532, 2010.

[69] K.-W. Lee, J.-Y. Im, J.-M. Woo et al., "Neuroprotective and anti-inflammatory properties of a coffee component in the MPTP model of Parkinson's disease," *Neurotherapeutics*, vol. 10, no. 1, pp. 143–153, 2013.

[70] I. A. Ludwig, M. N. Clifford, M. E. J. Lean, H. Ashihara, and A. Crozier, "Coffee: biochemistry and potential impact on health," *Food and Function*, vol. 5, no. 8, pp. 1695–1717, 2014.

[71] B. Muqaku, A. Tahir, P. Klepeisz et al., "Coffee consumption modulates inflammatory processes in an individual fashion," *Molecular Nutrition and Food Research*, vol. 60, no. 12, pp. 2529–2541, 2016.

[72] M. Lazarus, H.-Y. Shen, Y. Cherasse et al., "Arousal effect of caffeine depends on adenosine A_{2A} receptors in the shell of the nucleus accumbens," *Journal of Neuroscience*, vol. 31, no. 27, pp. 10067–10075, 2011.

[73] L. Connole, A. Harkin, and M. Maginn, "Adenosine A1 receptor blockade mimics caffeine's attenuation of ethanol-induced motor incoordination," *Basic & Clinical Pharmacology & Toxicology*, vol. 95, no. 6, pp. 299–304, 2004.

[74] M. El Yacoubi, C. Ledent, M. Parmentier, J. Costentin, and J.-M. Vaugeois, "Caffeine reduces hypnotic effects of alcohol through adenosine A 2A receptor blockade," *Neuropharmacology*, vol. 45, no. 7, pp. 977–985, 2003.

[75] A. P. Simões, N. J. Machado, N. Gonçalves et al., "Adenosine A2A receptors in the amygdala control synaptic plasticity and contextual fear memory," *Neuropsychopharmacology*, vol. 41, no. 12, pp. 2862–2871, 2016.

[76] N. J. Machado, A. P. Simões, H. B. Silva et al., "Caffeine reverts memory but not mood impairment in a depression-prone mouse strain with up-regulated adenosine a2a receptor in hippocampal glutamate synapses," *Molecular Neurobiology*, pp. 1–12, 2016.

[77] R. A. Cunha and P. M. Agostinho, "Chronic caffeine consumption prevents memory disturbance in different animal models of memory decline," *Journal of Alzheimer's Disease*, vol. 20, no. 1, pp. S95–S116, 2010.

[78] M. P. Kaster, N. J. Machado, H. B. Silva et al., "Caffeine acts through neuronal adenosine A_{2A} receptors to prevent mood and memory dysfunction triggered by chronic stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 25, pp. 7833–7838, 2015.

[79] K. Xu, D. G. Di Luca, M. Orrú, Y. Xu, J.-F. Chen, and M. A. Schwarzschild, "Neuroprotection by caffeine in the MPTP model of parkinson's disease and its dependence on adenosine A2A receptors," *Neuroscience*, vol. 322, pp. 129–137, 2016.

[80] M. Rivera-Oliver and M. Díaz-Ríos, "Using caffeine and other adenosine receptor antagonists and agonists as therapeutic tools against neurodegenerative diseases: a review," *Life Sciences*, vol. 101, no. 1-2, pp. 1–9, 2014.

[81] M. Morelli, A. R. Carta, A. Kachroo, and M. A. Schwarzschild, "Pathophysiological roles for purines. Adenosine, caffeine and urate," *Progress in Brain Research*, vol. 183, no. C, pp. 183–208, 2010.

[82] J.-F. Chen and Y. Chern, "Impacts of methylxanthines and adenosine receptors on neurodegeneration: human and experimental studies," *Handbook of Experimental Pharmacology*, vol. 200, pp. 267–310, 2011.

[83] C.-H. Kang, R. G. P. T. Jayasooriya, M. G. Dilshara et al., "Caffeine suppresses lipopolysaccharide-stimulated BV2 microglial cells by suppressing Akt-mediated NF-κB activation and ERK phosphorylation," *Food and Chemical Toxicology*, vol. 50, no. 12, pp. 4270–4276, 2012.

[84] P.-B. Andersson, V. H. Perry, and S. Gordon, "The acute inflammatory response to lipopolysaccharide in cns parenchyma differs from that in other body tissues," *Neuroscience*, vol. 48, no. 1, pp. 169–186, 1992.

[85] R.-H. Ke, J. Xiong, Y. Liu, and Z.-R. Ye, "Adenosine A_{2A} receptor induced gliosis via Akt/NF-κB pathway in vitro," *Neuroscience Research*, vol. 65, no. 3, pp. 280–285, 2009.

[86] H. M. Brothers, Y. Marchalant, and G. L. Wenk, "Caffeine attenuates lipopolysaccharide-induced neuroinflammation," *Neuroscience Letters*, vol. 480, no. 2, pp. 97–100, 2010.

[87] M. H. Madeira, A. Ortin-Martinez, F. Nadal-Nícolas et al., "Caffeine administration prevents retinal neuroinflammation and loss of retinal ganglion cells in an animal model of glaucoma," *Scientific Reports*, vol. 6, Article ID 27532, 2016.

[88] R. Steger, A. Kamal, S. Lutchman, L. Intrabartolo, R. Sohail, and J. C. Brumberg, "Chronic caffeine ingestion causes microglia activation, but not proliferation in the healthy brain," *Brain Research Bulletin*, vol. 106, pp. 39–46, 2014.

[89] C. Reitz and R. Mayeux, "Alzheimer disease: epidemiology, diagnostic criteria, risk factors and biomarkers," *Biochemical Pharmacology*, vol. 88, no. 4, pp. 640–651, 2014.

[90] A. Lloret, T. Fuchsberger, E. Giraldo, and J. Viña, "Molecular mechanisms linking amyloid β toxicity and Tau hyperphosphorylation in Alzheimers disease," *Free Radical Biology and Medicine*, vol. 83, pp. 186–191, 2015.

[91] M. D. Ikonomovic, W. E. Klunk, E. E. Abrahamson et al., "Post-mortem correlates of in vivo PiB-PET amyloid imaging in a typical case of Alzheimer's disease," *Brain*, vol. 131, no. 6, pp. 1630–1645, 2008.

[92] M. Bobinski, M. J. De Leon, J. Wegiel et al., "The histological validation of post mortem magnetic resonance imaging-determined hippocampal volume in Alzheimer's disease," *Neuroscience*, vol. 95, no. 3, pp. 721–725, 1999.

[93] X.-D. Pan, Y.-G. Zhu, N. Lin et al., "Microglial phagocytosis induced by fibrillar β-amyloid is attenuated by oligomeric β-amyloid: implications for Alzheimer's disease," *Molecular Neurodegeneration*, vol. 6, no. 1, article 45, 2011.

[94] A. Ning, J. Cui, E. To, K. H. Ashe, and J. Matsubara, "Amyloid-β deposits lead to retinal degeneration in a mouse model of Alzheimer disease," *Investigative Ophthalmology and Visual Science*, vol. 49, no. 11, pp. 5136–5143, 2008.

[95] S. C. K. Liew, P. L. Penfold, J. M. Provis, M. C. Madigan, and F. A. Blllson, "Modulation of MHC class II expression in the absence of lymphocytic infiltrates in alzheimer's retinae," *Journal of Neuropathology and Experimental Neurology*, vol. 53, no. 2, pp. 150–157, 1994.

[96] M. Johnson-Kozlow, D. Kritz-Silverstein, E. Barrett-Connor, and D. Morton, "Coffee consumption and cognitive function

among older adults," *American Journal of Epidemiology*, vol. 156, no. 9, pp. 842–850, 2002.

[97] K. Ritchie, I. Carrière, A. De Mendonça et al., "The neuroprotective effects of caffeine: a prospective population study (the Three City Study)," *Neurology*, vol. 69, no. 6, pp. 536–545, 2007.

[98] B. M. van Gelder, B. Buijsse, M. Tijhuis et al., "Coffee consumption is inversely associated with cognitive decline in elderly European men: The FINE Study," *European Journal of Clinical Nutrition*, vol. 61, no. 2, pp. 226–232, 2007.

[99] L. Arab, F. Khan, and H. Lam, "Epidemiologic evidence of a relationship between tea, coffee, or caffeine consumption and cognitive decline," *Advances in Nutrition*, vol. 4, no. 1, pp. 115–122, 2013.

[100] G. W. Arendash, T. Mori, C. Cao et al., "Caffeine reverses cognitive impairment and decreases brain amyloid-β levels in aged alzheimer's disease mice," *Journal of Alzheimer's Disease*, vol. 17, no. 3, pp. 661–680, 2009.

[101] G. W. Arendash, W. Schleif, K. Rezai-Zadeh et al., "Caffeine protects Alzheimer's mice against cognitive impairment and reduces brain β-amyloid production," *Neuroscience*, vol. 142, no. 4, pp. 941–952, 2006.

[102] M. H. Eskelinen and M. Kivipelto, "Caffeine as a protective factor in dementia and Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 20, no. 1, pp. S167–S174, 2010.

[103] C. Cao, X. Cirrito Lin Jr., L. Wang et al., "Caffeine suppresses amyloid-beta levels in plasma and brain of Alzheimer's disease transgenic mice," *Journal of Alzheimer's Disease*, vol. 17, no. 3, pp. 681–697, 2009.

[104] C. Laurent, S. Eddarkaoui, M. Derisbourg et al., "Beneficial effects of caffeine in a transgenic model of Alzheimer's disease-like tau pathology," *Neurobiology of Aging*, vol. 35, no. 9, pp. 2079–2090, 2014.

[105] M. Ujiie, D. L. Dickstein, D. A. Carlow, and W. A. Jefferies, "Blood-brain barrier permeability precedes senile plaque formation in an Alzheimer disease model," *Microcirculation*, vol. 10, no. 6, pp. 463–470, 2003.

[106] X. Chen, J. W. Gawryluk, J. F. Wagener, O. Ghribi, and J. D. Geiger, "Caffeine blocks disruption of blood brain barrier in a rabbit model of Alzheimer's disease," *Journal of Neuroinflammation*, vol. 5, article no. 12, 2008.

[107] Y.-M. Kuo, T. A. Kokjohn, M. D. Watson et al., "Elevated Aβ42 in skeletal muscle of Alzheimer disease patients suggests peripheral alterations of AβPP metabolism," *American Journal of Pathology*, vol. 156, no. 3, pp. 797–805, 2000.

[108] X. Chen, O. Ghribi, and J. D. Geiger, "Caffeine protects against disruptions of the blood-brain barrier in animal models of Alzheimer's and Parkinson's diseases," *Journal of Alzheimer's Disease*, vol. 20, no. 1, pp. S127–S141, 2010.

[109] I. G. Farkas, A. Czigner, E. Farkas et al., "Beta-amyloid peptide-induced blood-brain barrier disruption facilitates T-cell entry into the rat brain," *Acta Histochemica*, vol. 105, no. 2, pp. 115–125, 2003.

[110] F. Ullah, T. Ali, N. Ullah, and M. O. Kim, "Caffeine prevents d-galactose-induced cognitive deficits, oxidative stress, neuroinflammation and neurodegeneration in the adult rat brain," *Neurochemistry International*, vol. 90, pp. 114–124, 2015.

[111] O. P. Dall'Igna, P. Fett, M. W. Gomes, D. O. Souza, R. A. Cunha, and D. R. Lara, "Caffeine and adenosine A2a receptor antagonists prevent β-amyloid (25–35)-induced cognitive deficits in mice," *Experimental Neurology*, vol. 203, no. 1, pp. 241–245, 2007.

[112] P. M. Canas, L. O. Porciúncula, G. M. A. Cunha et al., "Adenosine A_{2A} receptor blockade prevents synaptotoxicity and memory dysfunction caused by β-amyloid peptides via p38 mitogen-activated protein kinase pathway," *Journal of Neuroscience*, vol. 29, no. 47, pp. 14741–14751, 2009.

[113] G. W. Arendash and C. Cao, "Caffeine and coffee as therapeutics against Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 20, supplement 1, pp. S117–S126, 2010.

[114] T. Klockgether, "Parkinson's disease: clinical aspects," *Cell and Tissue Research*, vol. 318, no. 1, pp. 115–120, 2004.

[115] E. C. Hirsch, S. Hunot, and A. Hartmann, "Neuroinflammatory processes in Parkinson's disease," *Parkinsonism and Related Disorders*, vol. 11, supplement 1, pp. S9–S15, 2005.

[116] A. Hald and J. Lotharius, "Oxidative stress and inflammation in Parkinson's disease: is there a causal link?" *Experimental Neurology*, vol. 193, no. 2, pp. 279–290, 2005.

[117] R. Kortekaas, K. L. Leenders, J. C. H. Van Oostrom et al., "Blood-brain barrier dysfunction in Parkinsonian midbrain in vivo," *Annals of Neurology*, vol. 57, no. 2, pp. 176–179, 2005.

[118] P. Jenner, "Oxidative mechanisms in nigral cell death in Parkinson's disease," *Movement Disorders*, vol. 13, no. 1, pp. 24–34, 1998.

[119] P. Jenner and C. W. Olanow, "The pathogenesis of cell death in Parkinson's disease," *Neurology*, vol. 66, no. 10, supplement 4, pp. S24–S30, 2006.

[120] H. Miwa, T. Kubo, S. Morita, I. Nakanishi, and T. Kondo, "Oxidative stress and microglial activation in substantia nigra following striatal MPP+," *NeuroReport*, vol. 15, no. 6, pp. 1039–1044, 2004.

[121] P. L. McGeer, S. Itagaki, B. E. Boyes, and E. G. McGeer, "Reactive microglia are positive for HLA–DR in the substantia nigra of Parkinson's and Alzheimer's disease brains," *Neurology*, vol. 38, no. 8, pp. 1285–1291, 1988.

[122] P. S. Whitton, "Inflammation as a causative factor in the aetiology of Parkinson's disease," *British Journal of Pharmacology*, vol. 150, no. 8, pp. 963–976, 2007.

[123] E. C. Hirsch, T. Breidert, E. Rousselet, S. Hunot, A. Hartmann, and P. P. Michel, "The role of glial reaction and inflammation in Parkinson's disease," *Annals of the New York Academy of Sciences*, vol. 991, pp. 214–228, 2003.

[124] A. Hartmann, S. Hunot, and E. C. Hirsch, "Inflammation and dopaminergic neuronal loss in Parkinson's disease: a complex matter," *Experimental Neurology*, vol. 184, no. 2, pp. 561–564, 2003.

[125] M. Mogi, A. Togari, K.-I. Tanaka, N. Ogawa, H. Ichinose, and T. Nagatsu, "Increase in level of tumor necrosis factor (TNF)-α in 6- hydroxydopamine-lesioned striatum in rats without influence of systemic L- DOPA on the TNF-α induction," *Neuroscience Letters*, vol. 268, no. 2, pp. 101–104, 1999.

[126] D. Blum-Degena, T. Müller, W. Kuhn, M. Gerlach, H. Przuntek, and P. Riederer, "Interleukin-1β and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients," *Neuroscience Letters*, vol. 202, no. 1-2, pp. 17–20, 1995.

[127] K. Sriram, J. M. Matheson, S. A. Benkovic, D. B. Miller, M. I. Luster, and J. P. O'Callaghan, "Mice deficient in TNF receptors are protected against dopaminergic neurotoxicity: implications for Parkinson's disease," *The FASEB journal*, vol. 16, no. 11, pp. 1474–1476, 2002.

[128] M.-J. Bian, L.-M. Li, M. Yu, J. Fei, and F. Huang, "Elevated interleukin-1β induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine aggravating dopaminergic neurodegeneration in old male mice," *Brain Research*, vol. 1302, pp. 256–264, 2009.

[129] D. D. Lofrumento, C. Saponaro, A. Cianciulli et al., "MPTP-induced neuroinflammation increases the expression of pro-inflammatory cytokines and their receptors in mouse brain," *NeuroImmunoModulation*, vol. 18, no. 2, pp. 79–88, 2010.

[130] C. F. Orr, D. B. Rowe, and G. M. Halliday, "An inflammatory review of Parkinson's disease," *Progress in Neurobiology*, vol. 68, no. 5, pp. 325–340, 2002.

[131] M. A. Hernán, B. Takkouche, F. Caamaño-Isorna, and J. J. Gestal-Otero, "A meta-analysis of coffee drinking, cigarette smoking, and the risk of Parkinson's disease," *Annals of Neurology*, vol. 52, no. 3, pp. 276–284, 2002.

[132] L. S. Honig, G. W. Ross, R. D. Abbott, H. Petrovitch, L. R. White, and C. M. Tanner, "Relationship between caffeine intake and Parkinson disease," *JAMA*, vol. 284, no. 11, pp. 1378–1379, 2000.

[133] J. Costa, N. Lunet, C. Santos, J. Santos, and A. Vaz-Carneiro, "Caffeine exposure and the risk of Parkinson's disease: a systematic review and meta-analysis of observational studiess," *Journal of Alzheimer's Disease*, vol. 20, no. 1, pp. S221–S238, 2010.

[134] A. Wang, Y. Lin, Y. Wu, and D. Zhang, "Macronutrients intake and risk of Parkinson's disease: a meta-analysis," *Geriatrics and Gerontology International*, vol. 15, no. 5, pp. 606–616, 2015.

[135] X. Chen, X. Lan, I. Roche, R. Liu, and J. D. Geiger, "Caffeine protects against MPTP-induced blood-brain barrier dysfunction in mouse striatum," *Journal of Neurochemistry*, vol. 107, no. 4, pp. 1147–1157, 2008.

[136] K. Xu, Y.-H. Xu, J.-F. Chen, and M. A. Schwarzschild, "Caffeine's neuroprotection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity shows no tolerance to chronic caffeine administration in mice," *Neuroscience Letters*, vol. 322, no. 1, pp. 13–16, 2002.

[137] J. L. Bata-García, J. Villanueva-Toledo, G. Gutiérrez-Ospina, F. J. Álvarez-Cervera, F. J. Heredia-López, and J. L. Góngora-Alfaro, "Sustained improvement of motor function in hemiparkinsonian rats chronically treated with low doses of caffeine or trihexyphenidyl," *Pharmacology Biochemistry and Behavior*, vol. 86, no. 1, pp. 68–78, 2007.

[138] J. L. Bata-García, L. Tun-Cobá, F. J. Alvarez-Cervera, J. R. Villanueva-Toledo, F. J. Heredia-López, and J. L. Góngora-Alfaro, "Improvement of postural adjustment steps in hemiparkinsonian rats chronically treated with caffeine is mediated by concurrent blockade of A_1 and A_{2A} adenosine receptors," *Neuroscience*, vol. 166, no. 2, pp. 590–603, 2010.

[139] A. Kalda, L. Yu, E. Oztas, and J.-F. Chen, "Novel neuroprotection by caffeine and adenosine A_{2A} receptor antagonists in animal models of Parkinson's disease," *Journal of the Neurological Sciences*, vol. 248, no. 1-2, pp. 9–15, 2006.

[140] J. F. Chen, K. Xu, J. P. Petzer et al., "Neuroprotection by caffeine and A(2A) adenosine receptor inactivation in a model of Parkinson's disease," *The Journal of neuroscience*, vol. 21, no. 10, 2001.

[141] M. Pierri, E. Vaudano, T. Sager, and U. Englund, "KW-6002 protects from MPTP induced dopaminergic toxicity in the mouse," *Neuropharmacology*, vol. 48, no. 4, pp. 517–524, 2005.

[142] H. Kase, "Industry forum: progress in pursuit of therapeutic A2A antagonists—the adenosine A2A receptor selective antagonist KW6002: research and development toward a novel nondopaminergic therapy for Parkinson's disease," *Neurology*, vol. 61, no. 11, supplement 1, pp. S97–S100, 2003.

[143] R. Dungo and E. D. Deeks, "Istradefylline: first global approval," *Drugs*, vol. 73, no. 8, pp. 875–882, 2013.

[144] R. A. Hauser, F. Stocchi, O. Rascol et al., "Preladenant as an adjunctive therapy with levodopa in Parkinson disease: two randomized clinical trials and lessons learned," *JAMA Neurology*, vol. 72, no. 12, pp. 1491–1500, 2015.

[145] B. D. Trapp, L. Bö, S. Mörk, and A. Chang, "Pathogenesis of tissue injury in MS lesions," *Journal of Neuroimmunology*, vol. 98, no. 1, pp. 49–56, 1999.

[146] M.-A. Lécuyer, H. Kebir, and A. Prat, "Glial influences on BBB functions and molecular players in immune cell trafficking," *Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1862, no. 3, pp. 472–482, 2016.

[147] E. N. Benveniste, "Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis," *Journal of Molecular Medicine*, vol. 75, no. 3, pp. 165–173, 1997.

[148] F. L. Heppner, M. Greter, D. Marino et al., "Experimental autoimmune encephalomyelitis repressed by microglial paralysis," *Nature Medicine*, vol. 11, no. 2, pp. 146–152, 2005.

[149] A. R. Simard and S. Rivest, "Bone marrow stem cells have the ability to populate the entire central nervous system into fully differentiated parenchymal microglia," *The FASEB Journal*, vol. 18, no. 9, pp. 998–1000, 2004.

[150] Z. Yu, D. Sun, J. Feng et al., "MSX3 switches microglia polarization and protects from inflammation-induced demyelination," *Journal of Neuroscience*, vol. 35, no. 16, pp. 6350–6365, 2015.

[151] S. C. Starossom, I. D. Mascanfroni, J. Imitola et al., "Galectin-1 deactivates classically activated microglia and protects from inflammation-induced neurodegeneration," *Immunity*, vol. 37, no. 2, pp. 249–263, 2012.

[152] X. Chen, X. Ma, Y. Jiang, R. Pi, Y. Liu, and L. Ma, "The prospects of minocycline in multiple sclerosis," *Journal of Neuroimmunology*, vol. 235, no. 1-2, pp. 1–8, 2011.

[153] A. Wlodarczyk, O. Cédile, K. N. Jensen et al., "Pathologic and protective roles for microglial subsets and bone marrow- and blood-derived myeloid cells in central nervous system inflammation," *Frontiers in Immunology*, vol. 6, article 463, 2015.

[154] A. Shemer and S. Jung, "Differential roles of resident microglia and infiltrating monocytes in murine CNS autoimmunity," *Seminars in Immunopathology*, vol. 37, no. 6, pp. 613–623, 2015.

[155] Z. Jiang, J. X. Jiang, and G. X. Zhang, "Macrophages: a double-edged sword in experimental autoimmune encephalomyelitis," *Immunology Letters*, vol. 160, no. 1, pp. 17–22, 2014.

[156] G. Hasko and P. Pacher, "Regulation of macrophage function by adenosine," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, no. 4, pp. 865–869, 2012.

[157] M. Mayne, P. N. Shepel, Y. Jiang, J. D. Geiger, and C. Power, "Dysregulation of adenosine A_1 receptor-mediated cytokine expression in peripheral blood mononuclear cells from multiple sclerosis patients," *Annals of Neurology*, vol. 45, no. 5, pp. 633–639, 1999.

[158] J. B. Johnston, C. Silva, G. Gonzalez et al., "Diminished adenosine A1 receptor expression on macrophages in brain and blood of patients with multiple sclerosis," *Annals of Neurology*, vol. 49, no. 5, pp. 650–658, 2001.

[159] G. Q. Chen, Y. Y. Chen, X. S. Wang et al., "Chronic caffeine treatment attenuates experimental autoimmune encephalomyelitis induced by guinea pig spinal cord homogenates in Wistar rats," *Brain Research*, vol. 1309, pp. 116–125, 2010.

[160] S. Tsutsui, J. Schnermann, F. Noorbakhsh et al., "A1 adenosine receptor upregulation and activation attenuates neuroinflammation and demyelination in a model of multiple sclerosis," *Journal of Neuroscience*, vol. 24, no. 6, pp. 1521–1529, 2004.

[161] S. R. Jahromi, M. Toghae, M. J. Jahromi, and M. Aloosh, "Dietary pattern and risk of multiple sclerosis," *Iranian Journal of Neurology*, vol. 11, no. 2, pp. 47–53, 2012.

[162] E. Rissanen, J. R. Virta, T. Paavilainen et al., "Adenosine A2A receptors in secondary progressive multiple sclerosis: a [11C]TMSX brain PET study," *Journal of Cerebral Blood Flow and Metabolism*, vol. 33, no. 9, pp. 1394–1401, 2013.

[163] F. Vincenzi, C. Corciulo, M. Targa et al., "Multiple sclerosis lymphocytes upregulate A2A adenosine receptors that are antiinflammatory when stimulated," *European Journal of Immunology*, vol. 43, no. 8, pp. 2206–2216, 2013.

[164] G. Haskó, C. Szabó, Z. H. Németh, V. Kvetan, S. M. Pastores, and E. S. Vizi, "Adenosine receptor agonists differentially regulate IL-10, TNF-α, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice," *Journal of Immunology*, vol. 157, no. 10, pp. 4634–4640, 1996.

[165] S.-Q. Yao, Z.-Z. Li, Q.-Y. Huang et al., "Genetic inactivation of the adenosine A_{2A} receptor exacerbates brain damage in mice with experimental autoimmune encephalomyelitis," *Journal of Neurochemistry*, vol. 123, no. 1, pp. 100–112, 2012.

[166] J. H. Mills, L. F. Thompson, C. Mueller et al., "CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 27, pp. 9325–9330, 2008.

[167] J. Ingwersen, B. Wingerath, J. Graf et al., "Dual roles of the adenosine A2a receptor in autoimmune neuroinflammation," *Journal of Neuroinflammation*, vol. 13, no. 1, article no. 48, 2016.

[168] M. Sitkovsky, D. Lukashev, S. Deaglio, K. Dwyer, S. C. Robson, and A. Ohta, "Adenosine A2A receptor antagonists: blockade of adenosinergic effects and T regulatory cells," *British Journal of Pharmacology*, vol. 153, no. 1, pp. S457–S464, 2008.

[169] T. Wang, N.-N. Xi, Y. Chen et al., "Chronic caffeine treatment protects against experimental autoimmune encephalomyelitis in mice: therapeutic window and receptor subtype mechanism," *Neuropharmacology*, vol. 86, pp. 203–211, 2014.

[170] R. Boia, A. F. Ambrosio, and A. R. Santiago, "Therapeutic opportunities for caffeine and A2A receptor antagonists in retinal diseases," *Ophthalmic Research*, vol. 55, no. 4, pp. 212–218, 2016.

[171] A. R. Santiago, R. Boia, P. Tralhão et al., "Caffeine modulates retinal neuroinflammation and cell survival in retinal ischemia," in *Proceedings of the 12th European Meeting on Glial Cells in Health and Disease*, p. E178, Glia, Bilbao, Spain, 2015.

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Review Article

Neuroinflammation as Fuel for Axonal Regeneration in the Injured Vertebrate Central Nervous System

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Damage to the central nervous system (CNS) is one of the leading causes of morbidity and mortality in elderly, as repair after lesions or neurodegenerative disease usually fails because of the limited capacity of CNS regeneration. The causes underlying this limited regenerative potential are multifactorial, but one critical aspect is neuroinflammation. Although classically considered as harmful, it is now becoming increasingly clear that inflammation can also promote regeneration, if the appropriate context is provided. Here, we review the current knowledge on how acute inflammation is intertwined with axonal regeneration, an important component of CNS repair. After optic nerve or spinal cord injury, inflammatory stimulation and/or modification greatly improve the regenerative outcome in rodents. Moreover, the hypothesis of a beneficial role of inflammation is further supported by evidence from adult zebrafish, which possess the remarkable capability to repair CNS lesions and even restore functionality. Lastly, we shed light on the impact of aging processes on the regenerative capacity in the CNS of mammals and zebrafish. As aging not only affects the CNS, but also the immune system, the regeneration potential is expected to further decline in aged individuals, an element that should definitely be considered in the search for novel therapeutic strategies.

1. Introduction

Brain injuries and neurodegenerative disorders, such as Alzheimer's and Parkinson's disease, multiple sclerosis, or glaucoma, represent a growing social and economic burden and affect an increasing number of people in our aging society. Traumatic lesions and neurodegeneration drastically reduce life quality and lead to severe and often fatal impairments, largely because the central nervous system (CNS) of adult mammals retains only little capacity for regeneration into adulthood, which comprises the replacement of lost neurons (de novo neurogenesis) and/or the repair of damaged axons (axonal regeneration) [1, 2]. The lack of long-distance axonal regeneration in the mature mammalian CNS, on which will be focused here, has been attributed to an insufficient intrinsic growth capacity of its neurons and an inhibitory extrinsic environment [3, 4]. Our current knowledge of the underlying molecules and pathways mainly comes from two well characterized rodent injury models: optic nerve and spinal cord lesions. Damage to the optic nerve, which solely consists of axons originating from the retinal ganglion cells (RGC) located in the inner retina, results in apoptotic RGC death and consequently in vision loss [5–7]. Preservation of injured cells followed by axonal regeneration can be stimulated, both by intrinsic and by extrinsic factors, but full functional recovery has not yet been achieved [8–10]. Spinal cord injuries lead to death of the damaged cells at the epicenter of the lesion, including neurons, oligodendrocytes, and astrocytes. After the primary insult, secondary processes (excitotoxicity, oxidative stress, etc.) may cause additional loss of neurons and supporting cells. Furthermore, interrupted descending and ascending axonal tracts have debilitating consequences, and although proximal segments typically survive, they do not regenerate spontaneously [11–13]. Restoration of motor and sensory tracts via axonal regeneration is believed to be the most promising way to reverse paralysis after spinal cord injury [14]. Regenerative strategies known thus far, as well as identified intracellular pathways and growth-inhibiting factors, are largely similar to those characterised in optic nerve regeneration [15, 16].

In mammals, the acute inflammatory response that takes place rapidly after traumatic CNS lesions is put forward as one of the major elements affecting the regenerative outcome [17]. Microglia, the main mediators of inflammation in the CNS, are among the first cells to respond to damage. They become activated, thereby changing their morphology from ramified to amoeboid, proliferate, migrate to the injury site, and start to produce a variety of pro- and anti-inflammatory cytokines [18]. Furthermore, neutrophils and macrophages from the periphery are recruited to the injured area, and, together with reactive astrocytes, microglia/macrophages will contribute to the formation of a regeneration-inhibiting glial scar [4, 19]. Traditionally, the acute inflammatory response has been viewed as a detrimental orchestrator in CNS damage and pathology. After spinal cord injury, depletion of peripheral macrophages enhances axonal regeneration and improves functional recovery [20]. Administration of the anti-inflammatory drug minocycline gives similar results [21]. However, more recent evidence suggests that the inflammatory response can also positively contribute to regeneration [22, 23], as is exemplified by an improved behavioural outcome after spinal cord injury resulting from an increased number of monocyte-derived macrophages via adoptive transfer [24]. These conflicting results have led to substantial controversy regarding the negative or positive effect of acute inflammation in CNS regeneration. Ongoing and future investigations of its mediator cells and their key regulatory switches thus seem to be essential for a better understanding of how successful regeneration can be induced.

In sharp contrast to mammals, adult zebrafish are capable of extensive and successful regeneration throughout their body, including their fins, heart, liver, and CNS [25, 26]. This has sparked the interest of many neuroscientists, who turned to this small laboratory animal to understand the crucial molecules underlying successful CNS repair. Adult zebrafish retain the capacity of robust axonal regeneration and can morphologically and functionally recover from optic nerve and spinal cord injuries [27]. Moreover, similar to the situation in mammals, an acute inflammatory response occurs after CNS injury in zebrafish, which has recently been suggested to positively contribute to the regenerative process [28, 29]. Given the high degree of conservation between teleosts and mammals at both the molecular and genetic level, research in zebrafish can help to overcome the limitations of nonregenerative mammalian models, in which the regenerative outcome is always suboptimal, even when stimulated [25]. A thorough understanding of how zebrafish can couple acute inflammation to successful regeneration after injury may thus contribute to the development of regenerative therapies.

Since neurodegenerative diseases are age-related, regenerative therapies will often need to be achieved in the senescent CNS. Therefore, the effect of aging on the regenerative potential should not be overlooked. Indeed, aging processes affect CNS functioning, as is evidenced by, for instance, a reduced synaptic efficacy and neuronal loss in the senescent CNS [30, 31], and may further deteriorate the already poor regenerative outcome. Notably, also the immune system is subjected to aging [32–34], which may complicate its functioning during regeneration after CNS injuries. Despite its relevance, the impact of immune senescence on the regenerative capacity in the aged CNS remains poorly understood. Also in zebrafish, which has recently been established as a valuable model for human CNS aging [35], in-depth characterization of the interplay between acute inflammation and axonal regeneration in an aging context is still lacking.

This review aims at providing an overview of the current understanding of how inflammatory factors modify the regenerative outcome after damage in the adult CNS, in both mammals and zebrafish, thereby focusing on microglia and macrophages. Moreover, the effect of aging on inflammatory cell physiology and the implications this may have on the regenerative capacity will be discussed.

2. Acute Inflammation Promotes Axonal Regeneration in Mammals

Over the past decades, intensive research efforts have focused on the discovery of novel targets of which manipulation could enable regeneration after CNS trauma. Modulation of the acute inflammatory response has been proposed as promising strategy to induce axonal regeneration. Compelling evidence for a beneficial influence of different aspects of neuroinflammation has been gathered in various brain injury models [17, 36, 37]. Here, we will focus on the role of inflammation during axonal regeneration after optic nerve and spinal cord injuries.

2.1. Inflammatory Stimulation Improves the Regenerative Outcome. Stimulation of inflammation has proven to be one of the pivotal factors to induce a regenerative response in mammalian axonal regeneration models. For optic nerve injury, early studies have shown that a peripheral nerve graft, lens injury, or intravitreal injection of zymosan, a proinflammatory glucan molecule derived from the yeast cell wall, can induce RGC axon growth in rodents [38–41]. More recently, the smaller molecule Pam_3Cys, which acts on the Toll-like receptor 2 (TLR2), is shown to stimulate axonal regeneration as well [42, 43]. All these experimental procedures induce activation of retinal micro- and macroglia and are accompanied by an influx of neutrophils and macrophages to the vitreous. Therefore, these treatments can collectively be referred to as "inflammatory stimulation" [4, 43].

Similarly, inflammatory stimulation improves the regenerative outcome after spinal cord injury. For example, intraspinal injection of zymosan increases axon growth [44], and stimulation with Pam2CSK4, another TLR2 agonist, was found to reduce axonal loss after spinal cord injury. This neuroprotection is a prerequisite for growth cone formation and subsequent axonal regeneration [45]. Thus, multiple lines of evidence point towards a positive effect of inflammation on (neuroprotection and) axonal regeneration of damaged neurons. However, in order to gain a better understanding of this process, detailed characterization of the nature of inflammatory mediators is indispensable.

2.2. Mediators of Inflammation in Optic Nerve Regeneration

2.2.1. Inflammatory Events at the Injury Site. Similar to other CNS lesions, acute damage to the optic nerve causes changes in the microenvironment directly surrounding the site of the injury. Myelin, which is normally wrapped around the axons, becomes fragmented, leaving the axon tips exposed to myelin-derived inhibitory molecules such as Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp) [4, 7]. Very soon after the injury, resident microglia become reactivated and monocyte-derived macrophages are recruited to the lesion site. Microglia are involved in the reactivation of astrocytes, as they start secreting various cytokines and other factors [46]. Eventually, these glial cells all contribute to the formation of scar tissue, which represents an important barrier to regenerating axons [4, 47]. Furthermore, the actions of microglia/macrophages as well as astrocytes have been suggested to lead to the propagation of secondary degeneration, which contributes to spreading of damage beyond the initial (primary) lesion site [48, 49]. Alternatively, microglia/macrophages have also been suggested to exert beneficial functions at the lesion site, such as phagocytosis of myelin debris or protection against glutamate excitotoxicity. The outcome of microglial/macrophage activation, whether positive or negative, is supposed to be highly dependent on the timing and the precise pathological conditions [49, 50] and thus needs further elucidation.

2.2.2. Inflammatory Events in the Retina. Injury to the optic nerve induces acute inflammatory processes not only at the epicenter of the lesion, but also in the retina, where the RGC cell somata reside. Since stimulation of inflammation in the optic nerve as well as in the eye can protect RGCs, but only the latter promotes axonal regeneration [51], paradigms of inflammatory stimulation focus on altering retinal events.

After optic nerve injury, the resident retinal microglia respond rapidly, which can be considered a primary event resulting from the injury. However, microglial reactivation in the retina has also been linked to secondary degeneration of RGCs, although its exact role is unclear [49]. Yet, microglial reactivation does not occur uniformly across the retina. In the naive adult retina, surveying microglia are mainly found in four layers: the nerve fibre layer (NFL), the ganglion cell layer (GCL), and the inner and outer plexiform layers (IPL and OPL, resp.) [52, 53]. Upon injury, microglia in the OPL remain almost unaffected, while their cell number is increased and morphology is switched from ramified to amoeboid in the inner retinal layers, and most prominently in the GCL [53, 54]. In adult rats, microglial cell numbers augment dramatically from 3 days to 3 weeks after injury and return to almost normal levels by 6 weeks [53]. This increase coincides with the period during which RGC death occurs in response to the injury [5, 52, 53], and microglia actively phagocytize the debris from these RGCs and their axons [52–54]. Notably, the increase in glial cell number after optic nerve injury may either originate from local proliferation or from infiltration of blood-derived macrophages [53], but as it is very difficult to discriminate between these two cell populations [24], the relative contribution of both processes remains unclear.

Interestingly, microglial activation is also observed in the contralateral, uninjured eye. Although the increase in microglial density is not as high as in the ipsilateral retina and is mostly confined to the central retina, clear morphological changes can be observed [52, 53]. This suggests microglial activation in both eyes upon unilateral optic nerve damage, pointing towards cross-talk between both eyes, which may be orchestrated via the optic chiasm or hematogenous transference [53, 55]. Yet, its physiological function remains to be elucidated. Of note, this finding then also clearly indicates that the use of the contralateral eye as an internal control in retinal de- and regeneration studies should be reconsidered [53].

Next to the reactivated amoeboid microglia, another unique, so-called rod microglial morphology has recently been described in the mouse retina after optic nerve injury [56]. Rod microglia are first discovered after injury in the brain cortex [57] and are also described in the retina in a laser-induced ocular hypertension model for glaucoma [58, 59]. After optic nerve injury, the rod microglia are present from day 7 and are completely gone by 6 weeks. They are suggested to form the major group of phagocytic cells in the retina during this time [56].

The functional role of resident microglia in neuroprotection and axonal regeneration after optic nerve injury has not yet been studied in detail. Instead, most attention has been given to the role of retinal macroglia (mostly astrocytes) on the one hand and infiltrating leukocytes (neutrophils and macrophages) on the other hand. Nevertheless, some debate exists on their relative importance. Firstly, axonal regeneration can be stimulated via intravitreal injection of zymosan, which causes the infiltration of neutrophils and macrophages. These cells may in turn serve as a source for oncomodulin, a small calcium-binding protein that has been suggested to be one of the major mediators of the beneficial effect of the intraocular inflammation on axonal regeneration (Figure 1(a)). Its function is dependent on the presence of cAMP and mannose [60–62] and thought to result from its binding to a high-affinity receptor on RGCs [60, 63]. Administration of a peptide competing for oncomodulin receptor binding was found to prohibit axon growth after optic nerve injury in mice [16, 61, 63]. Furthermore, combined deletion of *dectin-1* and *TLR2*, both coding for pattern recognition receptors expressed by inflammatory cells and necessary to respond to inflammatory stimulation, completely abolishes the regeneration-promoting effect of zymosan, again pointing towards the importance of immune mediators in axonal regeneration [64].

Second, inflammatory stimulation also induces the release of cytokines from activated retinal macroglia. Three cytokines from the IL-6 superfamily, namely, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and IL-6, have been proposed as key mediators of the stimulating effect of inflammation on optic nerve regeneration [4, 65, 66] (Figure 1(a)). Indeed, the neuroprotective and axon growth stimulating effects of inflammatory stimulation are diminished in CNTF knock-out mice and abolished in CNTF/LIF double knock-out mice, thereby ascribing a

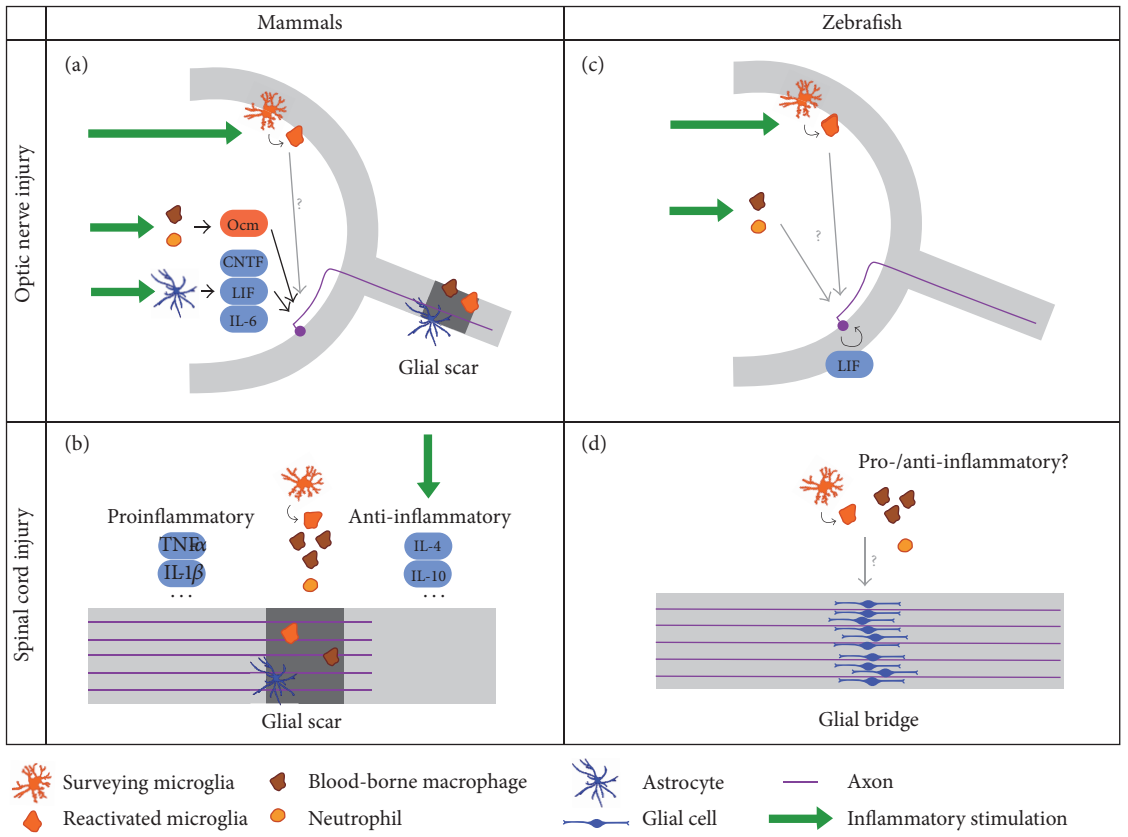

Figure 1: Summary of the current knowledge of the role of acute inflammation in axonal regeneration, in mammals and zebrafish. (a) After optic nerve injury in mammals, surveying retinal microglia become reactivated, proliferate, and transform into amoeboid microglia. Inflammatory stimulation (green arrows), which can be achieved via administration of TLR2 agonists or lens injury, further induces micro- and macroglial cell activation and an influx of neutrophils and blood-borne macrophages to the vitreous. Infiltrating macrophages and neutrophils secrete oncomodulin (Ocm), an inflammatory mediator that is thought to act on RGCs directly. Moreover, inflammatory stimulation elicits the secretion of IL-6 family cytokines from reactive astrocytes. Signal transduction of these cytokines is primarily mediated via the JAK/STAT3 and mTOR pathways in RGCs. Thus, inflammatory stimulation activates the intrinsic growth state of RGCs, and when combined with SOCS3 and/or PTEN deletion, feedback inhibitors of the JAK/STAT3 and mTOR pathway, respectively, axon regeneration beyond the glial scar can be obtained. (b) After an injury in the mammalian spinal cord microglia are activated, and neutrophils and blood-borne macrophages are recruited to the lesion site. Microglia/macrophages mostly adopt the proinflammatory phenotype and secrete proinflammatory cytokines such as TNF-α and IL-1β, while anti-inflammatory microglia/macrophages, which produce anti-inflammatory cytokines including IL-4 and IL-10, only represent a small percentage. Since the proinflammatory type is associated with adverse effects on regeneration, while anti-inflammatory cells are assumed to be protective and growth-promoting, treatments that stimulate anti-inflammatory activation at the expense of the proinflammatory type (green arrows) improve axonal growth beyond the glial scar and coincide with a better regenerative outcome. (c) Stimulation of acute inflammation after optic nerve injury in zebrafish activates microglia and induces recruitment of neutrophils and blood-borne macrophages, mirroring the situation in mammals. This results in an acceleration of the spontaneous regenerative process. Although it has already been shown that LIF and the JAK/STAT3 and mTOR pathways are implicated in optic nerve regeneration in zebrafish as well, the precise mechanism of how the positive effects of acute inflammation is mediated remains elusive. (d) After a spinal cord injury in zebrafish, microglia are activated and neutrophils and blood-borne macrophages infiltrate the lesion site, although their precise contribution to axonal regeneration is still unknown. Despite pro- and anti-inflammatory macrophages have been described in zebrafish injury models outside the CNS, the polarization of microglia/macrophages after spinal cord injury has not yet been studied. Strikingly, a growth-permissive glial bridge is formed at the lesion site, while glial scar is absent in zebrafish.

principal role to CNTF, with LIF as an additional contributing factor [65]. Of note, direct intravitreal administration of CNTF has only limited effects on axonal regeneration, yet this might be explained by the short half-life of the protein in the vitreous [40, 67, 68]. The continuous release of CNTF and LIF by astrocytes after inflammatory stimulation, ensuring prolonged supply to RGCs, can be mimicked via AAV-mediated expression of CNTF in RGCs [69–71] or in Müller glia [72]. This viral CNTF expression has stronger effects on neuroprotection and axonal regeneration than those achieved via intravitreal injection, eliciting long-distance regrowth of axons up to the optic chiasm, but rarely beyond [1, 72].

Altogether, these studies clearly indicate that this group of cytokines plays a determining role in optic nerve regeneration. To date, however, the relative importance of oncomodulin, CNTF, LIF, IL-6, and other inflammatory factors has not yet been fully elucidated.

The importance of acute inflammation as a positive determinant in optic nerve regeneration is also reflected in some of the downstream molecular mechanisms and pathways identified thus far. Signal transduction of IL-6 superfamily cytokines is primarily mediated via the Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway, which has been identified as an important positive player in optic nerve regeneration. The protein suppressor of cytokine signaling 3 (SOCS3), a feedback inhibitor of the JAK/STAT pathway, has counteracting effects on axon regeneration after optic nerve injury. Accordingly, deletion of the SOCS3 gene markedly enhances axon growth and improves the regenerative outcome of intravitreal CNTF administration [4, 10, 73]. Consistently, AAV-mediated overexpression of SOCS3 in RGCs almost completely abolishes RGC regeneration and suppresses the otherwise neurotrophic effect of intravitreal CNTF administration [74]. Of note, it has been shown that SOCS3 expression can be counteracted be delivering cAMP [75], which might explain the positive effect of elevating cAMP levels on axonal regeneration induced by inflammatory stimulation [4, 76]. Deletion of SOCS3, combined with a deletion of phosphatase and tensin homolog (PTEN), an upstream inhibitor of the mammalian target of rapamycin (mTOR) pathway that is also repeatedly shown to inhibit axonal regeneration, whether or not in combination with inflammatory stimulation [76–78], has been reported to induce a remarkable regenerative response [8–10, 76]. One study that uses PTEN deletion in combination with zymosan and a cAMP analog even reports scanty reinnervation of visual brain areas, including the lateral geniculate nucleus and the superior colliculus, and partial visual recovery [8]. Recently, it has also been shown that enhancing neural activity of RGCs via visual stimulation or chemogenetics, in combination with stimulation of mTOR activity by overexpressing the positive regulator Ras homolog enriched in brain 1 (Rheb1), enables long-distance and target-specific RGC axonal regeneration. This is accompanied by partial restoration of visual function [79]. Furthermore, continuous AAV-driven expression of hyper IL-6 cytokine (hIL-6), a designer cytokine that consist of the covalently linked bioactive parts of IL-6 and IL-6Rα, in RGCs has been recently described as the most potent unifactorial treatment to promote axonal regeneration known thus far. When combined with PTEN deletion, hIL-6 improves optic nerve regeneration even more, with some axons reaching the chiasm within 3 weeks after optic nerve injury [78]. Conclusively, novel therapeutic approaches based on recent insights in the beneficial role of inflammatory mediators in regenerative processes hold exciting promise.

2.3. Mediators of Inflammation in Spinal Cord Regeneration

2.3.1. Inflammatory Events at the Injury Site. Similar to other CNS injuries, damage to the spinal cord is followed by an acute inflammatory response. Resident microglia are activated, and neutrophils, macrophages, and lymphocytes infiltrate the lesion site. Also here, this inflammatory reaction eventually becomes chronic, and reactive astrocytes form a regeneration-inhibiting glial scar [19, 80, 81]. In the search for the exact contribution of inflammation to the regenerative potential, most studies in the field of spinal cord regeneration have focused on the role of microglia and blood-borne macrophages. Resident microglia are the first to respond to the injury, and infiltrating macrophages reach the injury site during the following days [82–86]. Most of these monocyte-derived macrophages originate from the spleen, while only a minority is mobilized from the bone marrow reservoir [86].

It has been proposed that different states of microglia/macrophage activation may influence the repair process [87]. Indeed, monocyte-derived macrophages polarize into different phenotypes, which are determined by the microenvironment and may change in response to new stimuli [88]. These functional states are generally divided into two main classes, based on the activation pathway, known as the "classically activated" proinflammatory M1 macrophages and the "alternatively activated" anti-inflammatory M2 macrophages. Later, additional subtypes of M2 (M2a, M2b, and M2c) have been described. However, macrophage activation is far more diverse than these simple categories. As such, the M1/M2 phenotypes rather represent two extreme poles, with in between a whole spectrum of activation states with overlapping properties [89–92]. A similar polarization has also been described for microglia [18]. Although there is now a general consensus that this M1/M2 classification of microglia/macrophage activation is an oversimplification, it nevertheless persists as a conceptual framework to facilitate our understanding of microglia/macrophage function [93].

After spinal cord injury, proinflammatory, M1-like macrophages are associated with secondary tissue damage, neuronal loss, axon retraction and demyelination, while anti-inflammatory, M2-like macrophages are assumed to be protective and promoting axon growth. In this regard, the balance between pro- and anti-inflammatory macrophages could be a major factor determining the regenerative outcome [19, 23]. Indeed, it has been demonstrated that most microglia/macrophages in the injured spinal cord display an M1-like activation state, with only a limited and transient presence of M2-like cells. Moreover, evidence suggests that lesion-derived factors (cytokines, chemokines, etc.) affect the microglial/macrophage phenotype, thereby favouring the proinflammatory state [94]. Therefore, it has been hypothesized that shifting macrophage activation towards the anti-inflammatory state may improve the regenerative outcome, mirroring successful tissue repair responses such as those occurring after skin or muscle injuries [87]. Some recent studies have indeed provided evidence for the beneficial effect of an augmented number of M2-like macrophages. For example, transfer of in vitro polarized M2-like macrophages to the damaged spinal cord improves functional recovery. Notably, this transfer of M2-like cells is suggested to alter the local microenvironment, thereby promoting the anti-inflammatory state [95]. Moreover, blocking of the IL-6 signaling pathway, via inhibition of the IL-6 receptor, results in

an increase in M2-like microglia/macrophages at the expense of the M1-like type. Indeed, this treatment inhibits classical activation and promotes the alternative pathway and is accompanied by an improved functional recovery [96]. Taken together, these studies suggest that promoting alternative microglial/macrophage activation is a promising strategy to induce spinal cord regeneration (Figure 1(b)). Of note, it has been argued that activated microglia and monocyte-derived macrophages form functionally distinct, nonredundant cell populations after spinal cord injury and do not contribute equally to the repair process. As such, it has been suggested that microglia rather than infiltrating macrophages unequivocally express markers for the pro- or anti-inflammatory phenotype [86]. Moreover, the beneficial secretion of the anti-inflammatory cytokine IL-10 is attributed to (a subset of) infiltrating macrophages but cannot be provided by the activated resident microglia [24]. Yet, the relative contribution of microglia and macrophages still needs further clarification.

2.3.2. Inflammatory Events at the Cell Soma of the Injured Neurons. Similar to the visual system, one should not only consider acute inflammation at the injury site itself but also turn to the cell soma of the axotomised neurons. However, the spinal cord is a much more complex structure than the optic nerve. While the latter only consists of RGC axons, the spinal cord is a well organised structure comprising neurons and axons of different types [97]. The cell bodies of the axotomised axons after spinal cord injury thus reside at different locations, challenging an unequivocal study of the inflammatory events that are provoked there. It has been repeatedly shown that spinal cord injuries induce widespread microglial activation in different brain areas, also outside the regions where the cell bodies of descending axon tracts are located [98–100]. However, this has mostly been associated with cognitive impairments and neuropathic pain as a result of the injury and not yet to regenerative processes. Thus, whether modification of these inflammatory responses would contribute to enhanced regeneration of the spinal cord remains elusive.

3. Successful Coupling of Neuroinflammation and CNS Regeneration in Zebrafish

Since adult zebrafish are capable of functional recovery after many CNS injuries, including optic nerve and spinal cord damage [27], they provide an attractive approach to study the interplay of inflammatory processes and CNS repair in a regenerative-supporting setting. Research in mammals may benefit from comparative studies in zebrafish. Amongst other advantages, this species provides an example of how acute inflammation can be linked to successful axonal regeneration. Importantly, the zebrafish immune system is highly comparable to its mammalian counterpart. The major immune cell lineages have been identified in zebrafish, and many of the immune receptor classes, signaling pathways, and inflammatory mediators are conserved as well [101].

3.1. Acute Inflammation in Zebrafish Optic Nerve Regeneration. Also in zebrafish, optic nerve injury models are well established. However, in contrast to mammals, in which a vast number of RGCs undergo apoptosis after optic nerve injury [5, 102], the large majority of their zebrafish counterparts seem protected and do survive the lesion [103, 104]. In a subsequent regenerative process, the damaged RGCs regrow their axons and reinnervate the target areas in the brain, of which the optic tectum is by far the most important [105–107]. The reestablished synaptic contacts are remarkably accurate and visual function eventually recovers, as shown by means of various vision-guided behavioural assays [27, 104].

Although the flawless regenerative responses after optic nerve injury in zebrafish are well known, the underlying cellular and molecular bases are less well understood. Nevertheless, analysis of gene expression patterns after optic nerve injury has already provided a framework for further functional studies [108, 109], and recent efforts have started to uncover the regulating mechanisms (recently reviewed by [110]). Noteworthy, no glial scar tissue is formed at the injury site. This may be attributed to the presumed absence of astrocytes in zebrafish. Instead, radial glial cells are assumed to take over at least part of the functions of mammalian astrocytes throughout the zebrafish CNS. Thus, the absence of a regeneration-inhibiting glial upon injury, may to some extent be explained by the functional differences between mammalian astrocytes and zebrafish radial glia [111].

Of importance, there is evidence to support the hypothesis that acute retinal neuroinflammation might provide the right context for the initiation of a regenerative response. Firstly, an acute inflammatory response is observed after optic nerve crush in fish, which has been suggested to positively contribute to regeneration. More specifically, the number of microglia/macrophages in the retina increases significantly from 3 days after optic nerve crush onwards but is resolved around 3 weeks after the injury [103]. Furthermore, intravitreal application of zymosan, which results in a massive number of neutrophils and microglia/macrophages in the retina, efficiently stimulates optic nerve regeneration in zebrafish-[103] (Figure 1(c)). Of note, application of zymosan has been associated with folding and retinal detachment of the mouse retina [112]. Although this cannot completely be ruled out, these effects have not been reported in zebrafish. In our laboratory, we have been able to confirm the positive effect of intravitreal zymosan injection on the regenerative process. Zymosan administration 3 days prior to optic nerve injury highly stimulated RGC axonal regeneration, assessed at 7 days after injury via anterograde biocytin tracing as described previously [113, 114]. Indeed, reinnervation of the optic tectum was found to be significantly increased in the zymosan-treated group, indicating that inflammatory stimulation accelerated the regenerative response after optic nerve injury in zebrafish (Figure 2). Thus, also in zebrafish inflammatory cells seem to have extensive effects on regeneration.

Second, recent evidence indicates that also in zebrafish the IL-6 cytokine superfamily stimulates axonal regeneration in an autocrine/paracrine manner, where especially LIF, rather than CNTF or IL-6, seems to be involved. LIF is upregulated upon injury in the fish retina and suggested to play a beneficial role in the early phase of the regenerative process [115, 116]. Strikingly and similar to mammals, endogenous

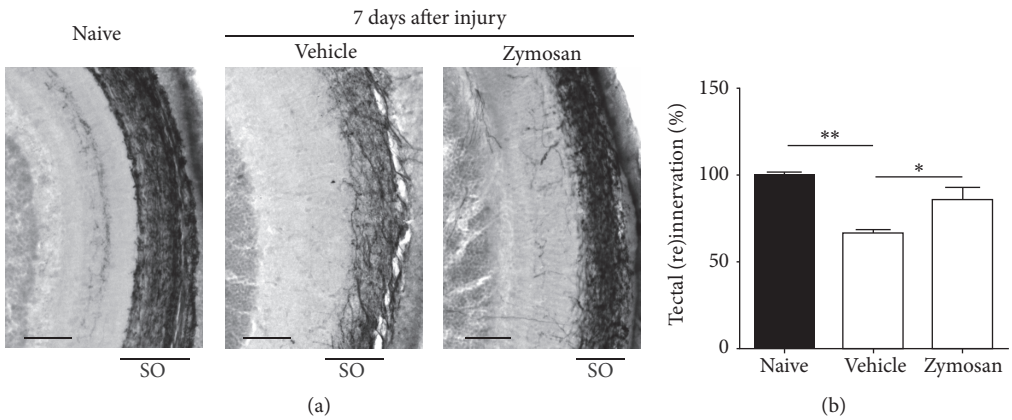

Figure 2: Intravitreal injection of zymosan accelerates axonal regeneration in zebrafish. (a) Representative images of biocytin-labeled axons in the contralateral optic tectum of naive fish (left) and fish treated with vehicle or zymosan, at 7 days after optic nerve injury (middle and right, resp.). The stratum opticum (SO), the layer through which the RGC axons innervate the tectum, is indicated. Scale bar = 50 μm. (b) Quantification of the reinnervated area of the optic tectum in fish treated with vehicle or zymosan at 7 days after injury, relative to naive fish. Intravitreal injection of zymosan significantly accelerates reinnervation, which is already close to naive levels in zymosan treated fish as compared to vehicle-injected fish. Data represent mean ± SEM ($n \geq 3$ animals per group, $^{*}p < 0.05$, and $^{**}p < 0.01$).

expression of zebrafish Socs3a also counteracts regeneration after optic nerve injury, as is evidenced by enhanced RGC axonal regeneration after Socs3a knockdown. However, despite the activation of this inhibitory pathway, zebrafish still possess the ability of robust axonal regeneration [115]. Furthermore, inhibiting mTOR activity compromises optic nerve regeneration. This indicates a supportive role for mTOR, although it seems ancillary rather than essential for zebrafish axonal regeneration [117]. Clearly, factors counteracting regeneration are not absent in zebrafish, yet evolution seems to have provided them with a way to overcome these inhibitory mechanisms. The key to successful regeneration in mammals may thus reside in finding a proper balance between growth inhibition and stimulation [1, 110].

3.2. Acute Inflammation in Zebrafish Spinal Cord Regeneration. Models to study spinal cord regeneration are well developed in adult zebrafish, where, again, damage to the spinal cord is followed by a spontaneous regeneration process. After a complete spinal cord transection, a growth-permissive glial cell bridge is formed between the rostral and caudal lesion site [118] and, by two weeks after injury, cerebrospinal axons have started to regenerate beyond the transection site. This axonal regeneration correlates with functional recovery, and most fish regain their swimming abilities by five to eight weeks after injury [27, 119]. Of note, the regenerative potential is not equal for all axons in the spinal cord, as some axon types show only poor regrowth [27]. Apart from axonal regeneration, regenerative neurogenesis has also been described after spinal cord injury in zebrafish. Motor neurons and different types of interneurons are generated from radial glial cells in the region adjacent to the injury site [120]. It has been demonstrated that some newly generated motor neurons may even be capable of connecting with their peripheral muscle targets, indicative of effective integration into the existing spinal network [121]. However, since this only applies to a small number of newborn motor neurons, it remains uncertain whether regenerative neurogenesis significantly contributes to functional recovery [122].

Damage to the zebrafish spinal cord induces an acute inflammatory response, including the activation of microglia and monocyte-derived macrophages [123, 124]. However, its functional contribution to the regenerative process remains largely unexplored [125] (Figure 1(d)). One study reports that lysophosphatidic acid has proinflammatory but antiregenerative effects after spinal cord injury, in zebrafish as well as in mice [126]. However, this does not completely rule out the possibility of a beneficial role of inflammatory cells, since their phenotype can differ upon different types of stimulation, as described above. Besides, it has been demonstrated that zebrafish Ptena negatively affects regeneration after spinal cord injury, a finding that mirrors the observations in mammals [127] and can be linked to inflammatory pathways, as described above. Lastly, in a model of motor neuron ablation in larval zebrafish, in which motor neurons regenerate from spinal progenitor cells, the microglia/macrophages that gather at the lesion site are suggested to play a beneficial role during regeneration. Indeed, suppressing the immune response via treatment with dexamethasone, a synthetic glucocorticoid with anti-inflammatory effects [128] significantly suppresses motor neuron regeneration [129]. Yet, in-depth characterization of the role of acute inflammation in zebrafish spinal cord regeneration is lacking.

3.3. Acute Inflammation in Other Zebrafish Regenerative Models. Neuroinflammation has been put forward as an important underpinning of successful regeneration in other zebrafish CNS injury models as well. In contrast to mammals, in which a stab injury in the CNS is followed by massive neuronal cell death, reactive gliosis, and the eventual formation of a growth-inhibiting glial scar [28, 130–132], zebrafish can

recover from such a lesion. Although cell death and reactive proliferation of microglia, oligodendrocytes, and other cells are observed in zebrafish as well, this resolves quickly and no evidence of glial scar tissue can be found [28, 29, 132]. Furthermore, zebrafish are able to initiate massive regenerative neurogenesis to compensate for the lost neurons. These newly generated neurons derive from proliferating radial glia cells [133]. Importantly, it has been proposed that acute neuroinflammation acts as a beneficial regulator of de novo neurogenesis in zebrafish. Systemic treatment with dexamethasone reduces the number of microglia/macrophages at the lesion site, coinciding with a diminished proliferation of radial glia and less newborn neurons, thus preventing regeneration after stab injury to the telencephalon [29]. Furthermore, the leukotriene signaling pathway appears to be a pivotal component of the inflammatory reaction and leukotriene C4 (LTC4), which signals through the cysteinyl leukotriene receptor 1 (CysLT1), is found to be necessary and sufficient for the initiation of neural progenitor proliferation and subsequent neurogenesis. Conclusively, inflammation appears to positively affect the reparative capacities in the zebrafish CNS [28, 29, 134, 135].

3.4. Macrophage Polarization in Zebrafish. Recently, M1- and M2-like macrophage subsets have also been observed in zebrafish. They express some of the markers that are typical for their mammalian counterparts. After caudal fin amputation or bacterial infection in larval fish, these macrophage phenotypes are activated in a time-dependent manner. Mirroring successful wound healing in mammals, monocyte-derived macrophages are recruited to the site of injury, where they first adopt a proinflammatory M1-like phenotype. In a later stage, this phenotype is progressively converted to M2-like, encompassing intermediate phenotypes in which both M1 and M2 markers are expressed [136]. This sequential M1-M2 response is supposed to be optimal for regeneration and contrasts the overwhelming presence of proinflammatory M1 microglia/macrophages observed after CNS injuries in mammals. Although supporting evidence from other zebrafish regeneration models is still needed, this study provides a first indication of functional similarities of microglia/macrophages in zebrafish and mammals.

4. The Impact of Inflammaging

To date, most studies aiming at the induction of CNS regeneration in mammals have been performed in young adult animals. However, the effect of aging on the regenerative mechanisms should not be overlooked. The limited regenerative capacity of the adult mammalian CNS further declines upon aging, and aging processes compromise the implementation of therapeutic strategies [137]. This is a major concern, especially since aging is one of the most important risk factors for many neurodegenerative diseases, and regenerative therapies are therefore most needed in elderly. Importantly, the immune system is subjected to aging as well, adding another level of complexity to this issue. Here, we will focus on the physiology of microglia and blood-borne macrophages in an aging context, and the consequences for repair after CNS injury.

4.1. Inflammaging in Mammals. It is well recognized that mammalian aging is accompanied by a low-grade, chronic proinflammatory state, which is also referred to as "inflammaging" and can be considered as a manifestation of immunosenescence [34, 138, 139]. Inflammaging is a systemic phenomenon that also affects the CNS. In the rodent retina, it is associated with morphological changes and functional impairments of microglia [32, 140]. This includes a slight but significant increase in microglial cell density, which is also observed in some other brain regions [141, 142], but not in all [143, 144]. One explanation for this phenomenon might be a reduced functionality of individual microglia, resulting in the necessity for more cells [32, 145]. Secondly, the ordered distribution of microglia throughout the retina seems to be deteriorating upon aging: they migrate from the inner retina towards the periphery and accumulate in the subretinal space, which is normally devoid of microglia [141, 142]. In addition, aged retinal microglia show reduced arborisation and slower process motilities, which likely compromise their dynamic surveying behaviour, further suggesting functional defects [141]. As similar observations are made in other brain regions as well [146, 147], there is increasing evidence for an age-related decline in the ability of microglia to perform their normal tasks in the CNS.

In addition, microglia develop age-related changes in their immune physiology. Evidence suggests that they adopt an altered, more inflammatory status, associated with increased expression of activation markers and proinflammatory cytokines [32]. This phenomenon has also been referred to as microglial sensitization or "priming." Indeed, the microglial responsiveness to stressors or damage cues from the local environment or the periphery is increased in aged individuals, and the resulting inflammatory reaction is more pronounced than in young adults [148, 149]. Thus, although seemingly contradictory, the functional impairments associated with microglial senescence may be accompanied by an exaggerated response to stress or injury because of microglial priming [149]. Of importance, these age-related changes in microglial function may render the CNS more vulnerable to neurodegeneration, but may also highly impact regenerative abilities [33, 150].

4.2. The Impact of Inflammaging on Mammalian CNS Regeneration. As a result of microglial priming, detrimental effects of the immune response upon injury may be promoted in aged individuals, thereby suppressing the beneficial aspects of inflammation and further restricting regenerative capacities. Some studies have already provided evidence for this hypothesis in different injury models. Firstly, after traumatic brain injury, the microglial response was indeed found to be more pronounced and prolonged in aged mice compared to young adults [151]. Another study shows that traumatic brain injury results in larger lesions in aged mice. Alongside, the immune activation is exaggerated, and an increased ratio of pro- to anti-inflammatory microglia/macrophages has been demonstrated [152]. Secondly, reduced functional recovery upon

aging is also observed after spinal cord injury in mice and has been correlated with impairments in the induction of IL-4 receptor α (IL-4Rα) expression in microglia. The authors suggest that the impaired recovery in aged mice is related to a reduced responsiveness of microglia/macrophages to IL-4 and thus a shift towards a proinflammatory cytokine activation [153]. Recently, this research group also demonstrated an age-related decline in macrophage IL-10 expression after spinal cord injury. Since IL-10 is a key indicator of M2-like activation, this points to a reduction in the number of anti-inflammatory macrophages, and thus a disrupted balance between macrophage phenotypes, which presumably underlies the observed impairment in functional recovery with age [154]. Lastly, age-related deteriorations of microglia have also been found after damage to the retina. In a laser-induced injury model, senescent microglia respond more slowly; that is, their process motility and migration rate are reduced. In addition, senescent microglia remain present at the injury site for a longer time period than in their young adult counterparts, indicating that resolution of the inflammatory reaction is retarded upon aging. Unfortunately, age-related differences in injury severity and functional recovery were not addressed in this study [141]. Conclusively, aging of microglia and blood-derived macrophages undoubtedly affects the regenerative capacity after CNS injuries.

4.3. The Effect of Inflammaging on the Zebrafish Regenerative Potential. Recently, the zebrafish has emerged as a valuable model for human aging. Substantial evidence points towards the presence of aging hallmarks in zebrafish, also in the CNS (reviewed by [35]). Therefore, it is an attractive model organism to shed light on the relationship between aging and regenerative capacities. An age-dependent decline in the regenerative capacity of the spinal cord has already been suggested [155], based on early findings in goldfish [156]. However, a comprehensive study of the effect of aging on axonal regeneration in the zebrafish CNS is still lacking, and the impact of inflammation herein has not yet been investigated. Currently, the only evidence that age-related alterations in the immune system may potentially underlie a diminished regenerative capacity in senescent fish comes from a model of optic nerve remyelination. Of note, in both rodents and zebrafish, the ability to restore myelin sheaths is high in young individuals but decreases upon aging. It has been suggested that after optic nerve demyelination in aged zebrafish, the reduced remyelination is a result of an impaired response of microglia/macrophages. Indeed, while microglia/macrophages are recruited to the lesion site in young adults at four days after injury, their number at this time point is not significantly different from the naive condition in aged fish [157]. Overall, although an association between altered inflammation and attenuated regeneration upon aging has already been put forward, the impact of (inflamm)aging on the normally flawless regenerative process in the zebrafish CNS is scarcely studied and awaits further in-depth characterization.

5. Conclusion

Over the past years, neurodegenerative diseases and CNS trauma have been a major focus on neuroscience research, with many studies dedicated to the elucidation of the cellular and molecular changes that underlie their pathology. The innate immune system is undoubtedly involved in the pathogenesis of many of these CNS conditions, yet increasing evidence suggests that it can also beneficially contribute to the regenerative process. Indeed, a balanced activity of inflammatory cell types, of which microglia and blood-borne macrophages are the most studied, has been shown to improve morphological and functional recovery after injury in optic nerve and spinal cord injury models.

Unlike mammals, zebrafish possess a powerful regenerative capacity after CNS lesions, which leads to successful repair and seems to coincide with a favourable inflammatory state. Further uncovering of the mechanisms that control inflammatory and regenerative processes might provide fruitful insights that may lead to the identification of innovative therapeutic targets for human patients.

Noteworthy, we foresee that an important hurdle will have to be taken in the development of novel CNS regenerative strategies from bench to bedside, being the fact that aging processes affect the already limited regenerative potential in mammals. Since the innate immune system is subjected to aging as well, it is assumed to react differently to injuries in aged individuals. Increasing evidence for a detrimental effect of inflammaging on the regenerative outcome is emerging, but further in-depth characterization in both mammals and zebrafish is highly warranted.

Abbreviations

AAV: Adeno-associated virus
cAMP: Cyclic adenosine monophosphate
CNS: Central nervous system
CNTF: Ciliary neurotrophic factor
CysLT1: Cysteinyl leukotriene receptor 1
GCL: Ganglion cell layer
hIL-6: Hyper IL-6 cytokine
IL-4Rα: IL-4 receptor α
IPL: Inner plexiform layer
JAK/STAT: Janus Kinase/Signal Transducers and Activators of Transcription
LIF: Leukemia inhibitory factor
LTC4: Leukotriene C4
mTOR: Mammalian target of rapamycin
NFL: Nerve fiber layer
OPL: Outer plexiform layer
PTEN: Phosphatase and tensin homolog
RGC: Retinal ganglion cell
Rheb1: Ras homolog enriched in brain 1
SOCS3: Suppressor of cytokine signaling 3
TLR2: Toll-like receptor 2
MAG: Myelinassociated glycoprotein
OMgp: Oligodendrocyte-myelin glycoprotein.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] V. Pernet and M. E. Schwab, "Lost in the jungle: new hurdles for optic nerve axon regeneration," *Trends in Neurosciences*, vol. 37, no. 7, pp. 381–387, 2014.

[2] M. H. Tuszynski and O. Steward, "Concepts and methods for the study of axonal regeneration in the CNS," *Neuron*, vol. 74, no. 5, pp. 777–791, 2012.

[3] E. A. Huebner and S. M. Strittmatter, "Axon regeneration in the peripheral and central nervous systems," *Results and Problems in Cell Differentiation*, vol. 48, pp. 339–351, 2009.

[4] D. Fischer and M. Leibinger, "Promoting optic nerve regeneration," *Progress in Retinal and Eye Research*, vol. 31, no. 6, pp. 688–701, 2012.

[5] M. Berkelaar, D. B. Clarke, Y.-C. Wang, G. M. Bray, and A. J. Aguayo, "Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats," *Journal of Neuroscience*, vol. 14, no. 7, pp. 4368–4374, 1994.

[6] S. Choudhury, Y. Liu, A. F. Clark, and I.-H. Pang, "Caspase-7: a critical mediator of optic nerve injury-induced retinal ganglion cell death," *Molecular Neurodegeneration*, vol. 10, article 40, 2015.

[7] M. Berry, Z. Ahmed, B. Lorber, M. Douglas, and A. Logan, "Regeneration of axons in the visual system," *Restorative Neurology and Neuroscience*, vol. 26, no. 2-3, pp. 147–174, 2008.

[8] S. De Lima, Y. Koriyama, T. Kurimoto et al., "Full-length axon regeneration in the adult mouse optic nerve and partial recovery of simple visual behaviors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 23, pp. 9149–9154, 2012.

[9] X. Luo, Y. Salgueiro, S. R. Beckerman, V. P. Lemmon, P. Tsoulfas, and K. K. Park, "Three-dimensional evaluation of retinal ganglion cell axon regeneration and pathfinding in whole mouse tissue after injury," *Experimental Neurology*, vol. 247, pp. 653–662, 2013.

[10] F. Sun, K. K. Park, S. Belin et al., "Sustained axon regeneration induced by co-deletion of PTEN and SOCS3," *Nature*, vol. 480, no. 7377, pp. 372–375, 2011.

[11] S. Thuret, L. D. F. Moon, and F. H. Gage, "Therapeutic interventions after spinal cord injury," *Nature Reviews Neuroscience*, vol. 7, no. 8, pp. 628–643, 2006.

[12] E. J. Bradbury and S. B. McMahon, "Spinal cord repair strategies: why do they work?" *Nature Reviews Neuroscience*, vol. 7, no. 8, pp. 644–653, 2006.

[13] M. Liu, W. Wu, H. Li et al., "Necroptosis, a novel type of programmed cell death, contributes to early neural cells damage after spinal cord injury in adult mice," *Journal of Spinal Cord Medicine*, vol. 38, no. 6, pp. 745–753, 2015.

[14] O. Steward and R. Willenberg, "Rodent spinal cord injury models for studies of axon regeneration," *Experimental Neurology*, vol. 287, pp. 374–383, 2017.

[15] W. Young, "Spinal cord regeneration," *Cell Transplantation*, vol. 23, no. 4-5, pp. 573–611, 2014.

[16] G. F. Butt, A. Habib, K. Mahgoub et al., "Optic nerve regeneration," *Expert Review of Ophthalmology*, vol. 7, no. 6, pp. 533–554, 2012.

[17] L. I. Benowitz and P. G. Popovich, "Inflammation and axon regeneration," *Current Opinion in Neurology*, vol. 24, no. 6, pp. 577–583, 2011.

[18] M. Czeh, P. Gressens, and A. M. Kaindl, "The yin and yang of microglia," *Developmental Neuroscience*, vol. 33, no. 3-4, pp. 199–209, 2011.

[19] D. Lee-Liu, G. Edwards-Faret, V. S. Tapia, and J. Larraín, "Spinal cord regeneration: lessons for mammals from non-mammalian vertebrates," *Genesis*, vol. 51, no. 8, pp. 529–544, 2013.

[20] P. G. Popovich, Z. Guan, P. Wei, I. Huitinga, N. van Rooijen, and B. T. Stokes, "Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury," *Experimental Neurology*, vol. 158, no. 2, pp. 351–365, 1999.

[21] D. P. Stirling, K. Khodarahmi, J. Liu et al., "Minocycline treatment reduces delayed oligodendrocyte death, attenuates axonal dieback, and improves functional outcome after spinal cord injury," *Journal of Neuroscience*, vol. 24, no. 9, pp. 2182–2190, 2004.

[22] V. W. Yong and S. Rivest, "Taking advantage of the systemic immune system to cure brain diseases," *Neuron*, vol. 64, no. 1, pp. 55–60, 2009.

[23] S. David and A. Kroner, "Repertoire of microglial and macrophage responses after spinal cord injury," *Nature Reviews Neuroscience*, vol. 12, no. 7, pp. 388–399, 2011.

[24] R. Shechter, A. London, C. Varol et al., "Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice," *PLoS Medicine*, vol. 6, no. 7, Article ID e1000113, 2009.

[25] M.-C. Keightley, C.-H. Wang, V. Pazhakh, and G. J. Lieschke, "Delineating the roles of neutrophils and macrophages in zebrafish regeneration models," *The International Journal of Biochemistry & Cell Biology*, vol. 56, pp. 92–106, 2014.

[26] W. Shi, Z. Fang, L. Li, and L. Luo, "Using zebrafish as the model organism to understand organ regeneration," *Science China Life Sciences*, vol. 58, no. 4, pp. 343–351, 2015.

[27] T. Becker and C. G. Becker, "Axonal regeneration in zebrafish," *Current Opinion in Neurobiology*, vol. 27, pp. 186–191, 2014.

[28] N. Kyritsis, C. Kizil, and M. Brand, "Neuroinflammation and central nervous system regeneration in vertebrates," *Trends in Cell Biology*, vol. 24, no. 2, pp. 128–135, 2014.

[29] N. Kyritsis, C. Kizil, S. Zocher et al., "Acute inflammation initiates the regenerative response in the adult zebrafish brain," *Science*, vol. 338, no. 6112, pp. 1353–1356, 2012.

[30] T. A. Rando, "Stem cells, ageing and the quest for immortality," *Nature*, vol. 441, no. 7097, pp. 1080–1086, 2006.

[31] M. Yeoman, G. Scutt, and R. Faragher, "Insights into CNS ageing from animal models of senescence," *Nature Reviews Neuroscience*, vol. 13, no. 6, pp. 435–445, 2012.

[32] W. T. Wong, "Microglial aging in the healthy CNS: phenotypes, drivers, and rejuvenation," *Frontiers in Cellular Neuroscience*, vol. 7, article 22, 2013.

[33] W. J. Streit and Q.-S. Xue, "Microglial senescence," *CNS and Neurological Disorders - Drug Targets*, vol. 12, no. 6, pp. 763–767, 2013.

[34] A. Salminen, K. Kaarniranta, and A. Kauppinen, "Inflammaging: disturbed interplay between autophagy and inflammasomes," *Aging*, vol. 4, no. 3, pp. 166–175, 2012.

[35] J. Van houcke, L. De Groef, E. Dekeyster, and L. Moons, "The zebrafish as a gerontology model in nervous system aging, disease, and repair," *Ageing Research Reviews*, vol. 24, pp. 358–368, 2015.

[36] G. Szalay, B. Martinecz, N. Lénárt et al., "Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke," *Nature Communications*, vol. 7, Article ID 11499, 2016.

[37] D. J. Loane and A. Kumar, "Microglia in the TBI brain: the good, the bad, and the dysregulated," *Experimental Neurology*, vol. 275, pp. 316–327, 2016.

[38] S. Leon, Y. Yin, J. Nguyen, N. Irwin, and L. I. Benowitz, "Lens injury stimulates axon regeneration in the mature rat optic nerve," *Journal of Neuroscience*, vol. 20, no. 12, pp. 4615–4626, 2000.

[39] D. Fischer, M. Pavlidis, and S. Thanos, "Cataractogenic lens injury prevents traumatic ganglion cell death and promotes axonal regeneration both in vivo and in culture," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 12, pp. 3943–3954, 2000.

[40] B. Lorber, M. Berry, and A. Logan, "Lens injury stimulates adult mouse retinal ganglion cell axon regeneration via both macrophage- and lens-derived factors," *European Journal of Neuroscience*, vol. 21, no. 7, pp. 2029–2034, 2005.

[41] M. Berry, J. Carlile, A. Hunter, W.-L. Tsang, P. Rosustrel, and J. Sievers, "Optic nerve regeneration after intravitreal peripheral nerve implants: trajectories of axons regrowing through the optic chiasm into the optic tracts," *Journal of Neurocytology*, vol. 28, no. 9, pp. 721–741, 1999.

[42] T. G. Hauk, M. Leibinger, A. Müller, A. Andreadaki, U. Knippschild, and D. Fischer, "Stimulation of axon regeneration in the mature optic nerve by intravitreal application of the toll-like receptor 2 agonist Pam3Cys," *Investigative Ophthalmology and Visual Science*, vol. 51, no. 1, pp. 459–464, 2010.

[43] D. Fischer, "Stimulating axonal regeneration of mature retinal ganglion cells and overcoming inhibitory signaling," *Cell and Tissue Research*, vol. 349, no. 1, pp. 79–85, 2012.

[44] J. C. Gensel, S. Nakamura, Z. Guan, N. van Rooijen, D. P. Ankeny, and P. G. Popovich, "Macrophages promote axon regeneration with concurrent neurotoxicity," *Journal of Neuroscience*, vol. 29, no. 12, pp. 3956–3968, 2009.

[45] D. P. Stirling, K. Cummins, M. Mishra, W. Teo, V. W. Yong, and P. Stys, "Toll-like receptor 2-mediated alternative activation of microglia is protective after spinal cord injury," *Brain*, vol. 137, no. 3, pp. 707–723, 2014.

[46] X.-T. Yang, G.-H. Huang, D.-F. Feng, and K. Chen, "Insight into astrocyte activation after optic nerve injury," *Journal of Neuroscience Research*, vol. 93, no. 4, pp. 539–548, 2015.

[47] J. M. Cregg, M. A. DePaul, A. R. Filous, B. T. Lang, A. Tran, and J. Silver, "Functional regeneration beyond the glial scar," *Experimental Neurology*, vol. 253, pp. 197–207, 2014.

[48] H.-Y. Li, Y.-W. Ruan, C.-R. Ren, Q. Cui, and K.-F. So, "Mechanisms of secondary degeneration after partial optic nerve transection," *Neural Regeneration Research*, vol. 9, no. 6, pp. 565–574, 2014.

[49] M. Fitzgerald, C. A. Bartlett, A. R. Harvey, and S. A. Dunlop, "Early events of secondary degeneration after partial optic nerve transection: an immunohistochemical study," *Journal of Neurotrauma*, vol. 27, no. 2, pp. 439–452, 2010.

[50] Y. Yin, Q. Cui, Y. Li et al., "Macrophage-derived factors stimulate optic nerve regeneration," *Journal of Neuroscience*, vol. 23, no. 6, pp. 2284–2293, 2003.

[51] Z. Ahmed, M. Aslam, B. Lorber, E. L. Suggate, M. Berry, and A. Logan, "Optic nerve and vitreal inflammation are both RGC neuroprotective but only the latter is RGC axogenic," *Neurobiology of Disease*, vol. 37, no. 2, pp. 441–454, 2010.

[52] P. Sobrado-Calvo, M. Vidal-Sanz, and M. P. Villegas-Pérez, "Rat retinal microglial cells under normal conditions, after optic nerve section, and after optic nerve section and intravitreal injection of trophic factors or macrophage inhibitory factor," *Journal of Comparative Neurology*, vol. 501, no. 6, pp. 866–878, 2007.

[53] L. Cen, M. Han, L. Zhou et al., "Bilateral retinal microglial response to unilateral optic nerve transection in rats," *Neuroscience*, vol. 311, pp. 56–66, 2015.

[54] E. Garcia-Valenzuela, S. C. Sharma, and A. L. Piña, "Multilayered retinal microglial response to optic nerve transection in rats," *Molecular Vision*, vol. 11, pp. 225–231, 2005.

[55] N. Bodeutsch, H. Siebert, C. Dermon, and S. Thanos, "Unilateral injury to the adult rat optic nerve causes multiple cellular responses in the contralateral site," *Journal of Neurobiology*, vol. 38, no. 1, pp. 116–128, 1999.

[56] T.-F. Yuan, Y.-X. Liang, B. Peng, B. Lin, and K.-F. So, "Local proliferation is the main source of rod microglia after optic nerve transection," *Scientific Reports*, vol. 5, Article ID 10788, 2015.

[57] J. M. Ziebell, S. E. Taylor, T. Cao, J. L. Harrison, and J. Lifshitz, "Rod microglia: elongation, alignment, and coupling to form trains across the somatosensory cortex after experimental diffuse brain injury," *Journal of Neuroinflammation*, vol. 9, article 247, 2012.

[58] B. Rojas, B. I. Gallego, A. I. Ramirez et al., "Microglia in mouse retina contralateral to experimental glaucoma exhibit multiple signs of activation in all retinal layers," *Journal of Neuroinflammation*, vol. 11, no. 1, article no. 133, 2014.

[59] R. de Hoz, B. I. Gallego, A. I. Ramírez et al., "Rod-like microglia are restricted to eyes with laser-induced ocular hypertension but absent from the microglial changes in the contralateral untreated eye," *PLoS ONE*, vol. 8, no. 12, Article ID e83733, 2013.

[60] Y. Yin, M. T. Henzl, B. Lorber et al., "Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells," *Nature Neuroscience*, vol. 9, no. 6, pp. 843–852, 2006.

[61] T. Kurimoto, Y. Yin, G. Habboub et al., "Neutrophils express oncomodulin and promote optic nerve regeneration," *Journal of Neuroscience*, vol. 33, no. 37, pp. 14816–14824, 2013.

[62] L. I. Benowitz, Z. He, and J. L. Goldberg, "Reaching the brain: advances in optic nerve regeneration," *Experimental Neurology*, vol. 287, part 3, pp. 365–373, 2017.

[63] Y. Yin, Q. Cui, H.-Y. Gilbert et al., "Oncomodulin links inflammation to optic nerve regeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 46, pp. 19587–19592, 2009.

[64] K. T. Baldwin, K. S. Carbajal, B. M. Segal, and R. J. Giger, "Neuroinflammation triggered by β-glucan/dectin-1 signaling enables CNS axon regeneration," *Proceedings of the National*

Academy of Sciences of the United States of America, vol. 112, no. 8, pp. 2581–2586, 2015.

[65] M. Leibinger, A. Müller, A. Andreadaki, T. G. Hauk, M. Kirsch, and D. Fischer, "Neuroprotective and axon growth-promoting effects following inflammatory stimulation on mature retinal ganglion cells in mice depend on ciliary neurotrophic factor and leukemia inhibitory factor," *Journal of Neuroscience*, vol. 29, no. 45, pp. 14334–14341, 2009.

[66] M. Leibinger, A. Müller, P. Gobrecht, H. Diekmann, A. Andreadaki, and D. Fischer, "Interleukin-6 contributes to CNS axon regeneration upon inflammatory stimulation," *Cell Death and Disease*, vol. 4, article e609, 2013.

[67] A. Müller, T. G. Hauk, M. Leibinger, R. Marienfeld, and D. Fischer, "Exogenous CNTF stimulates axon regeneration of retinal ganglion cells partially via endogenous CNTF," *Molecular and Cellular Neuroscience*, vol. 41, no. 2, pp. 233–246, 2009.

[68] A. Müller, T. G. Hauk, and D. Fischer, "Astrocyte-derived CNTF switches mature RGCs to a regenerative state following inflammatory stimulation," *Brain*, vol. 130, no. 12, pp. 3308–3320, 2007.

[69] S. G. Leaver, Q. Cui, O. Bernard, and A. R. Harvey, "Cooperative effects of bcl-2 and AAV-mediated expression of CNTF on retinal ganglion cell survival and axonal regeneration in adult transgenic mice," *European Journal of Neuroscience*, vol. 24, no. 12, pp. 3323–3332, 2006.

[70] S. G. Leaver, Q. Cui, G. W. Plant et al., "AAV-mediated expression of CNTF promotes long-term survival and regeneration of adult rat retinal ganglion cells," *Gene Therapy*, vol. 13, no. 18, pp. 1328–1341, 2006.

[71] M. Hellström, M. A. Pollett, and A. R. Harvey, "Post-injury delivery of rAAV2-CNTF combined with short-term pharmacotherapy is neuroprotective and promotes extensive axonal regeneration after optic nerve trauma," *Journal of Neurotrauma*, vol. 28, no. 12, pp. 2475–2483, 2011.

[72] V. Pernet, S. Joly, D. Dalkara et al., "Long-distance axonal regeneration induced by CNTF gene transfer is impaired by axonal misguidance in the injured adult optic nerve," *Neurobiology of Disease*, vol. 51, pp. 202–213, 2013.

[73] P. D. Smith, F. Sun, K. K. Park et al., "SOCS3 deletion promotes optic nerve regeneration in vivo," *Neuron*, vol. 64, no. 5, pp. 617–623, 2009.

[74] M. Hellström, J. Muhling, E. M. Ehlert et al., "Negative impact of rAAV2 mediated expression of SOCS3 on the regeneration of adult retinal ganglion cell axons," *Molecular and Cellular Neuroscience*, vol. 46, no. 2, pp. 507–515, 2011.

[75] K. K. Park, Y. Hu, J. Muhling et al., "Cytokine-induced SOCS expression is inhibited by cAMP analogue: impact on regeneration in injured retina," *Molecular and Cellular Neuroscience*, vol. 41, no. 3, pp. 313–324, 2009.

[76] T. Kurimoto, Y. Yin, K. Omura et al., "Long-distance axon regeneration in the mature optic nerve: contributions of oncomodulin, cAMP, and pten gene deletion," *Journal of Neuroscience*, vol. 30, no. 46, pp. 15654–15663, 2010.

[77] K. K. Park, K. Liu, Y. Hu et al., "Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway," *Science*, vol. 322, no. 5903, pp. 963–966, 2008.

[78] M. Leibinger, A. Andreadaki, P. Gobrecht, E. Levin, H. Diekmann, and D. Fischer, "Boosting central nervous system axon regeneration by circumventing limitations of natural cytokine signaling," *Molecular Therapy*, vol. 24, no. 10, pp. 1712–1725, 2016.

[79] J.-H. A. Lim, B. K. Stafford, P. L. Nguyen et al., "Neural activity promotes long-distance, target-specific regeneration of adult retinal axons," *Nature Neuroscience*, vol. 19, no. 8, pp. 1073–1084, 2016.

[80] H. Prüss, M. A. Kopp, B. Brommer et al., "Non-resolving aspects of acute inflammation after spinal cord injury (SCI): indices and resolution plateau," *Brain Pathology*, vol. 21, no. 6, pp. 652–660, 2011.

[81] B. S. Mietto, K. Mostacada, and A. M. B. Martinez, "Neurotrauma and inflammation: CNS and PNS responses," *Mediators of Inflammation*, vol. 2015, Article ID 251204, 14 pages, 2015.

[82] D. Davalos, J. Grutzendler, G. Yang et al., "ATP mediates rapid microglial response to local brain injury in vivo," *Nature Neuroscience*, vol. 8, no. 6, pp. 752–758, 2005.

[83] K. D. Beck, H. X. Nguyen, M. D. Galvan, D. L. Salazar, T. M. Woodruff, and A. J. Anderson, "Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment," *Brain*, vol. 133, no. 2, pp. 433–447, 2010.

[84] D. J. Donnelly, E. E. Longbrake, T. M. Shawler et al., "Deficient CX3CR1 signaling promotes recovery after mouse spinal cord injury by limiting the recruitment andactivation of Ly6Clo/iNOS+ macrophages," *Journal of Neuroscience*, vol. 31, no. 27, pp. 9910–9922, 2011.

[85] P. G. Popovich and W. F. Hickey, "Bone marrow chimeric rats reveal the unique distribution of resident and recruited macrophages in the contused rat spinal cord," *Journal of Neuropathology & Experimental Neurology*, vol. 60, no. 7, pp. 676–685, 2001.

[86] L. V. Blomster, F. H. Brennan, H. W. Lao, D. W. Harle, A. R. Harvey, and M. J. Ruitenberg, "Mobilisation of the splenic monocyte reservoir and peripheral CX3CR1 deficiency adversely affects recovery from spinal cord injury," *Experimental Neurology*, vol. 247, pp. 226–240, 2013.

[87] J. C. Gensel and B. Zhang, "Macrophage activation and its role in repair and pathology after spinal cord injury," *Brain Research*, vol. 1619, pp. 1–11, 2015.

[88] R. D. Stout and J. Suttles, "Functional plasticity of macrophages: reversible adaptation to changing microenvironments," *Journal of Leukocyte Biology*, vol. 76, no. 3, pp. 509–513, 2004.

[89] S. David, A. D. Greenhalgh, and A. Kroner, "Macrophage and microglial plasticity in the injured spinal cord," *Neuroscience*, vol. 307, pp. 311–318, 2015.

[90] P. Murray, J. Allen, S. Biswas et al., "Macrophage activation and polarization: nomenclature and experimental guidelines," *Immunity*, vol. 41, no. 1, pp. 14–20, 2014.

[91] R. Shechter and M. Schwartz, "Harnessing monocyte-derived macrophages to control central nervous system pathologies: no longer 'if' but 'how'," *The Journal of Pathology*, vol. 229, no. 2, pp. 332–346, 2013.

[92] D. M. Mosser and J. P. Edwards, "Exploring the full spectrum of macrophage activation," *Nature Reviews Immunology*, vol. 8, no. 12, pp. 958–969, 2008.

[93] X. Hu, R. K. Leak, Y. Shi et al., "Microglial and macrophage polarization—new prospects for brain repair," *Nature Reviews Neurology*, vol. 11, no. 1, pp. 56–64, 2015.

[94] K. A. Kigerl, J. C. Gensel, D. P. Ankeny, J. K. Alexander, D. J. Donnelly, and P. G. Popovich, "Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord," *Journal of Neuroscience*, vol. 29, no. 43, pp. 13435–13444, 2009.

[95] S.-F. Ma, Y.-J. Chen, J.-X. Zhang et al., "Adoptive transfer of M2 macrophages promotes locomotor recovery in adult rats after spinal cord injury," *Brain, Behavior, and Immunity*, vol. 45, pp. 157–170, 2015.

[96] A. R. Guerrero, K. Uchida, H. Nakajima et al., "Blockade of interleukin-6 signaling inhibits the classic pathway and promotes an alternative pathway of macrophage activation after spinal cord injury in mice," *Journal of Neuroinflammation*, vol. 9, article 40, 2012.

[97] M. C. Bear, B. W. Paradiso, and M. A. Neuroscience, *Neuroscience: Exploring the Brain*, Lippincott Williams & Wilkins, Philadelphia, Pa, USA, 2007.

[98] J. Wu, Z. Zhao, B. Sabirzhanov et al., "Spinal cord injury causes brain inflammation associated with cognitive and affective changes: role of cell cycle pathways," *Journal of Neuroscience*, vol. 34, no. 33, pp. 10989–11006, 2014.

[99] Z. Li, H. Wei, S. Piirainen et al., "Spinal versus brain microglial and macrophage activation traits determine the differential neuroinflammatory responses and analgesic effect of minocycline in chronic neuropathic pain," *Brain, Behavior, and Immunity*, vol. 58, pp. 107–117, 2016.

[100] M. R. Detloff, L. C. Fisher, V. McGaughy, E. E. Longbrake, P. G. Popovich, and D. M. Basso, "Remote activation of microglia and pro-inflammatory cytokines predict the onset and severity of below-level neuropathic pain after spinal cord injury in rats," *Experimental Neurology*, vol. 212, no. 2, pp. 337–347, 2008.

[101] N. Oosterhof, E. Boddeke, and T. J. van Ham, "Immune cell dynamics in the CNS: learning from the zebrafish," *GLIA*, vol. 63, no. 5, pp. 719–735, 2015.

[102] G. Kalesnykas, E. N. Oglesby, D. J. Zack et al., "Retinal ganglion cell morphology after optic nerve crush and experimental glaucoma," *Investigative Ophthalmology and Visual Science*, vol. 53, no. 7, pp. 3847–3857, 2012.

[103] S. Zou, C. Tian, S. Ge, and B. Hu, "Neurogenesis of retinal ganglion cells is not essential to visual functional recovery after optic nerve injury in adult zebrafish," *PLOS ONE*, vol. 8, no. 2, Article ID e57280, 2013.

[104] S. Kato, T. Matsukawa, Y. Koriyama, K. Sugitani, and K. Ogai, "A molecular mechanism of optic nerve regeneration in fish: the retinoid signaling pathway," *Progress in Retinal and Eye Research*, vol. 37, pp. 13–30, 2013.

[105] C. G. Becker and T. Becker, "Gradients of ephrin-A2 and ephrin-A5b mRNA during retinotopic regeneration of the optic projection in adult zebrafish," *Journal of Comparative Neurology*, vol. 427, no. 3, pp. 469–483, 2000.

[106] C. G. Becker and T. Becker, "Growth and pathfinding of regenerating axons in the optic projection of adult fish," *Journal of Neuroscience Research*, vol. 85, no. 12, pp. 2793–2799, 2007.

[107] A. T. McCurley and G. V. G. V. Callard, "Time course analysis of gene expression patterns in zebrafish eye during optic nerve regeneration," *Journal of Experimental Neuroscience*, vol. 4, pp. 17–33, 2010.

[108] P. I. Fuller-Carter, K. W. Carter, D. Anderson, A. R. Harvey, K. M. Giles, and J. Rodger, "Integrated analyses of zebrafish miRNA and mRNA expression profiles identify miR-29b and miR-223 as potential regulators of optic nerve regeneration," *BMC Genomics*, vol. 16, no. 1, article 591, 2015.

[109] A. T. McCurley and G. V. Callard, "Time course analysis of gene expression patterns in zebrafish eye during optic nerve regeneration," *Journal of Experimental Neuroscience*, vol. 4, no. 1, pp. 17–33, 2010.

[110] J. P. Rasmussen and A. Sagasti, "Learning to swim, again: axon regeneration in fish," *Experimental Neurology*, vol. 287, part 3, pp. 318–330, 2017.

[111] D. A. Lyons and W. S. Talbot, "Glial cell development and function in zebrafish," *Cold Spring Harbor Perspectives in Biology*, vol. 7, no. 2, Article ID a020586, 2015.

[112] A. Tassoni, A. Gutteridge, A. C. Barber, A. Osborne, and K. R. Martin, "Molecular mechanisms mediating retinal reactive gliosis following bone marrow mesenchymal stem cell transplantation," *Stem Cells*, vol. 33, no. 10, pp. 3006–3016, 2015.

[113] C. G. Becker and T. Becker, "Gradients of ephrin-A2 and ephrin-A5b mRNA during retinotopic regeneration of the optic projection in adult zebrafish," *Journal of Comparative Neurology*, vol. 427, no. 3, pp. 469–483, 2000.

[114] K. Lemmens, I. Bollaerts, S. Bhumika et al., "Matrix metalloproteinases as promising regulators of axonal regrowth in the injured adult zebrafish retinotectal system," *Journal of Comparative Neurology*, vol. 524, no. 7, pp. 1472–1493, 2016.

[115] F. Elsaeidi, M. A. Bemben, X.-F. Zhao, and D. Goldman, "Jak/Stat signaling stimulates zebrafish optic nerve regeneration and overcomes the inhibitory actions of Socs3 and Sfpq," *The Journal of Neuroscience*, vol. 34, no. 7, pp. 2632–2644, 2014.

[116] K. Ogai, A. Kuwana, S. Hisano et al., "Upregulation of Leukemia Inhibitory Factor (LIF) during the early stage of optic nerve regeneration in zebrafish," *PLoS ONE*, vol. 9, no. 8, Article ID e106010, 2014.

[117] H. Diekmann, P. Kalbhen, and D. Fischer, "Active mechanistic target of rapamycin plays an ancillary rather than essential role in zebrafish CNS axon regeneration," *Frontiers in Cellular Neuroscience*, vol. 9, article 251, 2015.

[118] Y. Goldshmit, T. E. Sztal, P. R. Jusuf, T. E. Hall, M. Nguyen-Chi, and P. D. Currie, "Fgf-dependent glial cell bridges facilitate spinal cord regeneration in zebrafish," *The Journal of Neuroscience*, vol. 32, no. 22, pp. 7477–7492, 2012.

[119] K. Vajn, D. Suler, J. A. Plunkett, and M. Oudega, "Temporal profile of endogenous anatomical repair and functional recovery following spinal cord injury in adult zebrafish," *PLOS ONE*, vol. 9, no. 8, Article ID e105857, 2014.

[120] C. G. Becker and T. Becker, "Neuronal regeneration from ependymo-radial glial cells: cook, little pot, cook!," *Developmental Cell*, vol. 32, no. 4, pp. 516–527, 2015.

[121] M. M. Reimer, I. Sörensen, V. Kuscha et al., "Motor neuron regeneration in adult zebrafish," *Journal of Neuroscience*, vol. 28, no. 34, pp. 8510–8516, 2008.

[122] T. B. Dias, Y.-J. Yang, K. Ogai, T. Becker, and C. G. Becker, "Notch signaling controls generation of motor neurons in the lesioned spinal cord of adult zebrafish," *Journal of Neuroscience*, vol. 32, no. 9, pp. 3245–3252, 2012.

[123] T. Becker and C. G. Becker, "Regenerating descending axons preferentially reroute to the gray matter in the presence of a general macrophage/microglial reaction caudal to a spinal transection in adult zebrafish," *Journal of Comparative Neurology*, vol. 433, no. 1, pp. 131–147, 2001.

[124] S. P. Hui, A. Dutta, and S. Ghosh, "Cellular response after crush injury in adult zebrafish spinal cord," *Developmental Dynamics*, vol. 239, no. 11, pp. 2962–2979, 2010.

[125] O. Bloom, "Non-mammalian model systems for studying neuro-immune interactions after spinal cord injury," *Experimental Neurology*, vol. 258, pp. 130–140, 2014.

[126] Y. Goldshmit, R. Matteo, T. Sztal et al., "Blockage of lysophosphatidic acid signaling improves spinal cord injury outcomes," *American Journal of Pathology*, vol. 181, no. 3, pp. 978–992, 2012.

[127] D. Liu, Y. Yu, and M. Schachner, "Ptena, but not Ptenb, reduces regeneration after spinal cord injury in adult zebrafish," *Experimental Neurology*, vol. 261, pp. 196–205, 2014.

[128] A. E. Coutinho and K. E. Chapman, "The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights," *Molecular and Cellular Endocrinology*, vol. 335, no. 1, pp. 2–13, 2011.

[129] J. Ohnmacht, Y. Yang, G. W. Maurer et al., "Spinal motor neurons are regenerated after mechanical lesion and genetic ablation in larval zebrafish," *Development*, vol. 143, no. 9, pp. 1464–1474, 2016.

[130] M. V. Sofroniew, "Molecular dissection of reactive astrogliosis and glial scar formation," *Trends in Neurosciences*, vol. 32, no. 12, pp. 638–647, 2009.

[131] S. Robel, B. Berninger, and M. Götz, "The stem cell potential of glia: lessons from reactive gliosis," *Nature Reviews Neuroscience*, vol. 12, no. 2, pp. 88–104, 2011.

[132] M. März, R. Schmidt, S. Rastegar, and U. Strahle, "Regenerative response following stab injury in the adult zebrafish telencephalon," *Developmental Dynamics*, vol. 240, no. 9, pp. 2221–2231, 2011.

[133] V. Kroehne, D. Freudenreich, S. Hans, J. Kaslin, and M. Brand, "Regeneration of the adult zebrafish brain from neurogenic radial glia-type progenitors," *Development*, vol. 138, no. 22, pp. 4831–4841, 2011.

[134] M. Gemberling, T. J. Bailey, D. R. Hyde, and K. D. Poss, "The zebrafish as a model for complex tissue regeneration," *Trends in Genetics*, vol. 29, no. 11, pp. 611–620, 2013.

[135] C. Kizil, N. Kyritsis, and M. Brand, "Effects of inflammation on stem cells: together they strive?" *EMBO Reports*, vol. 16, no. 4, pp. 416–426, 2015.

[136] M. Nguyen-Chi, B. Laplace-Builhe, J. Travnickova et al., "Identification of polarized macrophage subsets in zebrafish," *eLife*, vol. 4, Article ID e07288, 2015.

[137] I. P. Johnson, "Age-related neurodegenerative disease research needs aging models," *Frontiers in Aging Neuroscience*, vol. 7, article no. 168, 2015.

[138] C. Franceschi, M. Bonafè, S. Valensin et al., "Inflamm-aging. An evolutionary perspective on immunosenescence," *Annals of the New York Academy of Sciences*, vol. 908, pp. 244–254, 2000.

[139] B. Giunta, F. Fernandez, W. V. Nikolic et al., "Inflammaging as a prodrome to Alzheimer's disease," *Journal of Neuroinflammation*, vol. 5, article 51, 2008.

[140] S. L. Patterson, "Immune dysregulation and cognitive vulnerability in the aging brain: interactions of microglia, IL-1β, BDNF and synaptic plasticity," *Neuropharmacology*, vol. 96, part A, pp. 11–18, 2015.

[141] M. R. Damani, L. Zhao, A. M. Fontainhas, J. Amaral, R. N. Fariss, and W. T. Wong, "Age-related alterations in the dynamic behavior of microglia," *Aging Cell*, vol. 10, no. 2, pp. 263–276, 2011.

[142] H. Xu, M. Chen, and J. V. Forrester, "Para-inflammation in the aging retina," *Progress in Retinal and Eye Research*, vol. 28, no. 5, pp. 348–368, 2009.

[143] H. D. VanGuilder, G. V. Bixler, R. M. Brucklacher et al., "Concurrent hippocampal induction of MHC II pathway components and glial activation with advanced aging is not correlated with cognitive impairment," *Journal of Neuroinflammation*, vol. 8, article 138, 2011.

[144] S.-M. Ye and R. W. Johnson, "Increased interleukin-6 expression by microglia from brain of aged mice," *Journal of Neuroimmunology*, vol. 93, no. 1-2, pp. 139–148, 1999.

[145] W. J. Streit and Q.-S. Xue, "The brain's aging immune system," *Aging and Disease*, vol. 1, no. 3, pp. 254–261, 2010.

[146] P. R. Mouton, J. M. Long, D.-L. Lei et al., "Age and gender effects on microglia and astrocyte numbers in brains of mice," *Brain Research*, vol. 956, no. 1, pp. 30–35, 2002.

[147] M.-È. Tremblay, M. L. Zettel, J. R. Ison, P. D. Allen, and A. K. Majewska, "Effects of aging and sensory loss on glial cells in mouse visual and auditory cortices," *GLIA*, vol. 60, no. 4, pp. 541–558, 2012.

[148] D. M. Norden and J. P. Godbout, "Review: microglia of the aged brain: primed to be activated and resistant to regulation," *Neuropathology and Applied Neurobiology*, vol. 39, no. 1, pp. 19–34, 2013.

[149] D. M. Norden, M. M. Muccigrosso, and J. P. Godbout, "Microglial priming and enhanced reactivity to secondary insult in aging, and traumatic CNS injury, and neurodegenerative disease," *Neuropharmacology*, vol. 96, part A, pp. 29–41, 2015.

[150] W. J. Streit, N. W. Sammons, A. J. Kuhns, and D. L. Sparks, "Dystrophic microglia in the aging human brain," *GLIA*, vol. 45, no. 2, pp. 208–212, 2004.

[151] R. Sandhir, G. Onyszchuk, and N. E. J. Berman, "Exacerbated glial response in the aged mouse hippocampus following controlled cortical impact injury," *Experimental Neurology*, vol. 213, no. 2, pp. 372–380, 2008.

[152] A. Kumar, B. A. Stoica, B. Sabirzhanov, M. P. Burns, A. I. Faden, and D. J. Loane, "Traumatic brain injury in aged animals increases lesion size and chronically alters microglial/macrophage classical and alternative activation states," *Neurobiology of Aging*, vol. 34, no. 5, pp. 1397–1411, 2013.

[153] A. M. Fenn, J. C. E. Hall, J. C. Gensel, P. G. Popovich, and J. P. Godbout, "IL-4 signaling drives a unique arginase$^+$/IL-1β^+ microglia phenotype and recruits macrophages to the inflammatory CNS: consequences of age-related deficits in IL-4Rα after traumatic spinal cord injury," *Journal of Neuroscience*, vol. 34, no. 26, pp. 8904–8917, 2014.

[154] B. Zhang, W. M. Bailey, K. J. Braun, and J. C. Gensel, "Age decreases macrophage IL-10 expression: implications for functional recovery and tissue repair in spinal cord injury," *Experimental Neurology*, vol. 273, pp. 83–91, 2015.

[155] T. Becker, M. F. Wullimann, C. G. Becker, R. R. Bernhardt, and M. Schachner, "Axonal regrowth after spinal cord transection in adult zebrafish," *Journal of Comparative Neurology*, vol. 377, no. 4, pp. 577–595, 1997.

[156] J. J. Bernstein, "Relation of spinal cord regeneration to age in adult goldfish," *Experimental Neurology*, vol. 9, no. 2, pp. 161–174, 1964.

[157] E. J. Münzel, C. G. Becker, T. Becker, and A. Williams, "Zebrafish regenerate full thickness optic nerve myelin after demyelination, but this fails with increasing age," *Acta Neuropathologica Communications*, vol. 2, article 77, 2014.

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Review Article

Neuropeptides and Microglial Activation in Inflammation, Pain, and Neurodegenerative Diseases

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Microglial cells are responsible for immune surveillance within the CNS. They respond to noxious stimuli by releasing inflammatory mediators and mounting an effective inflammatory response. This is followed by release of anti-inflammatory mediators and resolution of the inflammatory response. Alterations to this delicate process may lead to tissue damage, neuroinflammation, and neurodegeneration. Chronic pain, such as inflammatory or neuropathic pain, is accompanied by neuroimmune activation, and the role of glial cells in the initiation and maintenance of chronic pain has been the subject of increasing research over the last two decades. Neuropeptides are small amino acidic molecules with the ability to regulate neuronal activity and thereby affect various functions such as thermoregulation, reproductive behavior, food and water intake, and circadian rhythms. Neuropeptides can also affect inflammatory responses and pain sensitivity by modulating the activity of glial cells. The last decade has witnessed growing interest in the study of microglial activation and its modulation by neuropeptides in the hope of developing new therapeutics for treating neurodegenerative diseases and chronic pain. This review summarizes the current literature on the way in which several neuropeptides modulate microglial activity and response to tissue damage and how this modulation may affect pain sensitivity.

1. Introduction

Microglial cells are the resident macrophage-like cells of the central nervous system (CNS). They are in charge of immune surveillance and of sampling the microenvironment through their numerous cellular processes. When faced with an insult or pathogenic agent they rapidly respond by developing a classic proinflammatory program (termed M1), releasing inflammatory mediators such as tumour necrosis factor- (TNF-) α, Interleukin- (IL-) 1β, IL-12, reactive oxygen species (ROS), nitric oxide (NO), prostaglandins (PGs), and chemokines that help recruit other immune cells to the injured zone and amplify the inflammatory response. Once the noxious stimulus has been dealt with, it is crucial that the inflammatory response be dampened and resolved; this is achieved by active redirection of microglial phenotype towards an alternative immunomodulatory or acquired deactivation profile (termed M2), characterized by release of anti-inflammatory cytokines such as transforming growth factor- (TGF-) β and IL-10, and expression of Arginase-1 (AG1) [1]. These immunomodulatory mediators inhibit the release of proinflammatory factors from immune and nonimmune cells and promote tissue regeneration, thereby facilitating the return to homeostasis. When the resolution phase of the inflammatory response is altered, excessive damage to the affected tissue may ensue, potentially leading to cell death and neurodegeneration. In fact, microglial proinflammatory activation has been implicated in the pathology of many neurodegenerative disorders such as Parkinson's disease (PD) [2], Alzheimer's disease (AD) [3], multiple sclerosis (MS), and AIDS dementia [4] where neuronal damage occurs as a consequence of a prolonged proinflammatory response

and more recently, in several neuropsychiatric conditions [5]. Thus, the modulation of microglial activation is of great importance in the context of inflammatory and degenerative diseases of the CNS.

Over the last two decades, increasing attention has been brought to the role of glial cells in the development and maintenance of chronic pain, cumulative evidence suggesting that chronic pain could be the result of dysregulated glial activity [6]. Mediators such as proinflammatory cytokines, chemokines, PGE_2, and NO produced mainly by microglial cells and by other nonneuronal cells of the nervous and immune systems are known to contribute to pain hypersensitivity by activating nociceptive neurons in the CNS and in the peripheral nervous system (PNS) [7]. Concordantly, changes in morphological features and expression of molecular markers characteristic of an activated microglial phenotype have been observed in different animal models of nerve injury and pain [8], strongly suggesting reactive microglia might be involved in these pathological processes of the nervous system. In particular, many studies have demonstrated a central role for microglial p38 mitogen-activated protein kinase (MAPK) activation in the pathogenesis of neuropathic pain [7]. The role of this kinase in microglial signaling is pivotal since it can be activated by multiple microglial receptors, and it also regulates the synthesis of many inflammatory mediators associated with pain facilitation. Moreover, p38 MAPK is selectively activated in spinal microglial cells after spinal nerve ligation (SNL), and administration of a p38 MAPK inhibitor significantly attenuates allodynia [7], underscoring its role in nociception. Another well-recognized mediator of microglial-neuron communication in neuropathic pain transmission is brain-derived neurotrophic factor (BDNF), which is induced and released from microglial cells upon ATP stimulation, and mediates the depolarizing shift in the anion reversal potential in spinal neurons underlying neuropathic pain [9]. To further underscore the role of microglia in nociception, administration of the microglial inhibitor minocycline has been shown to attenuate pain hypersensitivity in models of pain facilitation [10], spinal cord injury (SCI) [11], burn injury-induced pain [12], SNL [13], inflammation-evoked hyperalgesia [14], and chronic constriction injury (CCI) [15], among others. Altogether, evidence suggests that modulators of microglial activation could potentially be employed as antinociceptive agents in pain management.

Neuropeptides are small amino acidic molecules produced mainly, though not exclusively, by cells of the nervous system. Typically, they have the capacity to regulate neuronal activity and may affect a great variety of central and peripheral functions, such as thermoregulation, reproductive behavior, food and water intake, and circadian rhythms [16]. Neuropeptides are also major regulators of immune and inflammatory responses; they affect pain sensitivity and are known to play an important role in neurogenesis, acting not only on neuronal cells but also on glial cells. Microglial cells express a variety of neuropeptide and neurotransmitter receptors which modulate microglial function [17], and the last decade has witnessed growing interest in the study of microglial activation, their role in neuroinflammation and neurodegeneration, and their modulation by neuropeptides.

Here, we review the role of neuropeptides in the modulation of microglial function (Figure 1). We have selected neuropeptides for which microglial cells are known to express specific receptors (Table 1), which might therefore directly affect microglial activity and influence processes such as inflammation, neurodegeneration, and pain sensitivity. Although the main focus is set on microglial cells, some data obtained in macrophages is provided in order to discuss similarities and differences between these cell types. It is important to bear in mind that microglial cells comprise a very heterogeneous population, and this heterogeneity also applies to their ability to respond to neuropeptides; in vitro studies using isolated microglia have demonstrated that only a fraction of the whole population actually responds to a certain neuropeptide and that this fraction can be altered depending on the activation state of the cells [18]. Consequently, caution should be taken when interpreting data from isolated microglia, and more importantly, when comparing the behavior of primary microglia to that of peripheral macrophages or to that of a microglia/macrophage cell line.

2. POMC-Derived Peptides

Within the CNS, the proopiomelanocortin (POMC) gene is mainly expressed in neurons from the arcuate nucleus (ARC) of the hypothalamus which project into the paraventricular nucleus, lateral hypothalamus, and other regions of the brain such as amygdala, cortex, hippocampus, medulla, mesencephalon, and spinal cord [19]. Through the action of tissue-specific prohormone convertases, it is posttranslationally processed to yield adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), β-MSH, and γ-MSH, collectively known as melanocortins, and the endogenous opioid β-endorphin (β-END). The melanocortin system is involved in the regulation of a great variety of functions such as food intake and energy expenditure, sexual behavior, exocrine gland secretion, fever control, pigmentation, learning, and memory [20, 21]. This system is also tightly linked to control of inflammation, both centrally and peripherally, by exerting anti-inflammatory actions on cells of the immune system including lymphocytes, monocytes, and macrophages, as well as nonimmune cells such as melanocytes, keratinocytes, and adipocytes, among others [22]. Melanocortins may also regulate macrophage differentiation into tissue-specific cells such as osteoclasts, Kupffer cells, and microglia [23], further highlighting the role of these neuropeptides as modulators of immunity.

Despite the long recognized anti-inflammatory properties of melanocortins, little is known about their direct influence on microglial cells, which are the main component of innate immunity in the CNS. In addition, most of the studies on the influence of POMC products on microglia used cell lines instead of primary microglia and focused on the effect of ACTH and α-MSH, along with several synthetic analogs of these peptides, whereas the effects of γ-MSH, β-MSH, and β-END have been studied less. Almost two decades ago, α-MSH was demonstrated to inhibit brain production of TNF-α in a mouse model of brain inflammation caused by bacterial lipopolysaccharide (LPS) injection [24]. The authors

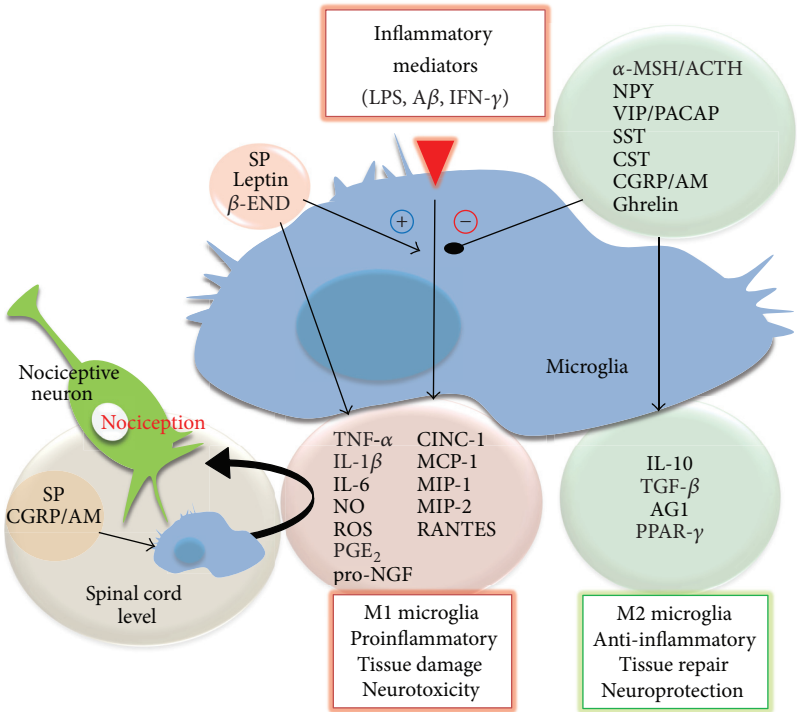

Figure 1: Neuropeptide modulation of microglial activation. In the presence of noxious stimuli such as LPS, Aβ, and IFN-γ or in the context of neuroinflammation or tissue damage, microglial cells produce a plethora of inflammatory mediators that promote and perpetuate the inflammatory response, potentially leading to neurodegeneration. Neuropeptides, acting through specific receptors present in microglial cells, are able to modulate microglial response and inhibit the release of inflammatory mediators while favoring development of an alternative activation program. On the other hand, neuropeptides such as SP, β-END, and leptin exert proinflammatory actions on microglial cells and may even potentiate the response to noxious stimuli. In experimental models of pain, certain neuropeptides such as SP, CGRP, and AM have been suggested to increase pain hypersensitivity partly by promoting proinflammatory activation of spinal cord microglia.

suggested that α-MSH was acting through the activation of MC1R present on astrocytes and microglia, the main producers of TNF-α within the brain. A year later, ACTH, α-MSH, and α-MSH COOH-terminal tripeptide (α-MSH$_{11-13}$) were shown to inhibit LPS + Interferon- (IFN-) γ-induced TNF-α, IL-6, and NO release in N9 microglial cells, most likely by a mechanism involving cAMP production [25]. The authors also demonstrated that microglial cells release α-MSH upon stimulation with LPS + IFN-γ, thereby creating an autocrine anti-inflammatory loop. This finding placed microglial cells in the group of nonneuronal immune cells expressing POMC mRNA and producing α-MSH as an autoregulatory factor, similar to what had been previously demonstrated in RAW 264.7 murine macrophages [26] and in human THP-1 myelomonocytic cells [27]. Subsequently, α-MSH and α-MSH$_{11-13}$ were also demonstrated to reduce the release of TNF-α and NO and the expression of TNF-α and inducible nitric oxide synthase (iNOS) induced by costimulation of N9 cells with Amyloid-β (Aβ) and IFN-γ [28], further supporting its role as an anti-inflammatory agent. Since then, melanocortins have proven to be anti-inflammatory and neuroprotective in vivo in several experimental models of nervous tissue damage, including brain ischemia [29–31] and traumatic brain injury, where neuroprotection exerted by α-MSH$_{(11-13)}$ was associated with decreased microglial activation [32], suggesting a contribution of local immunomodulation to the overall neuroprotective effect.

Melanocortins exert their actions through MCRs of which five subtypes have been described up to now (MCR1 to MCR5). These are G protein-coupled receptors (GPCRs) with seven transmembrane domains, all positively coupled with adenylate cyclase (AC) [33]. Nonetheless, cAMP-independent pathways have also been shown to mediate MCR signaling (reviewed in [34]). In the brain, the most abundant MCR subtypes are MC3R and MC4R [35, 36]. Expression of MC1R has been detected in RAW 264.7 cells [26], human monocytes [37], and murine macrophages [38]. MC3R expression was found in murine and rat macrophages [39, 40] and in RAW 264.7 cells [41] and expression of MC1R, MC3R, and MC5R was detected in THP-1 cells [27, 42]. Human monocytes express mRNA for MC1R, MC2R, MC3R, and MC5R [43]. MC4R, however, does not appear to be expressed in mouse macrophages [39] and RAW 264.7 cells [44]. On the other hand, MCR expression in microglial cells has not been thoroughly examined. Lindberg et al. showed that the human microglial cell line CHME-3 expresses MC1, MC3,

Table 1: Expression of neuropeptide receptors in microglial cells.

Receptor	Detection level	Microglial cell system	References
Melanocortin receptors			
MC1R	mRNA	CHME-3 cells	[45]
MC3R	mRNA	CHME-3 cells	[45]
MC4R	mRNA, protein	CHME-3 cells, rat microglia	[45, 47]
MC5R	mRNA	CHME-3 cells	[45]
Opioid receptors			
μ-receptors	mRNA, protein	Human fetal microglia, rat microglia	[55, 56]
κ-receptors	mRNA, protein	Human fetal microglia, rat microglia	[56, 57]
NPY receptors			
Y_1R	mRNA, protein	N9 cells, rat retinal microglia	[58, 59]
Y_2R	mRNA, protein	N9 cells, rat retinal microglia	[58, 59]
Y_5R	mRNA	N9 cells	[58]
VIP/PACAP receptors			
VPAC1	mRNA	Rat microglia, murine microglia, BV-2, and EOC13 microglial cell lines	[60, 61]
PAC1	mRNA	Rat microglia, murine microglia, BV-2, and EOC13 microglial cell lines	[60, 61]
STT/CST receptors			
sst2	mRNA, protein	Rat microglia, BV-2, N9, and murine microglia	[62, 63]
sst3	mRNA, protein	Rat microglia, BV-2, N9, and murine microglia	[62, 63]
sst4	mRNA, protein	Rat microglia, BV-2, N9, and murine microglia	[62, 63]
Tachykinin receptors			
NK-1R	mRNA, protein	Human fetal microglia, M4T.4, EOC13, and murine microglia	[64, 65]
NK-2R	mRNA	M4T.4 cells	[65]
NK-3R	*Functional evidence*	Rat microglia	[66]
CGRP/AM receptor			
CGRP/AM-R	*Functional evidence*	Rat microglia	[67–69]
Leptin receptors			
LepR	Protein	Rat microglia, BV-2 cells	[70, 71]
LepRa	mRNA	Rat microglia, BV-2 cells	[70, 71]
LepRb	mRNA	Rat microglia, BV-2 cells	[70, 71]
Ghrelin receptors			
CD36 (unacylated ghrelin receptor)	mRNA, Protein	N9 cells, human fetal microglia, and neonatal and adult murine microglia	[72–74]

MC4, and MC5 receptors and that α-MSH increases basal IL-6 secretion in these cells [45]. The authors suggested that this effect is mediated by either MC3R or MC5R, since agouti, an endogenous antagonist of the melanocortin system which has greater affinity for MC1R and MC4R than for other MCRs [46], did not block α-MSH induction of IL-6 but instead enhanced it. Contrary to what was described in macrophages, our group found MC4R expression in rat primary microglia, whereas expression of MC1R, MC3R, and MC5R was not detected by RT-PCR in these cells [47]. These data suggest that a certain degree of heterogeneity exists regarding MCR expression between microglia and peripheral macrophages and when comparing primary and transformed cell systems.

In RAW 264.7 macrophages, ACTH and melanotan II (MTII, a MC3/4R agonist) induce rapid cAMP accumulation and p38 MAPK phosphorylation but do not alter extracellular

signal-regulated kinases (ERK)1/2 and c-Jun N-terminal kinase (JNK) phosphorylation [41]. ACTH and MTII also stimulate IL-10 production through a protein kinase A- (PKA-) dependent mechanism [41]. The rapid increase in cAMP has also been observed in N9 cells and was proposed to mediate anti-inflammatory actions of melanocortins [25]. In CHME3 cells Lindberg and colleagues found that PKA was not involved in α-MSH-induced IL-6 release, but when α-MSH was combined with a PKA inhibitor, this led to a decrease in cell viability [45], indicating that certain mechanisms triggered by α-MSH do involve PKA activity in microglia. Our group found that [Nle_4, D-Phe_7]-α-MSH (NDP-MSH), a synthetic analog of α-MSH, induced a marked decrease in peroxisome proliferator-activated receptor- (PPAR-) β protein expression, which was prevented by inhibition of ERK1/2, thereby indicating a role for this kinase in melanocortin signaling in primary microglial cells [47].

α-MSH was shown to suppress Toll-like receptor (TLR) 4-induced (but not TLR2-induced) NO production in the macrophage cell line J774, where it also promoted IL-1 receptor associated kinase- (IRAK-) M binding to IRAK-1, thereby suppressing TLR4-dependent response to LPS at a very proximal stage in the signaling cascade [48]. NDP-MSH reduces TLR4 gene expression in rat microglial cells [49], and α-MSH reduces expression of the LPS coreceptor CD14 in THP-1-derived macrophages [50]. Evidence also indicates that melanocortins inhibit NF-κB activity by preventing IκB degradation and p65 nuclear translocation [51]. In the U-937 monocytic cell line these effects appear to be mediated by the cAMP-PKA pathway [51], whereas in RAW 264.7 cells inhibition of NF-κB by α-MSH seems to be a cAMP-independent mechanism [52]. Concordantly, NDP-MSH prevented LPS-induced p65 and c-Rel translocation to the nucleus in primary cultured rat microglial cells [49].

The importance of α-MSH in maintaining immunosuppression in the ocular microenvironment was identified long ago [53]. More recently, a study showed that α-MSH produced in the healthy retina is necessary for induction of AG1 expression in retinal microglial cells [54]. Since AG1 is a marker of alternatively activated M2 microglia and macrophages [1], α-MSH, in cooperation with neuropeptide Y (NPY) and other local neuropeptides, might be responsible for maintaining ocular immune privilege by alternatively activating local macrophages and microglia towards an immunosuppressive or tolerogenic phenotype [54]. On a similar note, our group studied the effects of NDP-MSH on rat microglial cells and found that it induces release of the anti-inflammatory cytokine IL-10 and expression of PPAR-γ [47] and of AG1 [49], all markers of alternative activation [1], further reinforcing a role for this neuropeptide in promoting an alternative activation program in microglial cells.

Phagocytosis of debris, pathogens, or apoptotic cells is an important part of the resolutory phase of inflammation. It is generally regarded as a beneficial phenomenon and its alteration has been linked to autoimmune disorders and neurodegenerative diseases [75]. However, phagocytosis can also activate the respiratory burst and increase generation of ROS, which may cause neurotoxicity [75]. In fact, both classical M1 (driven by IFN-γ) and alternative M2 (driven by IL-4 and IL-13) microglial activation paradigms have been found to induce phagocytosis of apoptotic cells and of purified myelin, respectively [76, 77]. In murine primary macrophages, the MCR agonist AP124 promotes phagocytosis of zymosan particles and of apoptotic neutrophils acting mainly through MC3R [78]. However, treatment of RAW 264.7 cells with α-MSH reduces phagocytosis of apoptotic cells [79] and *E. coli* bioparticles, and when combined with NPY it also reduces phagocytosis of gram-positive bioparticles [80]. Our group found that treatment of primary rat microglia with NDP-MSH does not affect basal or LPS-induced phagocytosis of latex beads; however, NDP-MSH inhibits phagocytosis induced by the TLR2 agonist Pam_3CSK_4 in microglial cells [49]. It is clear that the effects of neuropeptides on phagocytosis are highly dependent on variables such as the cell type involved, the stimulus, and the system of choice for studying phagocytosis (i.e., latex beads, opsonized versus unopsonized bioparticles, and apoptotic cells).

Evidence indicates that POMC-derived peptides play an important role in nociception and that at the spinal cord level melanocortin and opioid systems interact and cooperate with each other in pain perception, in that MCR activation seems to generally increase pain sensitivity whereas activation of opioid receptors by β-END induces analgesia [81]. Expression of POMC, β-END, ACTH, α-MSH, the endogenous antagonist Agouti-Related Protein (AgRP), and MC4R has been detected in areas of the spinal cord involved in nociception, underscoring the role of this system in nociceptive transmission [82–85]. Concordantly, several studies have shown that antagonism of the melanocortin system reduces neuropathic pain [86–88] and prevents morphine withdrawal hyperalgesia [89]. It has been established that spinal cord microglial cells play a crucial role in neuropathic pain by releasing mediators that activate nociceptive neurons [90]. However, they can also be a source of β-END and release it in response to diverse stimuli, such as corticotrophin-releasing hormone (CRH) or noradrenaline stimulation [91], and upon glucagon-like peptide-1 (GLP-1) receptor activation [92]. Induction of β-END by a GLP-1 receptor agonist was found to be mediated by a p38 MAPK-dependent mechanism in spinal dorsal horn microglia [93]. Microglial β-END, in turn, mediates GLP-1 antinociceptive effects in pain hypersensitivity states by activating opioid receptors located on neurons [92]. In addition, β-END (and other opioid receptor ligands) may act directly on microglia, although little is known about direct effects of β-END on these cells. Of the opioid receptors so far identified, microglia express μ- and κ-receptors, but not δ-receptors, and an additional opioid receptor-independent pathway may also exist for endogenous opioid receptor ligands [17, 55–57]. However, activation of the GLP-1 receptor/β-END pathway does not inhibit proinflammatory cytokine production in cultured microglia stimulated by LPS [94]. On the contrary, β-END has been shown to directly enhance basal and GP-120-induced TNF-α, IL-1β, and IL-6 release in brain perivascular microglia, where it also enhances replication of the human immunodeficiency virus (HIV), effects that are blocked by an opioid receptor antagonist [95]. Concordantly, prolonged treatment with

morphine induces microglial activation and upregulation of proinflammatory cytokines such as IL-6, TNF-α, and IL-1β in the rat spinal dorsal horn [96, 97]. Since these inflammatory mediators may in turn activate spinal nociceptive neurons and enhance pain sensitivity [7], the direct effect of opioids on microglial cells may partially account for the development of tolerance to long-term opioid treatment. This notion is supported by evidence showing that development of tolerance is mediated by activation of microglial p38 MAPK [98] and that treatment with the microglial inhibitor minocycline prevents morphine-induced tolerance [99].

3. NPY

NPY is a 36-amino acid neuropeptide derived from its precursor pre-pro-NPY. It belongs to the family of pancreatic peptides together with peptide YY (PYY) and pancreatic polypeptide (PP) and is widely distributed within the CNS and PNS. The physiological functions of NPY are varied and include regulation of blood pressure, circadian rhythms, feeding behavior, anxiety, and memory [100].

In mammals, five subtypes of Y receptors have been described (Y_1R, Y_2R, Y_4R, Y_5R, and Y_6R). Y_1R, Y_2R, and Y_5R are the preferential receptors for NPY and PYY, Y_4R binds PP, and Y_6R is a pseudogene in humans that has been lost in rats (reviewed in [101]). They are all GPCRs whose main signaling pathway involves coupling with $G_{i/0}$ proteins and inhibition of cAMP production, although other possible signaling pathways include intracellular calcium mobilization, modulation of Ca^{2+} and K^+ channel conductance, activation of PKC, PKA, phospholipase (PL) A2, MAPKs, guanylyl cyclase, and phosphatidylinositol 3-kinase (PI3K) [102].

Expression of NPY and of Y_1R, Y_2R, and Y_5R has been detected in N9 murine microglia [58]. In these cells, gene and protein expression of NPY and Y_1R increase with LPS treatment [58], suggesting the neuropeptide might have a regulatory function on LPS-induced microglial activation. In accordance with a proposed anti-inflammatory role, NPY inhibits LPS-induced iNOS expression, NO, and IL-1β release, and it also inhibits IL-1β-induced cell motility, iNOS expression, NO production, and NF-κB activation in N9 cells [58, 103]. NPY protected N9 microglia from methamphetamine-(METH-) induced apoptosis through a mechanism partially dependent on Y_2R and independent from Y_1R, and an Y_2R agonist reduced microglial CD11b immunoreactivity in hippocampal organotypic cultures incubated with METH, thereby suggesting a protective role for NPY via Y_2R in METH-induced microglial dysfunction [104]. Inflammatory cytokines released by glial cells are proposed to mediate local increase in neuronal excitability and apoptotic death in experimental models of seizures [105]. Concordantly, conditioned medium from LPS-treated rat microglial cells (with elevated levels of IL-1β and TNF-α) increase neuronal NMDA current, which when excessive may cause excitotoxic neuronal injury [106]. In the same study, cotreatment of microglial cells with LPS + NPY prevented the LPS-induced increase in IL-1β and TNF-α production through a mechanism involving Y_1R. Furthermore, the effect of microglial LPS-conditioned medium on NMDA current was not observed when neurons were incubated with medium from LPS + NPY cotreated microglia, suggesting that the anti-inflammatory effect of NPY on microglial cells might in turn be neuroprotective by preventing NMDA receptor hyperactivation [106].

NPY seems to play a special role in retinal physiology [102]. It is expressed in different neuronal and glial cells of the retina, including microglia [107]. The NPY receptors Y_1R and Y_2R are also expressed in retinal microglial cells [59], suggesting a possible modulatory role for this neuropeptide in inflammatory retinal dysfunctions. Moreover, it has been proposed that NPY released by the ocular posterior chamber and derived mainly from the retinal pigment epithelial cells promotes (in cooperation with α-MSH) the development of myeloid suppressor cell-like characteristics in retinal microglia and in migrating macrophages [54]. In addition, NPY modulates phagocytosis in professional phagocytes and antigen-presenting cells [108]. In RAW 264.7 cells, NPY suppresses phagocytosis of unopsonized bioparticles, phagolysosome activation, and Fc receptor-mediated generation of ROS [80]. Treatment of microglial cells with NPY inhibits LPS-induced actin cytoskeleton reorganization and phagocytosis of opsonized latex beads acting through Y_1R [109]. NPY also decreases LPS- and IL-1β-induced Fc receptor expression and prevents activation of p38 MAPK and HSP27 triggered by LPS in N9 cells [109]. The ability of NPY to act on microglial cells and to negatively regulate phagocytosis further supports a role for this neuropeptide (together with α-MSH) in maintaining the immune-privileged ocular microenvironment.

The NPY system is a key element in pain modulation. Evidence shows that intrathecal administration of NPY reduces behavioral signs of inflammatory and neuropathic pain, acting at spinal Y_1R and Y_2R [110, 111]. Considering that NPY decreases microglial proinflammatory activation, it would be interesting to study the potential role of microglia as mediators of NPY analgesic effects, an area which, to our knowledge, has not yet been addressed.

4. VIP and PACAP

The Vasoactive Intestinal Peptide (VIP) is a 28-amino acid polypeptide discovered in 1970 in intestinal extracts, able to induce vasodilation [112]. VIP is an immunoregulatory neuropeptide produced by neurons, endocrine, and immune cells, widely distributed in the CNS and PNS, and also found in heart, lung, thyroid, kidney, urinary and gastrointestinal tracts, genital organs, and the immune system [113]. VIP is structurally related to the secretin/glucagon family of peptides and hormones, sharing 70% sequence identity with the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) [114]. The precursor molecule pre-pro-VIP is processed into mature VIP and the related peptide C-terminal methionine amide (PHM) in humans and peptide with N-terminal histidine and C-terminal isoleucine amide (PHI) in other mammalian species [115].

PACAP was discovered nearly two decades later as a 38-amino acid hypothalamic neuropeptide that stimulated AC activity and increased cAMP levels in pituitary cells [116]. The sequence of PACAP-38 contains an internal cleavage site giving rise to a 27-amino acid fragment corresponding to the

N-terminal portion of PACAP-38, termed PACAP-27, which is also capable of inducing AC activity in pituitary cells [117]. In the CNS, the predominant form is PACAP-38, expressed mainly in the hypothalamus but also widely distributed in several extrahypothalamic regions. In the periphery, PACAP-38 is also the predominant form and has been found in numerous organs and tissues including the immune system [117].

Following the discovery of PACAP, it was found that VIP and PACAP act as endogenous ligands for the same receptors, which are three heterotrimeric GPCRs: PAC1, VPAC1 (or VIPR1), and VPAC2 (or VIPR2), each with a distinct expression pattern. PAC1 has much greater affinity for PACAP than for VIP, whereas VPAC1 and VPAC2 can bind PACAP and VIP with comparable affinity [118]. Expression of PAC1 is highest in the CNS, particularly in the thalamus, hypothalamus, hippocampus, olfactory bulb, and cerebellum. VPAC1 is also widely distributed in the CNS, particularly in the cerebral cortex and hippocampus, and in several peripheral organs [118]. VPAC2 expression has been detected in the thalamus, suprachiasmatic nucleus, hippocampus, brainstem, and dorsal root ganglia, and in some peripheral tissues such as smooth muscle of the cardiovascular, gastrointestinal, and reproductive systems [118]. These receptors are coupled primarily with Gs and activate AC and PKA, although VPAC/PAC1 activation has also been reported to activate or inhibit several other signaling pathways [119, 120].

VIP and PACAP proved to be neuroprotective in various models of nervous tissue damage [121]. Since microglial cells are the main source of inflammatory mediators in the CNS, it has been suggested that VIP and PACAP might be exerting their protective effects in part by acting directly on microglial cells as microglia-deactivating factors. Expression of VPAC1 and PAC1 (but not VPAC2) was detected in rat microglia, where these receptors mediate VIP and PACAP inhibition of LPS-induced TNF-α production in a cAMP-dependent manner [60]. Similarly, Delgado and colleagues found expression of VPAC1 and PAC1, but not VPAC2, in murine microglia, where VIP and PACAP inhibit LPS-stimulated production of the CXC chemokines macrophage-inflammatory protein-(MIP-) 2 and KC, and of the CC chemokines MIP-1α, MIP-1β, macrophage chemoattractant peptide (MCP)-1, and RANTES, and inhibit chemotactic activity of peripheral leukocytes [61]. VIP and PACAP also prevent the production of LPS-induced TNF-α, IL-1β, IL-6, and NO in murine microglia [122]. These effects are attained through activation of VPAC1, induction of cAMP production, and inhibition of NF-κB [61, 122]. The mechanism for VIP and PACAP inhibition of NF-κB in activated microglial cells involves a reduction in p65 binding to the coactivator CREB-binding protein (CBP) and parallel stimulation of CREB phosphorylation, thereby promoting CREB/CBP complex formation instead of p65/CBP interaction [123] and ultimately leading to reduced transcription of classic proinflammatory NF-κB-regulated genes. VIP inhibits cyclooxygenase- (COX-) 2 expression and PGE_2 production in LPS- and LPS + IFN-γ-treated murine microglial cells [124]. Activation of VPAC1-cAMP signaling by VIP and PACAP was also found to inhibit the MEKK1/MEK4/JNK pathway and subsequent binding of the transcription factor AP-1 in LPS-stimulated microglial cells [125]. PACAP was also shown to prevent LPS-induced BV-2 microglial activation by inducing cAMP production and inhibiting p38 MAPK signaling pathway [126]. This neuropeptide also attenuated microglial hypoxia-induced TNF-α, iNOS, and NO production and p38 MAPK activation and prevented neuronal death caused by microglial neurotoxicity [127]. In IFN-γ-treated microglial cells, activation of VPAC1 by VIP and PACAP inhibits expression of IFN-γ-inducible protein- (IP-) 10, iNOS, and CD40 through inhibition of the IFN-γ-induced JAK1-2/STAT1 pathway [128]. Another study demonstrated that PACAP and VIP downregulate mRNA and surface protein expression of costimulatory molecules CD40 and B7-2 in activated microglial cells. In this study, the effect of PACAP on CD40 was mediated by VPAC1 activation and, at least in part, by PACAP-induced IL-10 [129]. Altogether, evidence indicates a strong anti-inflammatory and immunosuppressive role for VIP and PACAP in microglial cells, mainly through VPAC1 and PAC1 activation.

Concordant with these neuroprotective and anti-inflammatory actions, treatment with VIP or PACAP protected mice from lethal endotoxemia, the protective effect being attributed to inhibition of endotoxin-induced TNF-α and IL-6 production [130]. Apart from inhibiting proinflammatory mediators, VIP and PACAP were found to rapidly increase IL-10 production in LPS-treated macrophages, primarily through activation of VPAC1, elevation of cAMP and increased CRE binding activity [131], and the stimulatory effect of VIP and PACAP on IL-10 production was also observed in vivo in LPS-treated mice [131].

A recent study suggested that endogenous PACAP may be neuroprotective during seizure by acting on microglial cells and inducing their polarization towards an alternative M2 phenotype [132]. VIP and PACAP also exert beneficial effects in several in vitro and in vivo models of neuroinflammation and neurodegeneration. Treatment with VIP prevented several features of mechanically induced brain trauma, such as microglial activation, proinflammatory cytokine secretion, leukocyte infiltration, and neurodegeneration [133]. In a model of brain focal ischemia, delayed intracerebroventricular delivery of PACAP-producing stem cells promoted fast and stable functional recovery which correlated with reduced inflammatory response and increased number of $AG1^+$ microglia, further supporting a role for PACAP in polarizing microglia towards an M2 neuroprotective phenotype [134]. In an in vitro model of ischemic damage by oxygen and glucose deprivation (OGD) and reoxygenation, pretreatment of BV-2 cells with PACAP alleviated hypoxic injury by preventing TLR4/MyD88/NF-κB signaling and decreasing proinflammatory cytokine levels and BV-2 apoptosis [135]. In a mouse model of glaucomatous retinal damage by intravitreal NMDA injection, PACAP-38 prevented NMDA-induced cell death in the ganglion cell layer, including retinal ganglion cells and $Iba1^+$ cells, presumably through a mechanism dependent on IL-10 release by retinal microglia/macrophages [136]. PACAP also increased mRNA levels of the anti-inflammatory mediators TGF-β and IL-10, which partially colocalized with $Iba1^+$ cells and $CD11b^+$ cells, providing additional evidence of PACAP inducing an acquired deactivation profile in microglia/macrophages.

Delgado and colleagues found that VIP inhibits Aβ-induced microglial activation, production of TNF-α, IL-1β, and NO, and activation of NADPH-oxidase and reduces neuronal death induced by fibrillar Aβ_{1-42} in murine neuron-microglia cocultures [137]. These effects were mediated by the VPAC1-cAMP-PKA signaling pathway and by inhibition of p38, ERK1/2, and NF-κB signaling cascades [137]. VIP also inhibited TNF-α and NO production in mouse primary microglia stimulated with Aβ_{42} + low dose IFN-γ, promoted microglial phagocytosis of Aβ_{1-42} through a PKC-dependent mechanism, and reduced Aβ deposits in the hippocampus of transgenic PS1/APP mice, a model of AD [138], suggesting a protective role for VIP in AD through modulation of microglial function.

PACAP-38 was shown to inhibit NADPH oxidase activity and ROS production in microglial cells. This mechanism appears to be involved in PACAP-induced neuroprotection and is not mediated by classic VPAC or PAC1 receptors [139]. This effect is of particular importance in PD, since dopaminergic neurons in the substantia nigra are highly vulnerable to oxidative stress [140]. In mice treated with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a model of PD, administration of VIP into the substantia nigra inhibited microglial activation and expression of the cytotoxic mediators TNF-α, IL-1β, iNOS, and NADPH-oxidase and reduced nigrostriatal nerve fiber loss and dopaminergic neuronal degeneration in the ipsilateral substantia nigra pars compacta [141]. PACAP and a VPAC1 agonist (but not a VPAC2 agonist) also prevented MPTP-induced microglial activation and dopaminergic neuronal degeneration with a potency similar to VIP, suggesting that the protective actions of these neuropeptides are primarily mediated by VPAC1 in this model of PD [141].

Treatment with PACAP showed beneficial effects in mice with experimental autoimmune encephalomyelitis (EAE), by delaying onset of the illness and reducing its severity [142]. The protective effect of PACAP was attributed, at least in part, to its ability to suppress production of proinflammatory mediators such as TNF-α, IL-1β, and IL-12 in microglia and macrophages and to reduce antigen-presenting efficacy and Th1 differentiation [142].

VIP and PACAP have been largely associated with neuropathic pain [143]. In a rat model of neuropathic pain caused by CCI, blockade of VPAC2 attenuated CCI-induced phosphorylation of p38 and ERK1/2 MAPK in the spinal cord and behavioral reflex sensitization. Conversely, agonist stimulation of VPAC2 in naïve rats increased activation of spinal p38 and ERK1/2 and thermal hyperalgesia [144]. Moreover, intrathecal addition of the glial inhibitor propentofylline reduced thermal hyperalgesia and mechanical allodynia in CCI rats and also reduced MAPK activation in CCI rats and in naïve rats treated with a VPAC2 agonist, suggesting involvement of glial cells in nerve injury-induced sensitization [144]. Astrocytes are known to express VPAC2 (along with VPAC1 and PAC1) [145], and VPAC2 mRNA was shown to be induced upon LPS treatment in mouse peritoneal macrophages [146], THP-1 cells [147], and RAW 264.7 cells [148]. However, expression of VPAC2 has not been detected in microglial cells, either resting or after LPS treatment [60, 61]. In summary, evidence implicates VPAC2-induced MAPK activation in spinal glial cells as a mechanism contributing to sensitization in chronic pain, although specific involvement of microglial cells is yet to be determined.

5. Somatostatin

Somatostatin (SST) is a cyclic peptide produced by neuroendocrine, inflammatory, and immune cells, which generally acts as an inhibitor of secretory and proliferative responses in a variety of widely distributed target cells. SST is synthesized from the precursor preprosomatostatin (preproSST), yielding in mammals two bioactive forms termed SST-14 and SST-28. SST receptors are GPCRs, of which five subtypes have been cloned (sst1 to sst5). The five receptors share common signaling pathways, such as inhibition of AC and cAMP formation, induction of tyrosine phosphatase phosphorylation, and modulation of MAPKs through G-protein-dependent mechanisms [149].

In rat primary cultured microglial cells, presence of mRNA for sst2, sst3, and sst4 has been demonstrated [62]. The receptors were functionally active, since SST-14 affected microglial protein phosphorylation and inhibited microglial proliferation induced by granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-3 [62]. Immunoreactivity for sst2, sst3, and sst4 was later detected in BV-2, N9, and mouse primary microglial cells, and SST induced migration in BV-2 and N9 cells [63]. Activation of sst2, sst3, and sst4 in neonatal rat microglia inhibited LPS-induced PGE_2 production [150], suggesting an anti-inflammatory role for SST in microglial cells. However, a study performed in cortical rat microglia cultures showed no effect of SST on basal or IL-1β-induced PGE_2 synthesis [151], the discrepancies in results being attributed to different experimental settings and to the type of inflammatory stimulus used.

SST stimulates expression of insulin-degrading-enzyme (IDE), an extracellular protease involved in Aβ degradation, in BV-2 and rat microglial cells [152]. Another study showed that SST dose-dependently induces Aβ_{1-42} phagocytosis in BV-2, N9, and rat primary microglial cells, supporting a protective role for SST in the development of AD by modulating microglial function [63]. However, this study showed no effect of SST either alone or in combination with Aβ on IDE or on neprilysin (another enzyme involved in Aβ degradation) expression in BV-2 and N9 cells [63]. The discrepancies may be due to the use in the second study of a much lower concentration of SST (100 nM) versus the 0.5–10 μM concentrations used in the first study or also to differences in incubation times before protein assessment.

In a recent study performed in a rat model of PD generated by injecting LPS into the brain's substantia nigra, SST pretreatment was able to dramatically decrease the number of activated microglial cells [153]. In this study, SST was also able to prevent neuronal cell death and to reduce production of TNF-α, IL-1β, PGE_2, and ROS by the substantia nigra [153], all of which are known to be produced by activated microglial cells in models of PD [154]. Therefore, it was suggested that protective effects of SST on neuronal survival in this model

of PD are associated with its ability to reduce microglial activation.

Apart from its anti-inflammatory actions STT plays an important role in pain sensitivity. It exerts analgesic effects both centrally and peripherally (reviewed in [155]). However, whether microglial modulation by STT mediates STT-induced analgesia is yet to be determined.

6. Cortistatin

Cortistatin (CST) is a cyclic peptide initially isolated from rat brain and named after its predominantly cortical expression and its ability to depress neuronal electrical activity [156]. Its precursor preprocortistatin (preproCST) is a 112-amino acid protein that may suffer proteolytic cleavage at multiple sites, yielding products of different lengths. CST belongs to the same family as SST; the 14-amino acid rat CST isoform and the 17-amino acid human isoform both share 11 amino acids with SST-14, including the motif responsible for SST receptor interaction, and are thereby able to bind all five known SST receptors in vitro with similar affinities to that of SST, acting as receptor agonists and inhibiting cAMP accumulation [157]. Although some of the biological actions of CST seem to be mediated by SST receptors, several effects of CST in the CNS are distinct from those of SST, such as induction of slow-wave sleep and reduction of locomotor activity, suggesting the existence of alternative signaling pathways for CST [157]. In support of this notion, other GPCRs have been shown to bind CST with selective affinity over SST, such as the ghrelin/growth hormone-secretagogue receptor (GHSR) which binds CST with similar affinity compared to ghrelin [158], and the human orphan receptor Mas-related G-protein-coupled receptor member X2 (MrgX2) [159] which also binds proadrenomedullin and related peptides [160]. More recently, truncated functional forms of sst5 have been described which show different signaling profiles in response to CST or SST [161, 162].

Expression of preproCST mRNA in the CNS appears to be restricted to the cortex and hippocampus [156]. In addition to its role as regulator of sleep rhythm and locomotor activity, evidence strongly suggests a role for CST in inflammation and immunomodulation. Expression of preproCST mRNA (but not of preproSST) was detected in human monocyte-derived macrophages and dendritic cells. In addition, CST was demonstrated to bind sst2, and both preproCST and sst2 are upregulated during macrophage differentiation and after LPS stimulation, suggesting CST might be an endogenous ligand for sst2 in the human immune system [163, 164]. In peritoneal macrophages, CST prevented the LPS-induced production of TNF-α, IL-12, IL-1β, IL-6, NO, MIP-2, and RANTES. Since these effects were only partially prevented by the sst antagonist cyclosomatostatin and were also partially blocked by a GHSR antagonist, evidence suggests CST is acting through sst-dependent and sst-independent pathways in these cells [165, 166]. CST was also shown to dose-dependently inhibit basal and IL-1β-induced PGE_2 release and to reduce IL-1β-stimulated COX-2 mRNA expression in primary rat microglial cells [151].

Protective effects of CST have been demonstrated in vivo in experimental models of ulcerative colitis, arthritis, and endotoxemia, where the anti-inflammatory effects of CST were attributed mainly to its ability to deactivate resident and infiltrating macrophages, and other immune cells [165–167]. CST also showed beneficial effects in a model of meningoencephalitis caused by *Klebsiella pneumoniae* infection; it reduced white blood cell infiltration into the cerebrospinal fluid (CSF), attenuated clinical symptoms of illness, inhibited TNF-α, IL-1β, and IL-6 brain expression and release into the CSF, and decreased neuronal cell death in the cortex and hippocampus [168]. Interestingly, the decrease in *K. pneumoniae*-induced proinflammatory cytokine production after CST treatment was also observed in vitro in neuron-glia cocultures, suggesting direct downregulation of glial activity may partially account for the anti-inflammatory effects of CST [168].

CST also exerted beneficial long lasting effects in models of chronic and relapsing-remitting EAE, where systemic treatment with the neuropeptide reduced incidence and severity of the disease [169]. CST treatment reduced inflammatory spinal cord infiltrates, decreased activation of autoreactive Th1/Th17 cells, and inhibited expression of proinflammatory mediators, while promoting regulatory T cell differentiation. The protective effects of CST were associated with its ability to promote the development of a glial neuroprotective phenotype, by inducing BDNF and activity-dependent neuroprotector protein release from neuron-glial cocultures, reducing astrocyte and microglial IL-6, TNF-α, and NO release, and preventing oxidative stress-induced oligodendrocyte death [169].

CST is expressed in interneurons from the spinal cord and in nociceptive neurons from the dorsal root ganglia, where it colocalizes mostly with peptidergic calcitonin gene-related peptide (CGRP)/substance P- (SP-) expressing nociceptors. It is considered to be an endogenous analgesic factor, as nociceptive responses to inflammatory pain are exacerbated in CST knockout mice, and administration of CST induces analgesia and decreases nocifensive behavior in several experimental models of inflammatory pain [170]. However, the analgesic effects of CST appear to be independent of its anti-inflammatory properties and rather exerted by directly inhibiting release of nociceptive peptides such as CGRP and SP from primary nociceptors, mainly through activation of sst2 and GHSR1 [170–172]. Nonetheless, the possibility that CST anti-inflammatory effects on microglial cells may be contributing to its analgesic properties cannot be ruled out.

7. Tachykinins

Tachykinins are a family of structurally related peptides derived from proteolytic cleavage of pre-pro-tachykinins, encoded in three *tac* genes and expressed throughout the nervous and immune systems. They regulate many diverse physiological processes including inflammation and nociception and are also involved in many pathological conditions [173]. The major mammalian tachykinins are SP and Neurokinin A (NKA), derived from *tac1,* Neurokinin B (NKB) which is derived from *tac3,* and hemokinin-1 (HK-1), derived

from *tac4*. All tachykinins signal through three subtypes of GPCRs termed NK-1R, NK-2R, and NK-3R. NK-1R is the preferential receptor for SP [173]. In humans, *tac4* encodes a precursor protein that has four described splice variants named endokinin (EK) A, EKB, EKC, and EKD. HK-1, EKA, and EKB have SP-like biological actions and can interact with NKRs, whereas EKC and EKD have negligible affinity for NKRs and are considered tachykinin gene-related peptides rather than true tachykinins [173]. Although at first it was believed that *tac4* was expressed only in hematopoietic cells [174], it was later shown that *tac4* displays a broad expression pattern similar to *tac1* and *tac3* [175].

SP has long been recognized as a central and peripheral neuropeptide with stimulatory properties on immune cells. Production of SP and expression of the SP receptor NK-1R were detected in human fetal microglia [64], suggesting that SP can act as an autocrine modulator in these cells. NK-1R protein expression was also detected in M4T.4 and EOC13 microglial cell lines and in primary murine microglia [65]. In keeping with its role as an immune stimulator, SP stimulated IL-6 release in BV-2 cells [176], induced translocation of the NF-κB subunit p65 to the nucleus in M4T.4 cells [65], stimulated thromboxane release [177] and microglial chemotaxis [178], and enhanced LPS-induced IL-1β release in rat microglia, although it did not enhance LPS-induced TNF-α production or induce cytokine release per se in this study [179]. However, SP was recently shown to increase per se expression of the complement receptor 3, release of TNF-α and IL-6, and production of ROS in rat microglial cells, its effects mediated by NK-1R, NK-2R, and NK-3R [66]. SP potentiated class II MHC (MHCII) expression in microglial cells from the brainstem of IFN-γ-treated rats, an effect that was prevented by addition of a NK-1R antagonist [180]. However, SP had no significant effect on IFN-γ-induced MHCII expression in the hippocampus, suggesting regional differences in microglial response to this neuropeptide [180]. SP also enhanced microglial NF-κB activation, COX-2 expression, PGE_2 production, and expression of the PGE_2 receptors EP2 and EP4, induced by *Borrelia burgdorferi*, the causative agent of Lyme disease [181], as well as *B. burgdorferi*- and *Neisseria meningitidis*-induced microglial production of IL-6 and TNF-α, in a NK-1R-dependent manner [182].

Evidence suggests that SP may play an important role in the pathogenesis of PD [183]. This neuropeptide is found in particularly high levels in the substantia nigra of the brain [184] and binds NK-1R present in a variety of cells such as endothelial cells, glial cells, and dopaminergic neurons, where it potentiates the release of striatal dopamine [183]. SP was shown to enhance microglial extracellular superoxide and intracellular ROS production through activation of NADPH oxidase, leading to neurotoxicity of dopaminergic neurons [185]. Deletion of endogenous SP in mice was shown to attenuate LPS-induced dopaminergic degeneration, as well as nigral microglial activation and expression of proinflammatory factors [186]. Moreover, addition of SP to microglial cultures potentiated LPS-induced TNF-α and nitrite production, MAPK, and NF-κB activation and also enhanced LPS- and 1-methyl-4-phenylpyridinium-induced dopaminergic degeneration in mixed neuron-glia cultures [186].

NK-1R activation has been linked to increased pain sensitivity. In a model of long-term morphine administration, blockade of NK-1R attenuated morphine withdrawal-mediated hyperalgesia and activation of spinal cord microglia [187]. Also, intraperitoneal administration of a NK-1R antagonist alleviated fracture-induced allodynia and reduced spinal cord microglial activation in a rat model of complex regional pain syndrome [188]. In a rat model of spinal sensitization, intrathecal injection of SP induced thermal hyperalgesia and increased p38 phosphorylation in spinal microglial cells; the hyperalgesia was prevented by pretreatment with a p38 inhibitor, supporting a role for microglial p38 activation in nociceptive behavior [189]. In rats suffering from mechanical allodynia, the cytokine TNF-α, known to play a key role in neuropathic pain [190], was increased in spinal microglial cells and astrocytes [191]. Concordant with its pronociceptive role, treatment of microglial cells with SP induced production of TNF-α mRNA and of transmembrane full-length TNF-α (mTNF-α), which in turn activates microglia, demonstrated by increased expression of OX-42 and by release of MCP-1, suggesting a possible mechanism through which SP-induced microglial mTNF-α expression might create a feed-forward loop and thereby contribute to development of chronic pain [192].

Despite its well-recognized proinflammatory properties, recent reports have suggested an anti-inflammatory and wound healing promoter role for SP. In a rat model of SCI, SP enhanced functional recovery, decreased expression of the M1 markers iNOS, TNF-α, and CD86, and induced expression of the M2 markers IL-10, AG1, and CD206 at the injury site. Immunoreactivity of CD206 was colocalized with $CD11b^+$ cells in SP-treated animals, suggesting a shift from M1 to M2 microglia/macrophage phenotype [193]. SP also promoted wound healing in a murine model of type 1 diabetes and promoted development of the alternative activation program, determined by a decreased M1/M2 marker ratio in skin macrophages [194].

Expression of *tac4* was detected in a microglial cell line and its levels decreased after incubation with a *B. burgdorferi* lysate [195]. However, another report showed that treatment of rat primary cultured microglia with LPS increased *tac4* mRNA levels through a mechanism involving NF-κB and p38 MAPK [196]. In a rat model of CCI, blockade of NK-2R (and to a lesser extent, NK-1R) attenuated CCI-induced phosphorylation of p38 and ERK1/2 MAPK in the spinal cord and behavioral reflex sensitization. Conversely, treatment of naïve rats with a NK-2R agonist increased activation of spinal p38 and ERK1/2 and induced thermal hyperalgesia. MAPK activation after CCI or after NK-2R activation, as well as CCI-induced behavioral sensitization, was prevented by addition of the glial inhibitor propentofylline [144]. In another rat model of neuropathic pain caused by CCI of the sciatic nerve, *tac4* mRNA was increased in the dorsal horn and blockade of NK-1R prevented this effect and also inhibited associated pain behavior and microglial activation [197]. Altogether, data suggest a role for *tac4* in pathological conditions associated with microglial activation.

8. CGRP and Adrenomedullin

CGRP and adrenomedullin (AM) are neuropeptides that belong to the CGRP/calcitonin peptide superfamily, which also includes calcitonin, amylin, and intermedin [198–202]. They are widely distributed in the CNS and PNS and both have potent vasodilator activity [203]. They exert their actions through specific receptors which are GPCRs formed by three components: the 7-transmembrane calcitonin receptor-like receptor (CRLR), the receptor component protein (RCP), and the receptor activity-modifying protein (RAMP) 1–3 [204]. Interaction of CRLR with RAMP1 forms the CGRP1 receptor, which has greater affinity for CGRP than for AM, whereas association with RAMP2 or RAMP3 gives rise to the AM preferring receptors 1 and 2, respectively. CGRP/AM receptor activation is known to stimulate AC and cAMP production, although other signaling pathways have been described such as PLC, intracellular calcium increase, and NO production [203]. Functional evidence indicates microglial cells express CGRP/AM receptors, as treatment with CGRP upregulates immediate-early *c-fos* gene expression and induces cAMP accumulation [67, 68], and both CGRP and AM modulate expression of LPS-induced inflammatory mediators in cultured microglial cells [69].

Cumulative evidence suggests a strong protective anti-inflammatory role for CGRP and AM in several models of diseases, although some proinflammatory effects have also been described for both neuropeptides [205, 206]. Treatment with AM has shown protective effects in a variety of experimental models of disease such as arterial and pulmonary hypertension, heart failure, septic shock, and ischemia-reperfusion injury [207]. AM expression is induced after hypoxia in various cell types of the brain, including neurons, astrocytes, endothelial cells, and microglia [208–210], suggesting a role for the neuropeptide in modulating hypoxic damage in the CNS. In addition, several studies have demonstrated a potent antioxidant role for AM in different cell types, including macrophages [211–214]. In a transient focal ischemia model using AM knockout heterozygous ($AM^{(+/-)}$) mice, the authors showed increased iNOS expression in microglial cells from $AM^{(+/-)}$ mice compared to wild type mice [215]. Furthermore, supernatants of microglial cells exposed to OGD effectively protected neurons against OGD-induced death, and this effect was prevented by an AM receptor antagonist [209], suggesting a neuroprotective role for microglia-derived AM under hypoxic/ischemic stress.

CGRP and AM also inhibit LPS-induced TNF-α, IL-6, and NO release in rat cultured microglia and prevent LPS-induced expression of MIP-1α and MCP-1 in microglia/astrocyte cocultures. The anti-inflammatory effect appears to be stimulus-specific since neither CGRP nor AM inhibits IL-6 and NO release induced by a cytokine mix composed of TNF-α, IFN-γ, and IL-1β [69]. The ability of neuropeptides to inhibit cytokine and chemokine release in an inflammatory context is of particular relevance in neuroinflammatory diseases such as MS, in which local release of these factors promotes CNS infiltration of leukocytes and autoreactive T cells, which in turn perpetuate and amplify the inflammatory reaction [216]. Concordantly, in a murine model of chronic-progressive EAE, treatment with AM reduced inflammatory infiltration and demyelination in the CNS, partly by impairing activation of autoreactive Th1/Th17 cells and increasing the number of Th2 cells and regulatory T cells and also by preventing oxidative stress-induced oligodendrocyte death and inhibiting expression of astroglial and microglial-derived proinflammatory mediators such as IL-6, IL-12, TNF-α, and NO [217]. In another murine model of chronic EAE, infusion of CGRP also decreased clinical signs of disease and prevented microglial activation, evidenced by a reduced proportion of amoeboid $Iba1^{+}$ cells [218]. Altogether, evidence suggests that modulation of microglial activation by CGRP/AM signaling may account, at least in part, for the protective effects of these neuropeptides in neuroinflammatory diseases.

CGRP has been strongly linked to increased pain sensitivity in the spinal cord. Binding of CGRP to CGRP1 receptors in the rat spinal cord produces hyperalgesia through a mechanism involving activation of PKA and PKC signaling pathways [219]. Injection of CGRP stimulates activation of spinal cord microglia in a model of temporomandibular joint disorder, evidenced by increased OX-42 immunostaining [220]. Administration of the CGRP antagonist ($CGRP^{8-37}$) attenuates mechanical hypersensitivity and reduces microgliosis in a rat model of collagen-induced arthritis-induced hypersensitivity [221]. Furthermore, CGRP was demonstrated to contribute to the development of tolerance to morphine-induced analgesia partly through induction of p38 MAPK phosphorylation, upregulation of IL-6, and activation of the NF-κB signaling pathway in spinal cord microglial cells [222–224]. AM is also thought to act as a pronociceptive mediator at the spinal cord level. The pathogenesis of tolerance to chronic morphine treatment is known to involve AM signaling and has been linked to AM-induced proinflammatory activation of spinal microglia and astrocytes [96]. In summary, in experimental models of pain, evidence suggests a strong link between CGRP/AM activity and proinflammatory microglial activation.

9. Leptin

Leptin is a cytokine-like 167-amino acid peptide derived from the *ob* gene (or *lep* gene), produced primarily in adipose tissue, and released into the circulation [225]. The concentration of leptin in blood is finely regulated by the nutritional state; it increases following food intake to suppress appetite and decreases with fasting, leptin levels positively correlating with the degree of adiposity in rodents and humans [226, 227]. Given the size of the peptide, central access of leptin to the brain depends mainly on its passage from circulation across the blood-brain barrier (BBB) through a saturable transport system [228]. However, some studies have shown that leptin may also be synthesized in the brain, more specifically in the cerebellum, cortex, and hypothalamus, suggesting specific and local functions for leptin [229].

There are six known isoforms for the leptin receptor (LepR or Ob-R), LepRa-f, which derive from alternative splicing of the *lepr* (or *db*) gene. They all share a common leptin-binding domain but differ in their intracellular domains.

LepRe is the only soluble isoform that lacks a transmembrane domain, binds circulating leptin, and inhibits its central transport. LepRb, the long isoform of LepR, is a class I cytokine receptor and the major signaling form of the leptin receptor [225]. Ligand binding to LepRb leads to recruitment and activation of JAK-2, which in turn initiates downstream signaling pathways that may involve MAPKs or different members of the STAT family of transcription factors, such as STAT1, STAT3, and STAT5 [225, 230]. LepRb is widely expressed in the brain, particularly on specialized subsets of neurons in several hypothalamic and brainstem nuclei [231, 232]. In homeostatic conditions, leptin regulates production of POMC, AgRP, and NPY in the ARC [233–235] and inhibits food intake acting at the hypothalamus; it may also modulate neurogenesis, synaptogenesis, neuronal excitability, and neuroprotection in extrahypothalamic sites [236].

Leptin significantly influences the normal functioning of the immune system by stimulating a wide variety of functions such as cytokine production, chemotaxis, cytotoxicity, and survival of immune cells and also plays a role in modulating autoimmune responses in several models of disease (reviewed in [237]). In turn, leptin expression can be regulated by cytokines. Treatment of mice with the proinflammatory mediators TNF-α or IL-1 increases serum levels of leptin [238]. Rodent adipose tissue leptin mRNA and circulating levels of leptin are also elevated after systemic LPS injection [238, 239]. Furthermore, LPS-induced fever and loss of appetite were found to be mediated by leptin through induction of IL-1β in the brain [240], a finding later confirmed by others [241], all evidence supporting leptin as a mediator of anorexia and cachexia in inflammatory diseases.

The effects of leptin could be due, at least in part, to its actions on microglial cells. Expression of both the short (LepRa) and long (LepRb) isoforms of the leptin receptor was detected in mouse primary cultured glial cells [242] and in rat microglial cells where LepRb is activated by leptin and induces IL-1β through a STAT3-mediated mechanism that is independent of caspase-1-mediated cleavage [70]. Leptin treatment of BV-2 cells, which also express LepRa and LepRb, induces IL-6 release by a mechanism involving the insulin-receptor substrate-1/PI3K/Akt/NF-κB signaling pathways [71]. Leptin was also able to induce IL-6 and TNF-α release in mouse primary cultured hypothalamic microglia [243]. Preincubation of rat microglial cells with leptin before treatment with LPS potentiated production of IL-1β, TNF-α, and chemokines such as CINC-1 and MIP-2 [244]. Collectively, data indicate leptin can acutely activate microglial cells, which may be directly involved in the inflammatory effects of leptin in the CNS in pathologic conditions. This effect may be of particular interest in obesity or metabolic syndrome, where production of adipokines is deregulated. In such conditions adipokines can promote inflammation, ROS production, and disruption of BBB permeability and even affect different brain structures like the hippocampus, increasing the risk of developing dementia, such as AD [245]. Also, obesity is known to induce leptin resistance, leading to even higher circulating levels of leptin and further contributing to the pathogenesis of obesity-associated neurodegenerative diseases. In mice with impaired leptin signaling or mice fed a high-fat diet, microglial function is altered or impaired, illustrating a link between adipokines and immunity in the CNS [243]. Moreover, leptin levels are significantly elevated in CSF and in hippocampal tissue of AD patients, and leptin receptor mRNA is decreased, suggesting that leptin signaling is also deregulated in AD brains [246]. Thus, restoration of leptin signaling could result in better functional outcomes in neurodegenerative disease states, especially those associated with obesity and metabolic disorders [247].

Leptin has been shown to contribute to the pathogenesis of neuropathic pain. In a rat model of CCI, administration of a leptin antagonist prevented the development of injury-induced mechanical allodynia and thermal hyperalgesia [248]. In this study, leptin levels were increased in the ipsilateral spinal cord dorsal horn and CSF after CCI, and LepRb expression was also increased in the spinal cord. Interestingly, in vitro exposure of organotypic lumbar spinal cord cultures to leptin induced IL-1β production and increased OX-42 immunoreactivity, suggesting spinal microglia as a source of IL-1β following leptin treatment [248]. In mice with partial sciatic nerve ligation (PSL), the procedure induced leptin production from adipocytes present in the epineurium of the injured sciatic nerve, and leptin proved to be necessary for PSL-induced tactile allodynia. Furthermore, leptin enhanced production of pronociceptive mediators such as COX-2, iNOS, and matrix metalloprotease-9 from perineural macrophages, linking adipokines to development of neuropathic pain through macrophage activation [249]. However, another report showed protective effects of leptin in a model of SCI, where acute leptin administration after injury enhanced functional motor recovery, prevented development of thermal hyperalgesia and mechanical allodynia, enhanced expression of neuroprotective genes, reduced inflammatory mediators, and decreased spinal cord microglia/macrophage activation [250]. The divergence observed in the effects of leptin between this study and previous ones might be explained by the different experimental models. Thus, further study is needed to clarify the role of leptin in neuropathic pain and the possible involvement of spinal microglial cells as mediators of leptin's action.

10. Ghrelin

Ghrelin is a 28-amino acid peptide originally isolated from rat stomach as an endogenous ligand for the GHSR with the ability to stimulate growth hormone (GH) release from the pituitary [251]. It is expressed at high levels in the stomach and is also produced in the ARC of the hypothalamus [251]. Ghrelin mRNA expression in the stomach and circulating plasma levels increase after fasting and decrease after refeeding [252]. It acts as an orexigenic peptide, antagonizing the effects of leptin on food intake through activation of the hypothalamic NPY/Y_1R pathway [253]. The ghrelin receptor GHSR has two isoforms: GHSR1α and GHSR1β [254]. Ghrelin acylation in Ser3 is required for hormonal activity and for ghrelin to activate its cognate receptor GHSR1α [255], which is expressed in many tissues including pituitary gland and hypothalamus [256]. Nonetheless, unacylated ghrelin is found in circulation at greater concentrations than ghrelin, suggesting a

relevant physiological role [255]. Ghrelin can act directly on hypothalamic NPY and AgRP neurons, increasing food intake and body weight gain through a GHSR-dependent, GH-independent pathway [257]. However, certain effects of ghrelin have been observed in tissues where only the GHSR1β isoform is expressed, leading to the postulation of the existence of novel nonspecific ghrelin receptors called ghrelin receptor-like receptors and of specific unacylated ghrelin receptors. Finally, GHSR has been shown to form heterodimers with other GPCRs such as sst5, MC3R, and the dopamine receptors D1 and D2. Since receptor heterodimerization can alter G protein coupling as well as ligand potency, these findings add complexity to the pathways involved in ghrelin signaling [254].

Ghrelin has been demonstrated to exert neuroprotective effects in several models of neurodegenerative and inflammatory diseases, in which its protective effects have been associated, at least in part, with its ability to reduce microglial activation. In experimental SCI, microglial p38 MAPK activation followed by pronerve growth factor (proNGF) release is known to mediate oligodendrocyte death [258]. Ghrelin has been shown to promote functional recovery after SCI, partly by inhibiting microglial p38 MAPK activation and proNGF release in the spinal cord and by preventing apoptotic cell death of neurons and oligodendrocytes through a GHSR1α-dependent pathway [259]. The inhibitory effects of ghrelin on microglial activation have also been observed in vitro using BV-2 microglial cells. In this assay system, ghrelin prevented LPS-induced BV-2 p38 MAPK and JNK activation and attenuated proNGF and ROS production [260]. However, while expression of GHSR1α has been detected in spinal cord neurons and oligodendrocytes, it has not been detected in microglial cells or astrocytes by immunohistochemistry [259] or in BV-2 cells by RT-PCR and western blot [260], suggesting that the mechanism underlying the inhibitory effects of ghrelin on microglial activation may be either indirect or through a GHSR1α-independent pathway. In an in vitro assay system of OGD followed by reoxygenation (OGD/RO), modeling SCI, endothelial cell-derived MMP-3 was shown to mediate BV-2 microglial p38 MAPK activation and proNGF release. In this study, supernatant of OGD/RO endothelial cells transfected with MMP-3 siRNA failed to induce BV-2 microglial activation compared to supernatant from cells transfected with control siRNA, and microglial activation was also attenuated in MMP-3 knockout mice. Moreover, addition of ghrelin to OGD/RO endothelial cells inhibited MMP-3 production in a GHSR1α-dependent fashion [261], providing a possible mechanism for ghrelin-induced inhibition of microglial activation. In an in vivo model of kainic acid- (KA-) induced hippocampal neurodegeneration, systemic administration of ghrelin prevented KA-induced neuronal cell death, attenuated microglial and astroglial activation, and reduced TNF-α, IL-1β, and COX-2 expression in the hippocampus. Furthermore, ghrelin inhibited KA-induced MMP-3 expression in hippocampal neurons, and the protective effects of ghrelin were exerted through a GHSR1α-dependent pathway [262]. Once again, evidence supports a link between ghrelin-induced modulation of microglial activation and inhibition of MMP-3 expression. Ghrelin also attenuated motoneuron loss in organotypic rat spinal cord cultures exposed to threohydroxyaspartate (THA), a model of excitotoxic motoneuron degeneration, and prevented spinal cord microglia activation and expression of IL-1β and TNF-α, suggesting a possible therapeutic role for ghrelin in amyotrophic lateral sclerosis [263]. Ghrelin has also shown neuroprotective effects in animal models of PD. In a murine model of MPTP-induced dopaminergic neuron loss, systemic ghrelin administration improved dopaminergic neuron survival, prevented the loss of striatal dopaminergic fibers, and reduced MPTP-induced microglial activation, expression of TNF-α, IL-1β, MMP-3, and iNOS activation. While the inhibitory effects of ghrelin on microglial activation were prevented by a GHSR1α antagonist, expression of GHSR1α was not detected either in microglia from the substantia nigra pars compacta or in rat microglia-enriched cultures by immunohistochemistry and RT-PCR, respectively. Therefore, evidence again suggests that ghrelin-mediated inhibition of microglial activation in vivo may be indirect, possibly mediated by the reduction in MMP-3 expression in dopaminergic neurons [264]. In a murine model of EAE, administration of ghrelin reduced clinical signs of disease and inhibited expression of TNF-α, IL-1β, and IL-6 in spinal cord microglia and in infiltrating T cells from ghrelin-treated mice. The effects of ghrelin on EAE are specific and most likely mediated by GHSR1α, as unacylated ghrelin, which lacks the ability to bind GHSR1α, had no modulatory effect on EAE. Interestingly, ghrelin also inhibited LPS-induced TNF-α release in cultured microglial cells isolated from mouse brain, suggesting ghrelin can directly modulate LPS-induced microglial activation [265], but the specific mechanisms involved in the direct action of ghrelin on microglial cells were not investigated. Ghrelin has shown modulatory effects in memory and learning processes and therefore has been proposed to play a relevant role in AD [266]. CD36 is a scavenger receptor involved in microglial interaction with fibrillar Aβ and a mediator of Aβ-induced microglial activation [72, 73], which also functions as a receptor for GH secretagogues [267]. Bulgarelli and colleagues found that unacylated ghrelin and other synthetic GH secretagogues inhibit Aβ-induced IL-6 and IL-1β mRNA expression in N9 microglial cells, whereas acylated ghrelin does not modify Aβ-induced cytokine production in these cells. Moreover, N9 cells express CD36 but do not express GHSR1α. Thus, data indicate a role for GH secretagogues other than acylated ghrelin in modulation of Aβ-induced microglial activation through alternative GH receptors, such as CD36 [268]. Altogether, evidence suggests a potential therapeutic role for ghrelin in neurodegenerative disorders involving inflammation and excitotoxic cell death, partly through ghrelin's ability to suppress proinflammatory microglial activation.

Ghrelin has been shown to promote analgesia in models of neuropathic pain. In a rat model of CCI of the sciatic nerve, intrathecal administration of ghrelin delayed mechanical allodynia and thermal hyperalgesia, while reducing p38 MAPK and p65 NF-κB activation, and proinflammatory cytokine expression in the spinal dorsal horn, through a GHSR1α-mediated mechanism [269]. Taking into account the ample evidence demonstrating the anti-inflammatory

properties of ghrelin, it is possible that its analgesic actions may be due, at least in part, to its microglia-deactivating effect.

11. Conclusions

In the past few decades, microglial cells have come to be considered key participants in inflammatory processes within the CNS. They are known to be involved in the development and/or progression of many neuroinflammatory and neurodegenerative diseases, including AD, MS, and PD. Furthermore, research is uncovering an increasingly important role for activated microglia in various neuropsychiatric conditions such as schizophrenia, autism, depression, and anxiety disorders, among others.

The role of microglia in pain sensitivity is a relatively newer area of research, and there is still much to be learnt about how endogenous neuropeptides may influence this phenomenon by acting directly on microglial cells. Nonetheless, it is increasingly evident that these cells are pivotal in development of pathologic pain through the release of inflammatory mediators and neurotransmitters that induce sustained activation of neuronal sensory pathways.

In summary, cumulative evidence suggests a leading role for microglia in pathologies currently of the highest medical relevance. Therefore, the study of central immunomodulatory mediators or microglia-deactivating factors has become a major area of research in the biomedical field, unveiling the existence of precise endogenous mechanisms mediated by neuropeptides that regulate microglial activation, and providing new targets for treatment of neuroinflammatory diseases.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] R. Franco and D. Fernández-Suárez, "Alternatively activated microglia and macrophages in the central nervous system," *Progress in Neurobiology*, vol. 131, pp. 65–86, 2015.

[2] H.-M. Gao, B. Liu, W. Zhang, and J.-S. Hong, "Novel anti-inflammatory therapy for Parkinson's disease," *Trends in Pharmacological Sciences*, vol. 24, no. 8, pp. 395–401, 2003.

[3] P. L. McGeer and E. G. McGeer, "The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases," *Brain Research Reviews*, vol. 21, no. 2, pp. 195–218, 1995.

[4] A. Minagar, P. Shapshak, R. Fujimura, R. Ownby, M. Heyes, and C. Eisdorfer, "The role of macrophage/microglia and astrocytes in the pathogenesis of three neurologic disorders: HIV-associated dementia, Alzheimer disease, and multiple sclerosis," *Journal of the Neurological Sciences*, vol. 202, no. 1-2, pp. 13–23, 2002.

[5] L. R. Frick, K. Williams, and C. Pittenger, "Microglial dysregulation in psychiatric disease," *Clinical and Developmental Immunology*, vol. 2013, Article ID 608654, 10 pages, 2013.

[6] R.-R. Ji, T. Berta, and M. Nedergaard, "Glia and pain: is chronic pain a gliopathy?" *Pain*, vol. 154, pp. S10–S28, 2013.

[7] R.-R. Ji and M. R. Suter, "p38 MAPK, microglial signaling, and neuropathic pain," *Molecular Pain*, vol. 3, article 33, 2007.

[8] R.-R. Ji, T. Berta, and M. Nedergaard, "Glia and pain: is chronic pain a gliopathy?" *Pain*, vol. 154, supplement 1, pp. S10–S28, 2013.

[9] J. A. M. Coull, S. Beggs, D. Boudreau et al., "BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain," *Nature*, vol. 438, no. 7070, pp. 1017–1021, 2005.

[10] A. Ledeboer, E. M. Sloane, E. D. Milligan et al., "Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation," *Pain*, vol. 115, no. 1-2, pp. 71–83, 2005.

[11] B. C. Hains and S. G. Waxman, "Activated microglia contribute to the maintenance of chronic pain after spinal cord injury," *The Journal of Neuroscience*, vol. 26, no. 16, pp. 4308–4317, 2006.

[12] Y.-W. Chang and S. G. Waxman, "Minocycline attenuates mechanical allodynia and central sensitization following peripheral second-degree burn injury," *Journal of Pain*, vol. 11, no. 11, pp. 1146–1154, 2010.

[13] S. Pu, Y. Xu, D. Du et al., "Minocycline attenuates mechanical allodynia and expression of spinal NMDA receptor 1 subunit in rat neuropathic pain model," *Journal of Physiology and Biochemistry*, vol. 69, no. 3, pp. 349–357, 2013.

[14] X.-Y. Hua, C. I. Svensson, T. Matsui, B. Fitzsimmons, T. L. Yaksh, and M. Webb, "Intrathecal minocycline attenuates peripheral inflammation-induced hyperalgesia by inhibiting p38 MAPK in spinal microglia," *European Journal of Neuroscience*, vol. 22, no. 10, pp. 2431–2440, 2005.

[15] J. Mika, M. Osikowicz, W. Makuch, and B. Przewlocka, "Minocycline and pentoxifylline attenuate allodynia and hyperalgesia and potentiate the effects of morphine in rat and mouse models of neuropathic pain," *European Journal of Pharmacology*, vol. 560, no. 2-3, pp. 142–149, 2007.

[16] J. Bakos, M. Zatkova, Z. Bacova, and D. Ostatnikova, "The role of hypothalamic neuropeptides in neurogenesis and neuritogenesis," *Neural Plasticity*, vol. 2016, Article ID 3276383, 10 pages, 2016.

[17] J. M. Pocock and H. Kettenmann, "Neurotransmitter receptors on microglia," *Trends in Neurosciences*, vol. 30, no. 10, pp. 527–535, 2007.

[18] M. Pannell, F. Szulzewsky, V. Matyash, S. A. Wolf, and H. Kettenmann, "The subpopulation of microglia sensitive to neurotransmitters/neurohormones is modulated by stimulation with LPS, interferon-γ, and IL-4," *Glia*, vol. 62, no. 5, pp. 667–679, 2014.

[19] A. N. Eberle, *The Melanotropins: Chemistry, Physiology and Mechanisms of Action*, Karger, 1988.

[20] J. E. S. Wikberg, R. Muceniece, I. Mandrika et al., "New aspects on the melanocortins and their receptors," *Pharmacological Research*, vol. 42, no. 5, pp. 393–420, 2000.

[21] V. Caruso, M. C. Lagerström, P. K. Olszewski, R. Fredriksson, and H. B. Schiöth, "Synaptic changes induced by melanocortin signalling," *Nature Reviews Neuroscience*, vol. 15, no. 2, pp. 98–110, 2014.

[22] A. Catania, S. Gatti, G. Colombo, and J. M. Lipton, "Targeting melanocortin receptors as a novel strategy to control inflammation," *Pharmacological Reviews*, vol. 56, no. 1, pp. 1–29, 2004.

[23] H. B. Patel, T. Montero-Melendez, K. V. Greco, and M. Perretti, "Melanocortin receptors as novel effectors of macrophage responses in inflammation," *Frontiers in Immunology*, vol. 2, article 41, 2011.

[24] N. Rajora, G. Boccoli, D. Burns, S. Sharma, A. P. Catania, and J. M. Lipton, "Alpha-MSH modulates local and circulating tumor necrosis factor-alpha in experimental brain inflammation," *The Journal of Neuroscience*, vol. 17, no. 6, pp. 2181–2186, 1997.

[25] R. Delgado, A. Carlin, L. Airaghi et al., "Melanocortin peptides inhibit production of proinflammatory cytokines and nitric oxide by activated microglia," *Journal of Leukocyte Biology*, vol. 63, no. 6, pp. 740–745, 1998.

[26] R. A. Star, N. Rajora, J. Huang, R. C. Stock, A. Catania, and J. M. Lipton, "Evidence of autocrine modulation of macrophage nitric oxide synthase by α-melanocyte-stimulating hormone," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 17, pp. 8016–8020, 1995.

[27] N. Rajora, G. Ceriani, A. Catania, R. A. Star, M. T. Murphy, and J. M. Lipton, "α-MSH production, receptors, and influence on neopterin in a human monocyte/macrophage cell line," *Journal of Leukocyte Biology*, vol. 59, no. 2, pp. 248–253, 1996.

[28] D. Galimberti, P. Baron, L. Meda et al., "α-MSH peptides inhibit production of nitric oxide and tumor necrosis factor-α by microglial cells activated with β-amyloid and interferon γ," *Biochemical and Biophysical Research Communications*, vol. 263, no. 1, pp. 251–256, 1999.

[29] D. Giuliani, S. Leone, C. Mioni et al., "Broad therapeutic treatment window of [Nle^4, D-Phe^7]α-melanocyte-stimulating hormone for long-lasting protection against ischemic stroke, in Mongolian gerbils," *European Journal of Pharmacology*, vol. 538, no. 1–3, pp. 48–56, 2006.

[30] D. Giuliani, A. Ottani, C. Mioni et al., "Neuroprotection in focal cerebral ischemia owing to delayed treatment with melanocortins," *European Journal of Pharmacology*, vol. 570, no. 1–3, pp. 57–65, 2007.

[31] Å. Forslin Aronsson, S. Spulber, L. M. Popescu et al., "α-melanocyte-stimulating hormone is neuroprotective in rat global cerebral ischemia," *Neuropeptides*, vol. 40, no. 1, pp. 65–75, 2006.

[32] E.-V. Schaible, A. Steinsträßer, A. Jahn-Eimermacher et al., "Single Administration of Tripeptide α-MSH(11-13) Attenuates Brain Damage by Reduced Inflammation and Apoptosis after Experimental Traumatic Brain Injury in Mice," *PLoS ONE*, vol. 8, no. 8, Article ID e71056, 2013.

[33] M. Lasaga, L. Debeljuk, D. Durand, T. N. Scimonelli, and C. Caruso, "Role of α-melanocyte stimulating hormone and melanocortin 4 receptor in brain inflammation," *Peptides*, vol. 29, no. 10, pp. 1825–1835, 2008.

[34] C. Caruso, L. Carniglia, D. Durand, T. N. Scimonelli, and M. Lasaga, "Melanocortins: anti-inflammatory and neuroprotective peptides," in *Mental and Behavioural Disorders and Diseases of the Nervous System*, L. M. Martins and S. H. Y. Loh, Eds., INTECH, Rijeka, Croatia, 2012.

[35] L. Roselli-Rehfuss, K. G. Mountjoy, L. S. Robbins et al., "Identification of a receptor for γ melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 19, pp. 8856–8860, 1993.

[36] K. G. Mountjoy, M. T. Mortrud, M. J. Low, R. B. Simerly, and R. D. Cone, "Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain," *Molecular Endocrinology*, vol. 8, no. 10, pp. 1298–1308, 1994.

[37] R. Bhardwaj, E. Becher, K. Mahnke et al., "Evidence for the differential expression of the functional α-melanocyte-stimulating hormone receptor MC-1 on human monocytes," *Journal of Immunology*, vol. 158, no. 7, pp. 3378–3384, 1997.

[38] U. Raap, T. Brzoska, S. Sohl et al., "α-melanocyte-stimulating hormone inhibits allergic airway inflammation," *Journal of Immunology*, vol. 171, no. 1, pp. 353–359, 2003.

[39] S. J. Getting, L. Gibbs, A. J. L. Clark, R. J. Flower, and M. Perretti, "POMC gene-derived peptides activate melanocortin type 3 receptor on murine macrophages, suppress cytokine release, and inhibit neutrophil migration in acute experimental inflammation," *The Journal of Immunology*, vol. 162, no. 12, pp. 7446–7453, 1999.

[40] S. J. Getting, H. C. Christian, R. J. Flower, and M. Perretti, "Activation of melanocortin type 3 receptor as a molecular mechanism for adrenocorticotropic hormone efficacy in gouty arthritis," *Arthritis and Rheumatism*, vol. 46, no. 10, pp. 2765–2775, 2002.

[41] C. W. Lam, M. Perretti, and S. J. Getting, "Melanocortin receptor signaling in RAW264.7 macrophage cell line," *Peptides*, vol. 27, no. 2, pp. 404–412, 2006.

[42] S. Taherzadeh, S. Sharma, V. Chhajlani et al., "α-MSH and its receptors in regulation of tumor necrosis factor-α production by human monocyte/macrophages," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 276, no. 5, part 2, pp. R1289–R1294, 1999.

[43] G. N. Andersen, M. Hägglund, O. Nagaeva et al., "Quantitative measurement of the levels of melanocortin receptor subtype 1, 2, 3 and 5 and pro-opio-melanocortin peptide gene expression in subsets of human peripheral blood leucocytes," *Scandinavian Journal of Immunology*, vol. 61, no. 3, pp. 279–284, 2005.

[44] C. W. Lam, S. J. Getting, and M. Perretti, "In vitro and in vivo induction of heme oxygenase 1 in mouse macrophages following melanocortin receptor activation," *The Journal of Immunology*, vol. 174, no. 4, pp. 2297–2304, 2005.

[45] C. Lindberg, E. Hjorth, C. Post, B. Winblad, and M. Schultzberg, "Cytokine production by a human microglial cell line: effects of β-amyloid and α-melanocyte-stimulating hormone," *Neurotoxicity Research*, vol. 8, no. 3-4, pp. 267–276, 2005.

[46] D. M. Dinulescu and R. D. Cone, "Agouti and agouti-related protein: analogies and contrasts," *Journal of Biological Chemistry*, vol. 275, no. 10, pp. 6695–6698, 2000.

[47] L. Carniglia, D. Durand, C. Caruso, and M. Lasaga, "Effect of NDP-α-MSH on PPAR-γ and $-\beta$ expression and anti-inflammatory cytokine release in rat astrocytes and microglia," *PLoS ONE*, vol. 8, no. 2, Article ID e57313, 2013.

[48] A. W. Taylor, "The immunomodulating neuropeptide alpha-melanocyte-stimulating hormone (α-MSH) suppresses LPS-stimulated TLR4 with IRAK-M in macrophages," *Journal of Neuroimmunology*, vol. 162, no. 1-2, pp. 43–50, 2005.

[49] L. Carniglia, D. Ramírez, D. Durand et al., "[Nle4, D-Phe7]-α-MSH inhibits toll-like receptor (TLR)2- and TLR4-induced microglial activation and promotes a M2-like phenotype," *PLOS ONE*, vol. 11, no. 6, p. e0158564, 2016.

[50] A. Sarkar, Y. Sreenivasan, and S. K. Manna, "α-Melanocyte-stimulating hormone inhibits lipopolysaccharide-induced biological responses by downregulating CD14 from macrophages," *FEBS Letters*, vol. 553, no. 3, pp. 286–294, 2003.

[51] S. K. Manna and B. B. Aggarwal, "α-melanocyte-stimulating hormone inhibits the nuclear transcription factor NF-κB activation induced by various inflammatory agents," *Journal of Immunology*, vol. 161, no. 6, pp. 2873–2880, 1998.

[52] I. Mandrika, R. Muceniece, and J. E. S. Wikberg, "Effects of melanocortin peptides on lipopolysaccharide/interferon-gamma-induced NF-kappaB DNA binding and nitric oxide production in macrophage-like RAW 264.7 cells: evidence for dual mechanisms of action," *Biochemical Pharmacology*, vol. 61, no. 5, pp. 613–621, 2001.

[53] A. W. Taylor, J. W. Streilein, and S. W. Cousins, "Identification of α-melanocyte stimulating hormone as a potential immunosuppressive factor in aqueous humor," *Current Eye Research*, vol. 11, no. 12, pp. 1199–1206, 1992.

[54] N. Kawanaka and A. W. Taylor, "Localized retinal neuropeptide regulation of macrophage and microglial cell functionality," *Journal of Neuroimmunology*, vol. 232, no. 1-2, pp. 17–25, 2011.

[55] C. C. Chao, S. Hu, K. B. Shark, W. S. Sheng, G. Gekker, and P. K. Peterson, "Activation of Mu opioid receptors inhibits microglial cell chemotaxis," *Journal of Pharmacology and Experimental Therapeutics*, vol. 281, no. 2, pp. 998–1004, 1997.

[56] J. Mika, K. Popiolek-Barczyk, E. Rojewska, W. Makuch, K. Starowicz, and B. Przewlocka, "Delta-opioid receptor analgesia is independent of microglial activation in a rat model of neuropathic pain," *PLoS ONE*, vol. 9, no. 8, Article ID e104420, 2014.

[57] C. C. Chao, G. Gekker, S. Hu et al., "kappa opioid receptors in human microglia downregulate human immunodeficiency virus 1 expression," *Proceedings of the National Academy of Sciences*, vol. 93, no. 15, pp. 8051–8056, 1996.

[58] R. Ferreira, S. Xapelli, T. Santos et al., "Neuropeptide y modulation of interleukin-1β (IL-1β)-induced nitric oxide production in microglia," *The Journal of Biological Chemistry*, vol. 285, no. 53, pp. 41921–41934, 2010.

[59] A. Santos-Carvalho, C. A. Aveleira, F. Elvas, A. F. Ambrósio, and C. Cavadas, "Neuropeptide Y receptors Y_1 and Y_2 are present in neurons and glial cells in rat retinal cells in culture," *Investigative Ophthalmology and Visual Science*, vol. 54, no. 1, pp. 429–443, 2013.

[60] W.-K. Kim, Y. Kan, D. Ganea, R. P. Hart, I. Gozes, and G. M. Jonakait, "Vasoactive intestinal peptide and pituitary adenylyl cyclase-activating polypeptide inhibit tumor necrosis factor-α production in injured spinal cord and in activated microglia via a cAMP-dependent pathway," *Journal of Neuroscience*, vol. 20, no. 10, pp. 3622–3630, 2000.

[61] M. Delgado, G. M. Jonakait, and D. Ganea, "Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit chemokine production in activated microglia," *Glia*, vol. 39, no. 2, pp. 148–161, 2002.

[62] J. Feindt, A. Schmidt, and R. Mentlein, "Receptors and effects of the inhibitory neuropeptide somatostatin in microglial cells," *Molecular Brain Research*, vol. 60, no. 2, pp. 228–233, 1998.

[63] S. Fleisher-Berkovich, T. Filipovich-Rimon, S. Ben-Shmuel, C. Hülsmann, M. P. Kummer, and M. T. Heneka, "Distinct modulation of microglial amyloid β phagocytosis and migration by neuropeptides[i]," *Journal of Neuroinflammation*, vol. 7, article 61, 2010.

[64] J.-P. Lai, G.-X. Zhan, D. E. Campbell, S. D. Douglas, and W.-Z. Ho, "Detection of substance P and its receptor in human fetal microglia," *Neuroscience*, vol. 101, no. 4, pp. 1137–1144, 2000.

[65] A. Rasley, K. L. Bost, J. K. Olson, S. D. Miller, and I. Marriott, "Expression of functional NK-1 receptors in murine microglia," *Glia*, vol. 37, no. 3, pp. 258–267, 2002.

[66] J. Zhu, C. Qu, X. Lu, and S. Zhang, "Activation of microglia by histamine and substance P," *Cellular Physiology and Biochemistry*, vol. 34, no. 3, pp. 768–780, 2014.

[67] J. Priller, C. A. Haas, M. Reddington, and G. W. Kreutzberg, "Calcitonin gene-related peptide and ATP induce immediate early gene expression in cultured rat microglial cells," *Glia*, vol. 15, no. 4, pp. 447–457, 1995.

[68] M. Reddington, J. Priller, J. Treichel, C. Haas, and G. W. Kreutzberg, "Astrocytes and microglia as potential targets for calcitonin gene related peptide in the central nervous system," *Canadian Journal of Physiology and Pharmacology*, vol. 73, no. 7, pp. 1047–1049, 1995.

[69] A. Consonni, S. Morara, F. Codazzi, F. Grohovaz, and D. Zacchetti, "Inhibition of lipopolysaccharide-induced microglia activation by calcitonin gene related peptide and adrenomedullin," *Molecular and Cellular Neuroscience*, vol. 48, no. 2, pp. 151–160, 2011.

[70] E. Pinteaux, W. Inoue, L. Schmidt, F. Molina-Holgado, N. J. Rothwell, and G. N. Luheshi, "Leptin induces interleukin-1β release from rat microglial cells through a caspase 1 independent mechanism," *Journal of Neurochemistry*, vol. 102, no. 3, pp. 826–833, 2007.

[71] C.-H. Tang, D.-Y. Lu, R.-S. Yang et al., "Leptin-induced IL-6 production is mediated by leptin receptor, insulin receptor substrate-1, phosphatidylinositol 3-kinase, Akt, NF-κB, and p300 pathway in microglia," *Journal of Immunology*, vol. 179, no. 2, pp. 1292–1302, 2007.

[72] M. E. Bamberger, M. E. Harris, D. R. McDonald, J. Husemann, and G. E. Landreth, "A cell surface receptor complex for fibrillar β-amyloid mediates microglial activation," *The Journal of Neuroscience*, vol. 23, no. 7, pp. 2665–2674, 2003.

[73] I. S. Coraci, J. Husemann, J. W. Berman et al., "CD36, a class B scavenger receptor, is expressed on microglia in Alzheimer's disease brains and can mediate production of reactive oxygen species in response to β-amyloid fibrils," *The American Journal of Pathology*, vol. 160, no. 1, pp. 101–112, 2002.

[74] J. B. El Khoury, K. J. Moore, T. K. Means et al., "CD36 mediates the innate host response to β-amyloid," *The Journal of Experimental Medicine*, vol. 197, no. 12, pp. 1657–1666, 2003.

[75] A. Sierra, O. Abiega, A. Shahraz, and H. Neumann, "Janus-faced microglia: beneficial and detrimental consequences of microglial phagocytosis," *Frontiers in Cellular Neuroscience*, 2013.

[76] A. Chan, T. Magnus, and R. Gold, "Phagocytosis of apoptotic inflammatory cells by microglia and modulation by different cytokines: mechanism for removal of apoptotic cells in the inflamed nervous system," *GLIA*, vol. 33, no. 1, pp. 87–95, 2001.

[77] B. A. Durafourt, C. S. Moore, D. A. Zammit et al., "Comparison of polarization properties of human adult microglia and blood-derived macrophages," *Glia*, vol. 60, no. 5, pp. 717–727, 2012.

[78] T. Montero-Melendez, H. B. Patel, M. Seed, S. Nielsen, T. E. N. Jonassen, and M. Perretti, "The melanocortin agonist AP214 exerts anti-inflammatory and proresolving properties," *American Journal of Pathology*, vol. 179, no. 1, pp. 259–269, 2011.

[79] A. W. Taylor, "Alpha-melanocyte stimulating hormone (α-MSH) is a post-caspase suppressor of apoptosis in RAW 264.7 macrophages," *PLoS ONE*, vol. 8, no. 8, Article ID e74488, 2013.

[80] T. A. Phan and A. W. Taylor, "The neuropeptides α-MSH and NPY modulate phagocytosis and phagolysosome activation in RAW 264.7 cells," *Journal of Neuroimmunology*, vol. 260, no. 1-2, pp. 9–16, 2013.

[81] K. Starowicz and B. Przewłocka, "The role of melanocortins and their receptors in inflammatory processes, nerve regeneration and nociception," *Life Sciences*, vol. 73, no. 7, pp. 823–847, 2003.

[82] K. Tsou, H. Khachaturian, H. Akil, and S. J. Watson, "Immunocytochemical localization of pro-opiomelanocortin-derived

peptides in the adult rat spinal cord," *Brain Research*, vol. 378, no. 1, pp. 28–35, 1986.

[83] K. Starowicz, W. Bilecki, A. Sieja, B. Przewlocka, and R. Przewlocki, "Melanocortin 4 receptor is expressed in the dorsal root ganglions and down-regulated in neuropathic rats," *Neuroscience Letters*, vol. 358, no. 2, pp. 79–82, 2004.

[84] M. van der Kraan, J. B. Tatro, M. L. Entwistle et al., "Expression of melanocortin receptors and pro-opiomelanocortin in the rat spinal cord in relation to neurotrophic effects of melanocortins," *Molecular Brain Research*, vol. 63, no. 2, pp. 276–286, 1999.

[85] M. Beltramo, M. Campanella, G. Tarozzo et al., "Gene expression profiling of melanocortin system in neuropathic rats supports a role in nociception," *Molecular Brain Research*, vol. 118, no. 1-2, pp. 111–118, 2003.

[86] R. Bertorelli, S. Fredduzzi, G. Tarozzo et al., "Endogenous and exogenous melanocortin antagonists induce anti-allodynic effects in a model of rat neuropathic pain," *Behavioural Brain Research*, vol. 157, no. 1, pp. 55–62, 2005.

[87] D. H. Vrinten, W. H. Gispen, G. J. Groen, and R. A. H. Adan, "Antagonism of the melanocortin system reduces cold and mechanical allodynia in mononeuropathic rats," *Journal of Neuroscience*, vol. 20, no. 21, pp. 8131–8137, 2000.

[88] K. Starowicz, S. A. Mousa, I. Obara et al., "Peripheral antinociceptive effects of MC4 receptor antagonists in a rat model of neuropathic pain—A Biochemical and Behavioral Study," *Pharmacological Reports*, vol. 61, no. 6, pp. 1086–1095, 2009.

[89] A. S. Kalange, D. M. Kokare, P. S. Singru, M. A. Upadhya, C. T. Chopde, and N. K. Subhedar, "Central administration of selective melanocortin 4 receptor antagonist HS014 prevents morphine tolerance and withdrawal hyperalgesia," *Brain Research*, vol. 1181, no. 1, pp. 10–20, 2007.

[90] K. Inoue and M. Tsuda, "Microglia and neuropathic pain," *GLIA*, vol. 57, no. 14, pp. 1469–1479, 2009.

[91] P. Sacerdote, S. Denis Donini, P. Paglia, F. Granucci, A. E. Panerai, and P. Ricciardicastagnoli, "Cloned microglial cells but not macrophages synthesize beta-endorphin in response to CRH activation," *Glia*, vol. 9, no. 4, pp. 305–310, 1993.

[92] N. Gong, Q. Xiao, B. Zhu et al., "Activation of spinal glucagon-like peptide-1 receptors specifically suppresses pain hypersensitivity," *The Journal of Neuroscience*, vol. 34, no. 15, pp. 5322–5334, 2014.

[93] H. Fan, T.-F. Li, N. Gong, and Y.-X. Wang, "Shanzhiside methylester, the principle effective iridoid glycoside from the analgesic herb *Lamiophlomis rotata*, reduces neuropathic pain by stimulating spinal microglial β-endorphin expression," *Neuropharmacology*, vol. 101, pp. 98–109, 2016.

[94] H. Fan, N. Gong, T.-F. Li et al., "The non-peptide GLP-1 receptor agonist WB4-24 blocks inflammatory nociception by stimulating β-endorphin release from spinal microglia," *British Journal of Pharmacology*, vol. 172, no. 1, pp. 64–79, 2015.

[95] K. S. Sundar, L. S. Kamaraju, J. Dingfelder et al., "Beta-endorphin enhances the replication of neurotropic human immunodeficiency virus in fetal perivascular microglia," *Journal of Neuroimmunology*, vol. 61, no. 1, pp. 97–104, 1995.

[96] X. Zeng, M. Y. Lin, D. Wang, Y. Zhang, and Y. Hong, "Involvement of adrenomedullin in spinal glial activation following chronic administration of morphine in rats," *European Journal of Pain (United Kingdom)*, vol. 18, no. 9, pp. 1323–1332, 2014.

[97] V. Raghavendra, M. D. Rutkowski, and J. A. Deleo, "The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats," *Journal of Neuroscience*, vol. 22, no. 22, pp. 9980–9989, 2002.

[98] Y. Cui, Y. Chen, J.-L. Zhi, R.-X. Guo, J.-Q. Feng, and P.-X. Chen, "Activation of p38 mitogen-activated protein kinase in spinal microglia mediates morphine antinociceptive tolerance," *Brain Research*, vol. 1069, no. 1, pp. 235–243, 2006.

[99] Y. Cui, X.-X. Liao, W. Liu et al., "A novel role of minocycline: attenuating morphine antinociceptive tolerance by inhibition of p38 MAPK in the activated spinal microglia," *Brain, Behavior, and Immunity*, vol. 22, no. 1, pp. 114–123, 2008.

[100] A. Thorsell and M. Heilig, "Diverse functions of neuropeptide Y revealed using genetically modified animals," *Neuropeptides*, vol. 36, no. 2-3, pp. 182–193, 2002.

[101] D. Larhammar and E. Salaneck, "Molecular evolution of NPY receptor subtypes," *Neuropeptides*, vol. 38, no. 4, pp. 141–151, 2004.

[102] A. Santos-Carvalho, A. R. Álvaro, J. Martins, A. F. Ambrósio, and C. Cavadas, "Emerging novel roles of neuropeptide Y in the retina: from neuromodulation to neuroprotection," *Progress in Neurobiology*, vol. 112, pp. 70–79, 2014.

[103] R. Ferreira, T. Santos, L. Cortes et al., "Neuropeptide y inhibits interleukin-1 beta-induced microglia motility," *Journal of Neurochemistry*, vol. 120, no. 1, pp. 93–105, 2012.

[104] J. Gonçalves, C. F. Ribeiro, J. O. Malva, and A. P. Silva, "Protective role of neuropeptide Y Y_2 receptors in cell death and microglial response following methamphetamine injury," *European Journal of Neuroscience*, vol. 36, no. 9, pp. 3173–3183, 2012.

[105] A. Vezzani, S. Balosso, and T. Ravizza, "The role of cytokines in the pathophysiology of epilepsy," *Brain, Behavior, and Immunity*, vol. 22, no. 6, pp. 797–803, 2008.

[106] Q. J. Li, C. Z. Dong, W. L. Li, W. Bu, J. Wu, and W. Q. Zhao, "Neuropeptide Y protects cerebral cortical neurons by regulating microglial immune function," *Neural Regeneration Research*, vol. 9, no. 9, pp. 959–967, 2014.

[107] A. R. Álvaro, J. Rosmaninho-Salgado, A. R. Santiago et al., "NPY in rat retina is present in neurons, in endothelial cells and also in microglial and Müller cells," *Neurochemistry International*, vol. 50, no. 5, pp. 757–763, 2007.

[108] S. Bedoui, S. von Hörsten, and T. Gebhardt, "A role for neuropeptide Y (NPY) in phagocytosis: implications for innate and adaptive immunity," *Peptides*, vol. 28, no. 2, pp. 373–376, 2007.

[109] R. Ferreira, T. Santos, M. Viegas et al., "Neuropeptide Y inhibits interleukin-1β-induced phagocytosis by microglial cells," *Journal of Neuroinflammation*, vol. 8, article 169, 2011.

[110] A. B. Intondi, M. N. Dahlgren, M. A. Eilers, and B. K. Taylor, "Intrathecal neuropeptide Y reduces behavioral and molecular markers of inflammatory or neuropathic pain," *Pain*, vol. 137, no. 2, pp. 352–365, 2008.

[111] P. A. Smith, T. D. Moran, F. Abdulla, K. K. Tumber, and B. K. Taylor, "Spinal mechanisms of NPY analgesia," *Peptides*, vol. 28, no. 2, pp. 464–474, 2007.

[112] S. I. Said and V. Mutt, "Polypeptide with broad biological activity: isolation from small intestine," *Science*, vol. 169, no. 3951, pp. 1217–1218, 1970.

[113] D. Ganea, K. M. Hooper, and W. Kong, "The neuropeptide vasoactive intestinal peptide: direct effects on immune cells and involvement in inflammatory and autoimmune diseases," *Acta Physiologica*, vol. 213, no. 2, pp. 442–452, 2015.

[114] S. I. Said, "Vasoactive intestinal peptide," *Journal of Endocrinological Investigation*, vol. 9, no. 2, pp. 191–200, 1986.

[115] N. Itoh, K. Obata, N. Yanaihara, and H. Okamoto, "Human preprovasoactive intestinal polypeptide contains a novel PHI-27-like peptide, PHM-27," *Nature*, vol. 304, no. 5926, pp. 547–549, 1983.

[116] A. Miyata, A. Arimura, R. R. Dahl et al., "Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells," *Biochemical and Biophysical Research Communications*, vol. 164, no. 1, pp. 567–574, 1989.

[117] D. Vaudry, A. Falluel-Morel, S. Bourgault et al., "Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery," *Pharmacological Reviews*, vol. 61, no. 3, pp. 283–357, 2009.

[118] A. J. Harmar, J. Fahrenkrug, I. Gozes et al., "Pharmacology and functions of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide: IUPHAR review 1," *British Journal of Pharmacology*, vol. 166, no. 1, pp. 4–17, 2012.

[119] L. Dickson and K. Finlayson, "VPAC and PAC receptors: from ligands to function," *Pharmacology and Therapeutics*, vol. 121, no. 3, pp. 294–316, 2009.

[120] J. A. Waschek, "VIP and PACAP: neuropeptide modulators of CNS inflammation, injury, and repair," *British Journal of Pharmacology*, vol. 169, no. 3, pp. 512–523, 2013.

[121] A. Dejda, P. Sokołowska, and J. Z. Nowak, "Neuroprotective potential of three neuropeptides PACAP, VIP and PHI," *Pharmacological Reports*, vol. 57, no. 3, pp. 307–320, 2005.

[122] M. Delgado, J. Leceta, and D. Ganea, "Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit the production of inflammatory mediators by activated microglia," *Journal of Leukocyte Biology*, vol. 73, no. 1, pp. 155–164, 2003.

[123] M. Delgado, "Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit CBP-NF-κB interaction in activated microglia," *Biochemical and Biophysical Research Communications*, vol. 297, no. 5, pp. 1181–1185, 2002.

[124] E. Gonzalez-Rey and M. Delgado, "Vasoactive intestinal peptide inhibits cyclooxygenase-2 expression in activated macrophages, microglia, and dendritic cells," *Brain, Behavior, and Immunity*, vol. 22, no. 1, pp. 35–41, 2008.

[125] M. Delgado, "Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit the MEKK1/MEK4/JNK signaling pathway in endotoxin-activated microglia," *Biochemical and Biophysical Research Communications*, vol. 293, no. 2, pp. 771–776, 2002.

[126] H. Lee and K. Suk, "Selective modulation of microglial signal transduction by PACAP," *NeuroReport*, vol. 15, no. 9, pp. 1469–1474, 2004.

[127] K. Suk, J.-H. Park, and W.-H. Lee, "Neuropeptide PACAP inhibits hypoxic activation of brain microglia: a protective mechanism against microglial neurotoxicity in ischemia," *Brain Research*, vol. 1026, no. 1, pp. 151–156, 2004.

[128] M. Delgado, "Inhibition of interferon (IFN) γ-induced Jak-STAT1 activation in microglia by vasoactive intestinal peptide. Inhibitory effect on CD40, IFN-induced protein-10, and inducible nitric-oxide synthase expression," *The Journal of Biological Chemistry*, vol. 278, no. 30, pp. 27620–27629, 2003.

[129] W.-K. Kim, D. Ganea, and G. M. Jonakait, "Inhibition of microglial CD40 expression by pituitary adenylate cyclase-activating polypeptide is mediated by interleukin-10," *Journal of Neuroimmunology*, vol. 126, no. 1-2, pp. 16–24, 2002.

[130] M. Delgado, C. Martinez, D. Pozo et al., "Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activation polypeptide (PACAP) protect mice from lethal endotoxemia through the inhibition of TNF-α and IL-6," *Journal of Immunology*, vol. 162, no. 2, pp. 1200–1205, 1999.

[131] M. Delgado, E. J. Munoz-Elias, R. P. Gomariz, and D. Ganea, "Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide enhance IL-10 production by murine macrophages: in vitro and in vivo studies," *Journal of Immunology*, vol. 162, no. 3, pp. 1707–1716, 1999.

[132] A. M. Bhandare, S. Mohammed, P. M. Pilowsky, and M. M. J. Farnham, "Antagonism of PACAP or microglia function worsens the cardiovascular consequences of kainic-acid-induced seizures in rats," *Journal of Neuroscience*, vol. 35, no. 5, pp. 2191–2199, 2015.

[133] M. Delgado, "Vasoactive intestinal peptide prevents activated microglia-induced neurodegeneration under inflammatory conditions: potential therapeutic role in brain trauma," *The FASEB Journal*, vol. 17, no. 13, pp. 1922–1924, 2003.

[134] C. Brifault, M. Gras, D. Liot, V. May, D. Vaudry, and O. Wurtz, "Delayed pituitary adenylate cyclase-activating polypeptide delivery after brain stroke improves functional recovery by inducing M2 microglia/macrophage polarization," *Stroke*, vol. 46, no. 2, pp. 520–528, 2015.

[135] X. Qin, Z.-Q. Sun, X.-J. Dai et al., "Toll-like receptor 4 signaling is involved in PACAP-induced neuroprotection in BV2 microglial cells under OGD/reoxygenation," *Neurological Research*, vol. 34, no. 4, pp. 379–389, 2012.

[136] Y. Wada, T. Nakamachi, K. Endo et al., "PACAP attenuates NMDA-induced retinal damage in association with modulation of the microglia/macrophage status into an acquired deactivation subtype," *Journal of Molecular Neuroscience*, vol. 51, no. 2, pp. 493–502, 2013.

[137] M. Delgado, N. Varela, and E. Gonzalez-Rey, "Vasoactive intestinal peptide protects against β-amyloid-induced neurodegeneration by inhibiting microglia activation at multiple levels," *Glia*, vol. 56, no. 10, pp. 1091–1103, 2008.

[138] M. Song, J.-X. Xiong, Y.-Y. Wang, J. Tang, B. Zhang, and Y. Bai, "VIP enhances phagocytosis of fibrillar beta-amyloid by microglia and attenuates amyloid deposition in the brain of APP/PS1 mice," *PLoS ONE*, vol. 7, no. 2, Article ID e29790, 2012.

[139] S. Yang, J. Yang, Z. Yang et al., "Pituitary adenylate cyclase-activating polypeptide (PACAP) 38 and PACAP4-6 are neuroprotective through inhibition of NADPH oxidase: Potent regulators of microglia-mediated oxidative stress," *Journal of Pharmacology and Experimental Therapeutics*, vol. 319, no. 2, pp. 595–603, 2006.

[140] P. Jenner, "Oxidative stress in Parkinson's disease," *Annals of Neurology*, vol. 53, no. S3, pp. S26–S38, 2003.

[141] M. Delgado and D. Ganea, "Neuroprotective effect of vasoactive intestinal peptide (VIP) in a mouse model of Parkinson's disease by blocking microglial activation," *The FASEB Journal*, vol. 17, no. 8, pp. 944–946, 2003.

[142] H. Kato, A. Ito, J. Kawanokuchi et al., "Pituitary adenylate cyclase-activating polypeptide (PACAP) ameliorates experimental autoimmune encephalomyelitis by suppressing the functions of antigen presenting cells," *Multiple Sclerosis*, vol. 10, no. 6, pp. 651–659, 2004.

[143] T. Dickinson and S. M. Fleetwood-Walker, "VIP and PACAP: very important in pain?" *Trends in Pharmacological Sciences*, vol. 20, no. 8, pp. 324–329, 1999.

[144] E. M. Garry, A. Delaney, G. Blackburn-Munro et al., "Activation of p38 and p42/44 MAP kinase in neuropathic pain: involvement of VPAC2 and NK2 receptors and mediation by spinal glia," *Molecular and Cellular Neuroscience*, vol. 30, no. 4, pp. 523–537, 2005.

[145] M. Grimaldi and S. Cavallaro, "Functional and molecular diversity of PACAP/VIP receptors in cortical neurons and type I astrocytes," *European Journal of Neuroscience*, vol. 11, no. 8, pp. 2767–2772, 1999.

[146] M. Delgado, E. J. Munoz-Elias, R. P. Gomariz, and D. Ganea, "VIP and PACAP inhibit IL-12 production in LPS-stimulated macrophages. Subsequent effect on IFNγ synthesis by T cells," *Journal of Neuroimmunology*, vol. 96, no. 2, pp. 167–181, 1999.

[147] M. Delgado and D. Ganea, "Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit nuclear factor-κB-dependent gene activation at multiple levels in the human monocytic cell line THP-1," *The Journal of Biological Chemistry*, vol. 276, no. 1, pp. 369–380, 2001.

[148] M. Delgado, E. J. Munoz-Elias, Y. Kan et al., "Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit tumor necrosis factor α transcriptional activation by regulating nuclear factor-kB and cAMP response element-binding protein/c-Jun," *Journal of Biological Chemistry*, vol. 273, no. 47, pp. 31427–31436, 1998.

[149] Y. C. Patel, "Somatostatin and its receptor family," *Frontiers in Neuroendocrinology*, vol. 20, no. 3, pp. 157–198, 1999.

[150] J. Grinshpun, L. Tveria, and S. Fleisher-Berkovich, "Differential regulation of prostaglandin synthesis in neonatal rat microglia and astrocytes by somatostatin," *European Journal of Pharmacology*, vol. 584, no. 2-3, pp. 312–317, 2008.

[151] C. Dello Russo, L. Lisi, P. Navarra, and G. Tringali, "Diverging effects of cortistatin and somatostatin on the production and release of prostanoids from rat cortical microglia and astrocytes," *Journal of Neuroimmunology*, vol. 213, no. 1-2, pp. 78–83, 2009.

[152] G. Tundo, C. Ciaccio, D. Sbardella et al., "Somatostatin modulates insulin-degrading-enzyme metabolism: implications for the regulation of microglia activity in AD," *PLoS ONE*, vol. 7, no. 4, Article ID e34376, 2012.

[153] L. Bai, X. Zhang, X. Li et al., "Somatostatin prevents lipopolysaccharide-induced neurodegeneration in the rat substantia nigra by inhibiting the activation of microglia," *Molecular Medicine Reports*, vol. 12, no. 1, pp. 1002–1008, 2015.

[154] S. V. More, H. Kumar, I. S. Kim, S.-Y. Song, and D.-K. Choi, "Cellular and molecular mediators of neuroinflammation in the pathogenesis of Parkinson's disease," *Mediators of Inflammation*, vol. 2013, Article ID 952375, 12 pages, 2013.

[155] E. Pintér, Z. Helyes, and J. Szolcsányi, "Inhibitory effect of somatostatin on inflammation and nociception," *Pharmacology and Therapeutics*, vol. 112, no. 2, pp. 440–456, 2006.

[156] L. De Lecea, J. R. Criado, Ó. Prospero-Garcia et al., "A cortical neuropeptide with neuronal depressant and sleep-modulating properties," *Nature*, vol. 381, no. 6579, pp. 242–245, 1996.

[157] L. de Lecea, "Cortistatin—functions in the central nervous system," *Molecular and Cellular Endocrinology*, vol. 286, no. 1-2, pp. 88–95, 2008.

[158] R. Deghenghi, M. Papotti, E. Ghigo, and G. Muccioli, "Cortistatin, but not somatostatin, binds to growth hormone secretagogue (GHS) receptors of human pituitary gland," *Journal of Endocrinological Investigation*, vol. 24, no. 1, pp. RC1–RC3, 2001.

[159] N. Robas, E. Mead, and M. Fidock, "MrgX2 is a high potency cortistatin receptor expressed in dorsal root ganglion," *Journal of Biological Chemistry*, vol. 278, no. 45, pp. 44400–44404, 2003.

[160] M. Kamohara, A. Matsuo, J. Takasaki et al., "Identification of MrgX2 as a human G-protein-coupled receptor for proadrenomedullin N-terminal peptides," *Biochemical and Biophysical Research Communications*, vol. 330, no. 4, pp. 1146–1152, 2005.

[161] J. Córdoba-Chacón, M. D. Gahete, M. Duran-Prado et al., "Identification and characterization of new functional truncated variants of somatostatin receptor subtype 5 in rodents," *Cellular and Molecular Life Sciences*, vol. 67, no. 7, pp. 1147–1163, 2010.

[162] J. Córdoba-Chacón, M. D. Gahete, M. Durán-Prado, R. M. Luque, and J. P. Castaño, "Truncated somatostatin receptors as new players in somatostatin-cortistatin pathophysiology," *Annals of the New York Academy of Sciences*, vol. 1220, no. 1, pp. 6–15, 2011.

[163] V. A. S. H. Dalm, P. M. Van Hagen, P. M. Van Koetsveld et al., "Expression of somatostatin, cortistatin, and somatostatin receptors in human monocytes, macrophages, and dendritic cells," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 285, no. 2, pp. E344–E353, 2003.

[164] V. A. Dalm, P. M. Van Hagen, P. M. Van Koetsveld et al., "Cortistatin rather than somatostatin as a potential endogenous ligand for somatostatin receptors in the human immune system," *The Journal of Clinical Endocrinology & Metabolism*, vol. 88, no. 1, pp. 270–276, 2003.

[165] E. Gonzalez-Rey, N. Varela, A. F. Sheibanie, A. Chorny, D. Ganea, and M. Delgado, "Cortistatin, an antiinflammatory peptide with therapeutic action in inflammatory bowel disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 11, pp. 4228–4233, 2006.

[166] E. Gonzalez-Rey, A. Chorny, G. Robledo, and M. Delgado, "Cortistatin, a new antiinflammatory peptide with therapeutic effect on lethal endotoxemia," *The Journal of Experimental Medicine*, vol. 203, no. 3, pp. 563–571, 2006.

[167] E. Gonzalez-Rey, A. Chorny, R. G. Del Moral, N. Varela, and M. Delgado, "Therapeutic effect of cortistatin on experimental arthritis by downregulating inflammatory and Th1 responses," *Annals of the Rheumatic Diseases*, vol. 66, no. 5, pp. 582–588, 2007.

[168] C.-T. Chiu, L.-L. Wen, H.-P. Pao, and J.-Y. Wang, "Cortistatin is induced in brain tissue and exerts neuroprotection in a rat model of bacterial meningoencephalitis," *Journal of Infectious Diseases*, vol. 204, no. 10, pp. 1563–1572, 2011.

[169] L. Souza-Moreira, M. Morell, V. Delgado-Maroto et al., "Paradoxical effect of cortistatin treatment and its deficiency on experimental autoimmune encephalomyelitis," *The Journal of Immunology*, vol. 191, no. 5, pp. 2144–2154, 2013.

[170] M. Morell, M. Camprubí-Robles, M. D. Culler, L. de Lecea, and M. Delgado, "Cortistatin attenuates inflammatory pain via spinal and peripheral actions," *Neurobiology of Disease*, vol. 63, pp. 141–154, 2014.

[171] M. Morell, L. Souza-Moreira, M. Caro et al., "Analgesic effect of the neuropeptide cortistatin in murine models of arthritic inflammatory pain," *Arthritis & Rheumatism*, vol. 65, no. 5, pp. 1390–1401, 2013.

[172] A. Capuano, D. Currò, P. Navarra, and G. Tringali, "Cortistatin modulates calcitonin gene-related peptide release from neuronal tissues of rat. Comparison with somatostatin," *Peptides*, vol. 32, no. 1, pp. 138–143, 2011.

[173] M. S. Steinhoff, B. von Mentzer, P. Geppetti, C. Pothoulakis, and N. W. Bunnett, "Tachykinins and their receptors: contributions

to physiological control and the mechanisms of disease," *Physiological Reviews*, vol. 94, no. 1, pp. 265–301, 2014.

[174] Y. Zhang, L. Lu, C. Furlonger, G. E. Wu, and C. J. Paige, "Hemokinin is a hematopoietic-specific tachykinin that regulates B lymphopoiesis," *Nature Immunology*, vol. 1, no. 5, pp. 392–397, 2000.

[175] M. M. Kurtz, R. Wang, M. K. Clements et al., "Identification, localization and receptor characterization of novel mammalian substance P-like peptides," *Gene*, vol. 296, no. 1-2, pp. 205–212, 2002.

[176] M. A. Laurenzi, C. Arcuri, R. Rossi, P. Marconi, and V. Bocchini, "Effects of microenvironment on morphology and function of the microglial cell line BV-2," *Neurochemical Research*, vol. 26, no. 11, pp. 1209–1216, 2001.

[177] D. Giulian, M. Corpuz, B. Richmond, E. Wendt, and E. R. Hall, "Activated microglia are the principal glial source of thromboxane in the central nervous system," *Neurochemistry International*, vol. 29, no. 1, pp. 65–76, 1996.

[178] K. Maeda, M. Nakai, S. Maeda, T. Kawamata, T. Yamaguchi, and C. Tanaka, "Possible different mechanism between amyloid-beta (25–35)- and substance P-induced chemotaxis of murine microglia," *Gerontology*, vol. 43, supplement 1, pp. 11–15, 1997.

[179] F. C. Martin, P. A. Anton, J. A. Gornbein, F. Shanahan, and J. E. Merrill, "Production of interleukin-1 by microglia in response to substance P: role for a non-classical NK-1 receptor," *Journal of Neuroimmunology*, vol. 42, no. 1, pp. 53–60, 1993.

[180] L. P. McCluskey and L. A. Lampson, "Local immune regulation in the central nervous system by substance P vs. glutamate," *Journal of Neuroimmunology*, vol. 116, no. 2, pp. 136–146, 2001.

[181] A. Rasley, I. Marriott, C. R. Halberstadt, K. L. Bost, and J. Anguita, "Substance P augments Borrelia burgdorferi-induced prostaglandin E_2 production by murine microglia," *The Journal of Immunology*, vol. 172, no. 9, pp. 5707–5713, 2004.

[182] V. S. Chauhan, D. G. Sterka, D. L. Gray, K. L. Bost, and I. Marriott, "Neurogenic exacerbation of microglial and astrocyte responses to *Neisseria meningitidis* and *Borrelia burgdorferi*," *The Journal of Immunology*, vol. 180, no. 12, pp. 8241–8249, 2008.

[183] E. Thornton and R. Vink, "Substance p and its tachykinin NK1 receptor: a novel neuroprotective target for parkinson's disease," *Neural Regeneration Research*, vol. 10, no. 9, pp. 1403–1405, 2015.

[184] M. J. Brownstein, E. A. Mroz, J. Stephen Kizer, M. Palkovits, and S. E. Leeman, "Regional distribution of substance P in the brain of the rat," *Brain Research*, vol. 116, no. 2, pp. 299–305, 1976.

[185] M. L. Block, G. Li, L. Qin et al., "Potent regulation of microglia-derived oxidative stress and dopaminergic neuron survival: substance P vs. dynorphin," *The FASEB Journal*, vol. 20, no. 2, pp. 251–258, 2006.

[186] Q. Wang, C.-H. Chu, L. Qian et al., "Substance P exacerbates dopaminergic neurodegeneration through neurokinin-1 receptor-independent activation of microglial NADPH oxidase," *Journal of Neuroscience*, vol. 34, no. 37, pp. 12490–12503, 2014.

[187] S. Tumati, T. M. Largent-Milnes, A. I. Keresztes et al., "Tachykinin NK_1 receptor antagonist co-administration attenuates opioid withdrawal-mediated spinal microglia and astrocyte activation," *European Journal of Pharmacology*, vol. 684, no. 1–3, pp. 64–70, 2012.

[188] W.-W. Li, T.-Z. Guo, X. Shi et al., "Substance P spinal signaling induces glial activation and nociceptive sensitization after fracture," *Neuroscience*, vol. 310, pp. 73–90, 2015.

[189] C. I. Svensson, M. Marsala, A. Westerlund et al., "Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing," *Journal of Neurochemistry*, vol. 86, no. 6, pp. 1534–1544, 2003.

[190] L. Leung and C. M. Cahill, "TNF-α and neuropathic pain—a review," *Journal of Neuroinflammation*, vol. 7, article no. 27, 2010.

[191] N. Kiguchi, T. Maeda, Y. Kobayashi, and S. Kishioka, "Up-regulation of tumor necrosis factor-alpha in spinal cord contributes to vincristine-induced mechanical allodynia in mice," *Neuroscience Letters*, vol. 445, no. 2, pp. 140–143, 2008.

[192] Z. Zhou, X. Peng, J. Hagshenas, R. Insolera, D. J. Fink, and M. Mata, "A novel cell-cell signaling by microglial transmembrane TNFα with implications for neuropathic pain," *Pain*, vol. 151, no. 2, pp. 296–306, 2010.

[193] M. H. Jiang, E. Chung, G. F. Chi et al., "Substance P induces M2-type macrophages after spinal cord injury," *NeuroReport*, vol. 23, no. 13, pp. 786–792, 2012.

[194] E. C. Leal, E. Carvalho, A. Tellechea et al., "Substance P promotes wound healing in diabetes by modulating inflammation and macrophage phenotype," *American Journal of Pathology*, vol. 185, no. 6, pp. 1638–1648, 2015.

[195] D. A. Nelson, I. Marriott, and K. L. Bost, "Expression of hemokinin 1 mRNA by murine dendritic cells," *Journal of Neuroimmunology*, vol. 155, no. 1-2, pp. 94–102, 2004.

[196] A. Sakai, K. Takasu, M. Sawada, and H. Suzuki, "Hemokinin-1 gene expression is upregulated in microglia activated by lipopolysaccharide through NF-κB and p38 MAPK signaling pathways," *PLoS ONE*, vol. 7, no. 2, Article ID e32268, 2012.

[197] T. Matsumura, A. Sakai, M. Nagano et al., "Increase in hemokinin-1 mRNA in the spinal cord during the early phase of a neuropathic pain state," *British Journal of Pharmacology*, vol. 155, no. 5, pp. 767–774, 2008.

[198] S. G. Amara, V. Jonas, M. G. Rosenfeld, E. S. Ong, and R. M. Evans, "Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products," *Nature*, vol. 298, no. 5871, pp. 240–244, 1982.

[199] J. Roh, C. L. Chang, A. Bhalla, C. Klein, and S. Y. T. Hsu, "Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes," *Journal of Biological Chemistry*, vol. 279, no. 8, pp. 7264–7274, 2004.

[200] K. Kitamura, K. Kangawa, M. Kawamoto et al., "Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma," *Biochemical and Biophysical Research Communications*, vol. 192, no. 2, pp. 553–560, 1993.

[201] P. Westermark, C. Wernstedt, E. Wilander, and K. Sletten, "A novel peptide in the calcitonin gene related peptide family as an amyloid fibril protein in the endocrine pancreas," *Biochemical and Biophysical Research Communications*, vol. 140, no. 3, pp. 827–831, 1986.

[202] G. J. S. Cooper, A. C. Willis, A. Clark, R. C. Turner, R. B. Sim, and K. B. M. Reid, "Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 23, pp. 8628–8632, 1987.

[203] S. D. Brain and A. D. Grant, "Vascular actions of calcitonin gene-related peptide and adrenomedullin," *Physiological Reviews*, vol. 84, no. 3, pp. 903–934, 2004.

[204] L. M. McLatchie, N. J. Fraser, M. J. Main et al., "RAMPS regulate the transport and ligand specificity of the calcitonin-receptor-like receptor," *Nature*, vol. 393, no. 6683, pp. 333–339, 1998.

[205] L. Y. F. Wong, B. M. Y. Cheung, Y.-Y. Li, and F. Tang, "Adrenomedullin is both proinflammatory and antiinflammatory: its effects on gene expression and secretion of cytokines and macrophage migration inhibitory factor in NR8383 macrophage cell line," *Endocrinology*, vol. 146, no. 3, pp. 1321–1327, 2005.

[206] J. Springer, P. Geppetti, A. Fischer, and D. A. Groneberg, "Calcitonin gene-related peptide as inflammatory mediator," *Pulmonary Pharmacology & Therapeutics*, vol. 16, no. 3, pp. 121–130, 2003.

[207] J. Bełtowski and A. Jamroz, "Adrenomedullin—what do we know 10 years since its discovery?" *Polish Journal of Pharmacology*, vol. 56, no. 1, pp. 5–27, 2004.

[208] A. Ladoux and C. Frelin, "Coordinated up-regulation by hypoxia of adrenomedullin and one of its putative receptors (RDC-1) in cells of the rat blood-brain barrier," *Journal of Biological Chemistry*, vol. 275, no. 51, pp. 39914–39919, 2000.

[209] E. Tixier, C. Leconte, O. Touzani, S. Roussel, E. Petit, and M. Bernaudin, "Adrenomedullin protects neurons against oxygen glucose deprivation stress in an autocrine and paracrine manner," *Journal of Neurochemistry*, vol. 106, no. 3, pp. 1388–1403, 2008.

[210] M. Bernaudin, Y. Tang, M. Reilly, E. Petit, and F. R. Sharp, "Brain genomic response following hypoxia and re-oxygenation in the neonatal rat: identification of genes that might contribute to hypoxia-induced ischemic tolerance," *Journal of Biological Chemistry*, vol. 277, no. 42, pp. 39728–39738, 2002.

[211] S. Oba, M. Hino, and T. Fujita, "Adrenomedullin protects against oxidative stress-induced podocyte injury as an endogenous antioxidant," *Nephrology Dialysis Transplantation*, vol. 23, no. 2, pp. 510–517, 2008.

[212] T. Yoshimoto, N. Fukai, R. Sato et al., "Antioxidant effect of adrenomedullin on angiotensin II-induced reactive oxygen species generation in vascular smooth muscle cells," *Endocrinology*, vol. 145, no. 7, pp. 3331–3337, 2004.

[213] T. Yoshimoto, N. Gochou, N. Fukai, T. Sugiyama, M. Shichiri, and Y. Hirata, "Adrenomedullin inhibits angiotensin II-induced oxidative stress and gene expression in rat endothelial cells," *Hypertension Research*, vol. 28, no. 2, pp. 165–172, 2005.

[214] E. N. Chini, C. C. S. Chini, C. Bolliger et al., "Cytoprotective effects of adrenomedullin in glomerular cell injury: central role of cAMP signaling pathway," *Kidney International*, vol. 52, no. 4, pp. 917–925, 1997.

[215] N. Miyamoto, R. Tanaka, T. Shimosawa et al., "Protein kinase A-dependent suppression of reactive oxygen species in transient focal ischemia in adrenomedullin-deficient mice," *Journal of Cerebral Blood Flow & Metabolism*, vol. 29, no. 11, pp. 1769–1779, 2009.

[216] B. Engelhardt and R. M. Ransohoff, "The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms," *Trends in Immunology*, vol. 26, no. 9, pp. 485–495, 2005.

[217] M. Pedreño, M. Morell, G. Robledo et al., "Adrenomedullin protects from experimental autoimmune encephalomyelitis at multiple levels," *Brain, Behavior, and Immunity*, vol. 37, pp. 152–163, 2014.

[218] C. Sardi, L. Zambusi, A. Finardi et al., "Involvement of calcitonin gene-related peptide and receptor component protein in experimental autoimmune encephalomyelitis," *Journal of Neuroimmunology*, vol. 271, no. 1-2, pp. 18–29, 2014.

[219] R.-Q. Sun, Y.-J. Tu, N. B. Lawand, J.-Y. Yan, Q. Lin, and W. D. Willis, "Calcitonin gene-related peptide receptor activation produces PKA- and PKC-dependent mechanical hyperalgesia and central sensitization," *Journal of Neurophysiology*, vol. 92, no. 5, pp. 2859–2866, 2004.

[220] R. J. Cady, J. R. Glenn, K. M. Smith, and P. L. Durham, "Calcitonin gene-related peptide promotes cellular changes in trigeminal neurons and glia implicated in peripheral and central sensitization," *Molecular Pain*, vol. 7, article 94, 2011.

[221] F. R. Nieto, A. K. Clark, J. Grist, V. Chapman, and M. Malcangio, "Calcitonin gene-related peptide-expressing sensory neurons and spinal microglial reactivity contribute to pain states in collagen-induced arthritis," *Arthritis & Rheumatology*, vol. 67, no. 6, pp. 1668–1677, 2015.

[222] Z. Wang, W. Ma, J.-G. Chabot, and R. Quirion, "Morphological evidence for the involvement of microglial p38 activation in CGRP-associated development of morphine antinociceptive tolerance," *Peptides*, vol. 31, no. 12, pp. 2179–2184, 2010.

[223] Z. Wang, W. Ma, J.-G. Chabot, and R. Quirion, "Cell-type specific activation of p38 and ERK mediates calcitonin gene-related peptide involvement in tolerance to morphine-induced analgesia," *FASEB Journal*, vol. 23, no. 8, pp. 2576–2586, 2009.

[224] Z. Wang, W. Ma, J.-G. Chabot, and R. Quirion, "Calcitonin gene-related peptide as a regulator of neuronal CaMKII-CREB, microglial p38-NFκB and astroglial ERK-Stat1/3 cascades mediating the development of tolerance to morphine-induced analgesia," *Pain*, vol. 151, no. 1, pp. 194–205, 2010.

[225] H. Münzberg and C. D. Morrison, "Structure, production and signaling of leptin," *Metabolism: Clinical and Experimental*, vol. 64, no. 1, pp. 13–23, 2015.

[226] R. C. Frederich, A. Hamann, S. Anderson, B. Lollmann, B. B. Lowell, and J. S. Flier, "Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action," *Nature Medicine*, vol. 1, no. 12, pp. 1311–1314, 1995.

[227] M. W. Schwartz, E. Peskind, M. Raskind, E. J. Boyko, and D. Porte Jr., "Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans," *Nature Medicine*, vol. 2, no. 5, pp. 589–593, 1996.

[228] W. A. Banks, A. J. Kastin, W. Huang, J. B. Jaspan, and L. M. Maness, "Leptin enters the brain by a saturable system independent of insulin," *Peptides*, vol. 17, no. 2, pp. 305–311, 1996.

[229] B. Morash, A. Li, P. R. Murphy, M. Wilkinson, and E. Ur, "Leptin gene expression in the brain and pituitary gland," *Endocrinology*, vol. 140, no. 12, pp. 5995–5998, 1999.

[230] P. Hekerman, J. Zeidler, S. Bamberg-Lemper et al., "Pleiotropy of leptin receptor signalling is defined by distinct roles of the intracellular tyrosines," *The FEBS Journal*, vol. 272, no. 1, pp. 109–119, 2005.

[231] C. F. Elias, J. F. Kelly, C. E. Lee et al., "Chemical characterization of leptin-activated neurons in the rat brain," *Journal of Comparative Neurology*, vol. 423, no. 2, pp. 261–281, 2000.

[232] M. M. Scott, J. L. Lachey, S. M. Sternson et al., "Leptin targets in the mouse brain," *Journal of Comparative Neurology*, vol. 514, no. 5, pp. 518–532, 2009.

[233] M. W. Schwartz, R. J. Seeley, S. C. Woods et al., "Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus," *Diabetes*, vol. 46, no. 12, pp. 2119–2123, 1997.

[234] M. W. Schwartz, D. G. Baskin, T. R. Bukowski et al., "Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice," *Diabetes*, vol. 45, no. 4, pp. 531–535, 1996.

[235] T. M. Mizuno and C. V. Mobbs, "Hypothalamic agouti-related protein messenger ribonucleic acid is inhibited by leptin and stimulated by fasting," *Endocrinology*, vol. 140, no. 2, pp. 814–817, 1999.

[236] S. G. Bouret, "Neurodevelopmental actions of leptin," *Brain Research*, vol. 1350, pp. 2–9, 2010.

[237] A. La Cava and G. Matarese, "The weight of leptin in immunity," *Nature Reviews Immunology*, vol. 4, no. 5, pp. 371–379, 2004.

[238] P. Sarraf, R. C. Frederich, E. M. Turner et al., "Multiple cytokines and acute inflammation raise mouse leptin levels: potential role in inflammatory anorexia," *Journal of Experimental Medicine*, vol. 185, no. 1, pp. 171–175, 1997.

[239] C. Grunfeld, C. Zhao, J. Fuller et al., "Endotoxin and cytokines induce expression of leptin, the ob gene product, in hamsters: A role for leptin in the anorexia of infection," *Journal of Clinical Investigation*, vol. 97, no. 9, pp. 2152–2157, 1996.

[240] C. Sachot, S. Poole, and G. N. Luheshi, "Circulating leptin mediates lipopolysaccharide-induced anorexia and fever in rats," *Journal of Physiology*, vol. 561, no. 1, pp. 263–272, 2004.

[241] L. M. Harden, I. du Plessis, S. Poole, and H. P. Laburn, "Interleukin-6 and leptin mediate lipopolysaccharide-induced fever and sickness behavior," *Physiology and Behavior*, vol. 89, no. 2, pp. 146–155, 2006.

[242] T. Hosoi, Y. Okuma, and Y. Nomura, "Expression of leptin receptors and induction of IL-1β transcript in glial cells," *Biochemical and Biophysical Research Communications*, vol. 273, no. 1, pp. 312–315, 2000.

[243] Y. Gao, N. Ottaway, S. C. Schriever et al., "Hormones and diet, but not body weight, control hypothalamic microglial activity," *GLIA*, vol. 62, no. 1, pp. 17–25, 2014.

[244] V. Lafrance, W. Inoue, B. Kan, and G. N. Luheshi, "Leptin modulates cell morphology and cytokine release in microglia," *Brain, Behavior, and Immunity*, vol. 24, no. 3, pp. 358–365, 2010.

[245] A. J. Kiliaan, I. A. C. Arnoldussen, and D. R. Gustafson, "Adipokines: a link between obesity and dementia?" *The Lancet Neurology*, vol. 13, no. 9, pp. 913–923, 2014.

[246] D. J. Bonda, J. G. Stone, S. L. Torres et al., "Dysregulation of leptin signaling in Alzheimer disease: evidence for neuronal leptin resistance," *Journal of Neurochemistry*, vol. 128, no. 1, pp. 162–172, 2014.

[247] C. Davis, J. Mudd, and M. Hawkins, "Neuroprotective effects of leptin in the context of obesity and metabolic disorders," *Neurobiology of Disease*, vol. 72, pp. 61–71, 2014.

[248] G. Lim, S. Wang, Y. Zhang, Y. Tian, and J. Mao, "Spinal leptin contributes to the pathogenesis of neuropathic pain in rodents," *Journal of Clinical Investigation*, vol. 119, no. 2, pp. 295–304, 2009.

[249] T. Maeda, N. Kiguchi, Y. Kobayashi, T. Ikuta, M. Ozaki, and S. Kishioka, "Leptin derived from adipocytes in injured peripheral nerves facilitates development of neuropathic pain via macrophage stimulation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 31, pp. 13076–13081, 2009.

[250] C. M. Fernández-Martos, P. González, and F. J. Rodriguez, "Acute leptin treatment enhances functional recovery after spinal cord injury," *PLoS ONE*, vol. 7, no. 4, Article ID e35594, 2012.

[251] M. Kojima, H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, and K. Kangawa, "Ghrelin is a growth-hormone-releasing acylated peptide from stomach," *Nature*, vol. 402, no. 6762, pp. 656–660, 1999.

[252] K. Toshinai, M. S. Mondal, M. Nakazato et al., "Upregulation of ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration," *Biochemical and Biophysical Research Communications*, vol. 281, no. 5, pp. 1220–1225, 2001.

[253] M. Shintani, Y. Ogawa, K. Ebihara et al., "Rapid publication ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway," *Diabetes*, vol. 50, no. 2, pp. 227–232, 2001.

[254] B. Callaghan and J. B. Furness, "Novel and conventional receptors for ghrelin, desacyl-ghrelin, and pharmacologically related compounds," *Pharmacological Reviews*, vol. 66, no. 4, pp. 984–1001, 2014.

[255] H. Hosoda, M. Kojima, H. Matsuo, and K. Kangawa, "Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue," *Biochemical and Biophysical Research Communications*, vol. 279, no. 3, pp. 909–913, 2000.

[256] X.-M. Guan, H. Yu, O. C. Palyha et al., "Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues," *Molecular Brain Research*, vol. 48, no. 1, pp. 23–29, 1997.

[257] M. Nakazato, N. Murakami, Y. Date et al., "A role for ghrelin in the central regulation of feeding," *Nature*, vol. 409, no. 6817, pp. 194–198, 2001.

[258] T. Y. Yune, J. Y. Lee, G. Y. Jung et al., "Minocycline alleviates death of oligodendrocytes by inhibiting pro-nerve growth factor production in microglia after spinal cord injury," *The Journal of Neuroscience*, vol. 27, no. 29, pp. 7751–7761, 2007.

[259] J. Y. Lee, H. Chung, Y. S. Yoo et al., "Inhibition of apoptotic cell death by ghrelin improves functional recovery after spinal cord injury," *Endocrinology*, vol. 151, no. 8, pp. 3815–3826, 2010.

[260] J. Y. Lee and T. Y. Yune, "Ghrelin inhibits oligodendrocyte cell death by attenuating microglial activation," *Endocrinology and Metabolism*, vol. 29, no. 3, pp. 371–378, 2014.

[261] J. Y. Lee, H. Y. Choi, and T. Y. Yune, "MMP-3 secreted from endothelial cells of blood vessels after spinal cord injury activates microglia, leading to oligodendrocyte cell death," *Neurobiology of Disease*, vol. 82, pp. 141–151, 2015.

[262] J. Lee, E. Lim, Y. Kim, E. Li, and S. Park, "Ghrelin attenuates kainic acid-induced neuronal cell death in the mouse hippocampus," *Journal of Endocrinology*, vol. 205, no. 3, pp. 263–270, 2010.

[263] S. Lee, Y. Kim, E. Li, and S. Park, "Ghrelin protects spinal cord motoneurons against chronic glutamate excitotoxicity by inhibiting microglial activation," *The Korean Journal of Physiology & Pharmacology*, vol. 16, no. 1, pp. 43–48, 2012.

[264] M. Moon, H. G. Kim, L. Hwang et al., "Neuroprotective effect of ghrelin in the 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine mouse model of parkinson's disease by blocking microglial activation," *Neurotoxicity Research*, vol. 15, no. 4, pp. 332–347, 2009.

[265] M.-M. Theil, S. Miyake, M. Mizuno et al., "Suppression of experimental autoimmune encephalomyelitis by Ghrelin," *The Journal of Immunology*, vol. 183, no. 4, pp. 2859–2866, 2009.

[266] M. D. Gahete, J. Córdoba-Chacón, R. D. Kineman, R. M. Luque, and J. P. Castaño, "Role of ghrelin system in neuroprotection and cognitive functions: implications in Alzheimer's disease," *Peptides*, vol. 32, no. 11, pp. 2225–2228, 2011.

[267] A. Demers, N. McNicoll, M. Febbraio et al., "Identification of the growth hormone-releasing peptide binding site in CD36: a

photoaffinity cross-linking study," *Biochemical Journal*, vol. 382, no. 2, pp. 417–424, 2004.

[268] I. Bulgarelli, L. Tamiazzo, E. Bresciani et al., "Desacyl-ghrelin and synthetic GH-secretagogues modulate the production of inflammatory cytokines in mouse microglia cells stimulated by β-amyloid fibrils," *Journal of Neuroscience Research*, vol. 87, no. 12, pp. 2718–2727, 2009.

[269] C.-H. Zhou, X. Li, Y.-Z. Zhu et al., "Ghrelin alleviates neuropathic pain through GHSR-1a-mediated suppression of the p38 MAPK/NF-κB pathway in a rat chronic constriction injury model," *Regional Anesthesia and Pain Medicine*, vol. 39, no. 2, pp. 137–148, 2014.

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Review Article

M1 and M2 Functional Imprinting of Primary Microglia: Role of P2X7 Activation and miR-125b

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Amyotrophic lateral sclerosis (ALS) is a most frequently occurring and severe form of motor neuron disease, causing death within 3–5 years from diagnosis and with a worldwide incidence of about 2 per 100,000 person-years. Mutations in over twenty genes associated with familial forms of ALS have provided insights into the mechanisms leading to motor neuron death. Moreover, mutations in two RNA binding proteins, TAR DNA binding protein 43 and fused in sarcoma, have raised the intriguing possibility that perturbations of RNA metabolism, including that of the small endogenous RNA molecules that repress target genes at the posttranscriptional level, that is, microRNAs, may contribute to disease pathogenesis. At present, the mechanisms by which microglia actively participate to both toxic and neuroprotective actions in ALS constitute an important matter of research. Among the pathways involved in ALS-altered microglia responses, in previous works we have uncovered the hyperactivation of P2X7 receptor by extracellular ATP and the overexpression of miR-125b, both leading to uncontrolled toxic M1 reactions. In order to shed further light on the complexity of these processes, in this short review we will describe the M1/M2 functional imprinting of primary microglia and a role played by P2X7 and miR-125b in ALS microglia activation.

1. Introductory Statement

The hardest challenge in microglia research is to decode a metamorphosis between the two ramified/surveilling and amoeboid/activated morphologic-functional end points and to establish a biological link between two forms of power, the anti- and the proinflammatory one. To this aim, molecular information becomes the master key to direct what has to be done in microglia, in other words if and how to respond to an internal or external stimulus with a consequent either aggressive inflammatory or compliant anti-inflammatory behavior. Along the flow of information that step by step instructs microglia on the "right" thing to do, understanding how the activation of a receptor sensing the environment translates an extracellular stimulus into an intracellular signal surely seems important. Under this regard, the P2X7 receptor activated by extracellular ATP possesses a privileged position. Extracellular ATP is not only a primordial precursor in the evolution of the chemical transmission and an ubiquitous intercellular communication agent, but also the chief endogenous microglia alarm signal. In addition, P2X7 is highly expressed in microglia, is involved in inflammatory responses, and possesses a dual either trophic and anti-inflammatory or toxic and proinflammatory function. In the regulation of microglial P2X7 by extracellular ATP, microRNAs, among which particularly miR-125b, are lately emerging as key modulators of receptor signaling and functional microglia commitment under basal or amyotrophic lateral sclerosis- (ALS-) evoked inflammatory conditions. In this work, we will describe the M1/M2 functional imprinting of primary microglia as a paradigm of pro/anti-inflammatory function and the role played by P2X7 and miR-125b in ALS microglia activation.

2. Basic Features of ALS

The two-century-old rare disease ALS is the most frequently occurring and severe form of progressive upper and lower

motor neuron disease, causing death within 3–5 years from diagnosis [1, 2], with a worldwide incidence of ~2 per 100,000 person-years [3] and without any curative treatment to date. Mutations within the superoxide dismutase 1 (*SOD1*) gene coding for the ubiquitously expressed enzyme SOD1 [4–6] are responsible for about a quarter of the inherited disease cases. Additional mutations in over twenty genes, among which the most frequent C9ORF72, TAR DNA binding protein 43 (TARDPB), and fused in sarcoma (FUS), have been associated with familial forms of ALS and account for about 10–15% of all ALS cases, providing insights into the complex genetic mechanisms that lead to motor neuron death [6]. Notably, mice that express mutant human SOD1 protein recapitulate most of the key features of ALS, comprising motor neuron degeneration and decreased life span, and are the most exploited animal model for studying the disease pathogenesis [7].

The hallmarks of ALS include genomic instability, epigenetic alterations, cellular senescence, stem cell exhaustion with decreased tissue regenerative potential, deregulated nutrient-sensing, mitochondrial dysfunction and oxidative stress, loss of proteostasis including autophagy impairment, altered intercellular communication and excitotoxicity, and central neuroinflammation sustained by microglia/macrophages and astrocytes [8]. Most recent advances have moreover recognized that the complicity of all types of glial and inflammatory cells at both peripheral and central level indeed contributes to endangering and compromising the motor neuron function [9, 10]. This in part explains the multigenic, multisystemic, multifactorial, and non-cell-autonomous nature of ALS.

Shared consent to explain the pathogenic mechanisms of ALS includes the release of proinflammatory factors and toxic agents and the prompt activation of glial cells, overall culminating into injury to motor neurons. Because extracellular ATP activating purinergic P2 receptors and particularly the P2X7 subtype [11] constitutes a lead neuron-to-microglia alarm signal, a role for purinergic receptor signaling in ALS neuroinflammation has been highlighted [12, 13]. The P2X7, since its discovery in lymphocytes and mast cells, has been mainly implicated in the processing and release of cytokines such as interleukin-1β (IL-1β) and involved in neuroinflammation and immunity, as well as in the initiation of cell death mediated by apoptosis, necrosis, and autophagy mechanisms [14]. Recent work provided evidence that modulation of microglia via the P2X7 also constitutes a novel pathway involved in ALS progression [15–19]. Moreover, Brilliant Blue G, a poorly centrally penetrant and low affinity P2X7 antagonist, was shown to reduce microgliosis but not astrocytosis in lumbar spinal cord, to modulate inflammatory genes such as nuclear factor kappa B (NF-kB), NADPH oxidase 2 (NOX2), IL-1β, interleukin-10 (IL-10), and brain derived neurotrophic factor (BDNF), to enhance motor neuron survival, and to induce a slightly delayed ALS disease onset with modest improvement of general conditions and motor performance, when provided to SOD1-G93A mice from late preonset [20]. Overall, these findings would suggest that P2X7 might be a susceptibility or modifying target in ALS pathogenesis.

3. Plasticity of Microglia

Since their discovery almost a century ago by del Río Hortega, microglia constituting about 10–15% of cells located in the brain and spinal cord are the first and main form of defense in the CNS, being responsible for homeostasis maintenance [21]. To this aim and in contrast to peripheral macrophages, microglia have developed a spatially and temporally tightly regulated mechanism of compliance to the extracellular environment, becoming extremely sensitive even to the smallest physiological perturbation or generic danger signal present in their surrounding area [22]. While the basic steady state of microglia is tolerant, prohomeostatic, and surveilling the environment, on the other hand there is no indication that neuronal damage might occur without activation of microglia. Accordingly, the main response of microglia to neuronal stress is rapid retraction of the fine processes and gradual transition from ramified/surveilling into a more amoeboid macrophage-like phagocytic cell. Only for investigation needs, the complexity of this transition has been simplified and categorized into a stereotypic process with basically two morphological ending phenotypes: ramified/surveilling and amoeboid/activated, often correlated with a migratory behavior and/or proliferation of the cells [23]. Accordingly to a functional scale, microglia activity can be then categorized into a classical proinflammatory and neurotoxic phenotype, known as M1, and the alternative anti-inflammatory M2 phenotype involved in the resolution of inflammation, phagocytosis and tissue repair [24]. These are considered as the two end points of a broad scale of microglia responses, of a continuous spectrum of functional heterogeneity. M1-like microglia generally express IL-1β, interleukin-6 (IL-6), tumor necrosis factor α (TNFα), nitric oxide synthase 2 (NOS2), and CD16/32, whereas M2-like microglia express lower levels of these markers, but higher levels of IL-10, BDNF, Arginase-1 (Arg1), and Mannose Receptor C, type 1 (MRC-1) [25]. Interestingly, the phenotypic identity of microglia is also age-dependent, but what is clear is that these cells are not fully committed to either an M1 or an M2 phenotype neither in newborn nor in adult mice. For instance, in early postnatal CNS, microglia express M1 markers such as iNOS and TNFα but also express the M2 marker Arg1 [26]. This pattern changes in old mice and microglia appear to be committed to a stronger M1 phenotype with higher levels of proinflammatory IL-1β and IL-6 mRNA than those observed, for instance, at 12 months of age [26]. The participation of microglia to ALS pathogenesis has been thoroughly investigated in sophisticated experiments in which mutated SOD1 was expressed in specific cell phenotypes. The main conclusion that arose from those studies was that several neurotransmitters can control neuroinflammation and neurotoxicity by activated microglia and that mutated SOD1 microglia do not initiate motor neuron degeneration but rather accelerate disease progression [27–29]. In aging SOD1 overexpressing ALS mice, a prevalence of M1-like microglia is indeed observed that is moreover neurotoxic in vitro [30]. However, in vivo/in vitro evidence about microglia being either M1 detrimental or M2 protective that is merely based on immunostaining

of morphologic markers and correlation studies is limitative and not standing on a solid ground. Additional information is absolutely required to explain the activation of these cells and particularly their role in the ALS brain.

4. Microglia versus Macrophages

Different combinations of inflammatory factors are known to be able to polarize microglia into a variety of activation states whose characterization is however still under scrutiny [31]. For instance, in vitro studies have determined that an environment dominated by proinflammatory stimuli, such as lipopolysaccharide (LPS) and interferon-γ (IFNγ), favors polarization toward M1 effector cells, while anti-inflammatory cytokines such interleukin-4 (IL-4) enable the M2 protective phenotype [32]. However, there is still a lot to be learned about the molecular mechanisms governing microglia plasticity across these different activation states [33]. In an attempt to understand microglia responsiveness to temporal combinations of M1 and M2 inflammatory stimuli and furthermore to establish potential discriminatory differences between central microglia and peripheral macrophages, we exploited an in vitro protocol based on the responsiveness of peritoneal macrophages to an inflammatory polarization gradient [34, 35]. This protocol generates a gradient of five polarity phenotypes. In particular, proinflammatory stimuli are used to differentiate cells into an M1 phenotype (state 1), while anti-inflammatory stimuli are used to differentiate them into M2 (state 4). A combination of both M1 and M2 stimuli is then used to differentiate microglia into an intermediate M1/M2 polarization phenotype (state 3). Finally, to study the polarity switch between M1 and M2 states, microglia are stimulated firstly with anti-inflammatory stimuli, washed, and then stimulated with proinflammatory ones and vice versa.

Similarly to peritoneal macrophages, we report here that primary microglia can polarize into M1-like phenotype characterized by high TNFα and low MRC-1 expression, when exposed to the inflammatory stimuli LPS alone (M1/state 1, Figures 1(a)–1(c)). Likewise, microglia polarize toward an M2-like phenotype characterized by low TNFα and high MRC-1 levels, when exposed to the anti-inflammatory cytokine IL-4 alone (M2/state 5, Figures 1(a)–1(c)). Surprisingly and unlike peripheral macrophages that are able to reversibly and dynamically swing among different activation states in vitro [34], the addition of either LPS after IL-4 washout (M2 > M1/state 2, Figures 1(a)–1(c)) or IL-4 after LPS washout (M1/state 4, Figures 1(a)–1(c)) is not able to counteract the M2 (low TNFα and high MRC-1 expressions) or M1 (high TNFα and low MRC-1 expressions) commitment initially elicited by microglia in vitro. Finally, the simultaneous addition of proinflammatory LPS and anti-inflammatory IL-4 (M1 > M2/state 3, Figures 1(a)–1(c)) directs microglia toward a preponderant M1 phenotype, while this same condition is known to induce hybrid M1/M2 phenotype in peritoneal macrophages [34]. Therefore, different from peripheral macrophages where an initially induced M1 or M2 phenotype can be reversibly and, respectively, redirected toward an M2 or M1 activation state, respectively, by IL-4 or LPS [34], primary microglia undergo an irreversible either pro- or anti-inflammatory "imprinting" once they are firstly challenged with, respectively, M1 or M2 inflammatory stimuli. In other words, central microglia appear less prone than peripheral macrophages to phenotypic redirection. These findings are in line with previous results indicating that stimulation of microglia with IL-4 prior to LPS prevents the LPS-mediated inhibition of the microglial neuroprotective effects and the release of neurotoxic factors [36, 37]. Moreover, they raise questions about the dynamics and versatility of microglia engagement into a specific functional phenotype, and most importantly they might contribute to explaining why microglia-targeted anti-inflammatory therapy has failed in ALS so far.

5. MiRNA-125b in ALS

MicroRNAs (miRNAs), endogenous small RNA molecules that have emerged as key regulators of target gene expression at the posttranscriptional level [38], behave as fine-tuners in controlling diverse biological processes at the molecular, cellular, and tissue level, including brain functioning. In the CNS, as much as 30% difference in miRNA expression profile exists among neurons, oligodendrocytes, astrocytes and microglia, thus explaining why cell-specific modulations of miRNA levels can easily contribute to orchestrating the complex network of interactions that regulate brain functioning [39]. In particular, miRNAs are also good candidates for distinguishing for instance brain resident microglia from infiltrating monocytes/macrophages or the different activation states of microglia [40, 41] thus becoming potential biomarkers and molecular targets during neuroinflammation and neurodegeneration. Not surprisingly, miRNAs dysregulations are well established also in ALS [42, 43] and in the last few years several works have demonstrated changes in the expression of selected miRNAs that are associated with both familial and sporadic cases [44–53]. In addition, studies performed with the SOD1-G93A mouse model have shed light on the role of specific miRNAs in ALS [52–54] and opened miRNA targeting as a new conceivable opportunity for ALS treatment [54–56].

In previous work, we have compared miRNAs transcriptional profiling of nontransgenic and ALS microglia under resting conditions and after inflammatory activation induced by extracellular ATP through P2X7 receptor, by identifying the upregulation of immune-enriched miR-22, miR-155, miR-125b, and miR-146b in ALS microglia. Moreover, we proved that miR-125b increases TNFα transcription by interfering with the STAT3 pathway. As in turn TNFα upregulates miR-125b and inhibitors of miR-125b reduce TNFα expression levels, we recognized the induction of miR-125b as a vicious gateway culminating in abnormal TNFα release [18]. Moreover, we have established an interplay between miR-125b and A20 protein in the modulation of classical NF-κB signaling sustained in microglia. In ALS, classical NF-κB pathway is indeed known to be related to persistent microglia activation and motor neuron injury [57] but mechanisms of negative control of NF-κB activity still remain unexplored. One of the major players in the termination of classical NF-κB

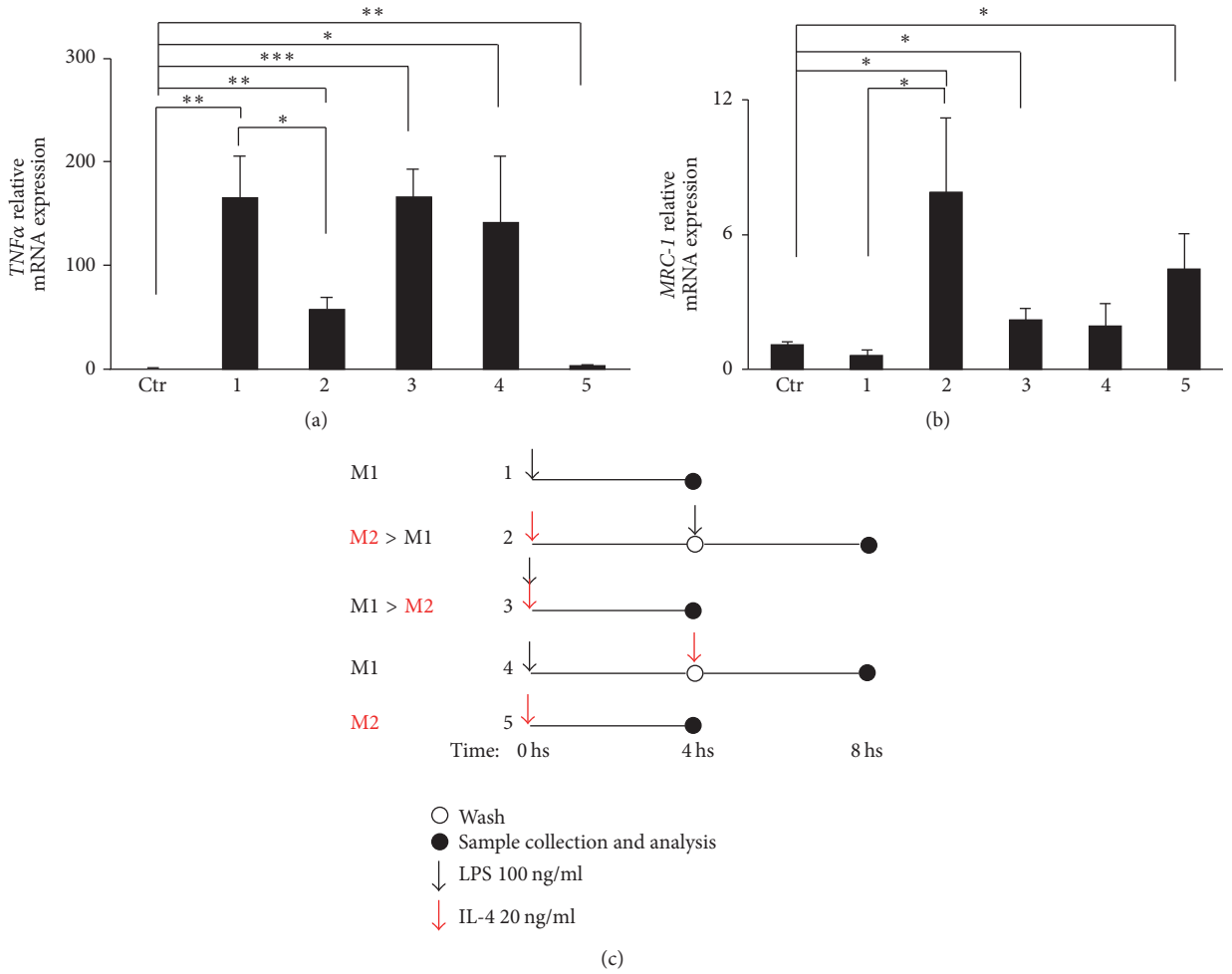

Figure 1: Microglia responsiveness to macrophages polarity gradient. Primary microglia obtained from brain cortex of P0/P1 C57BL/6 mice were prepared as previously described [15]. (a) *TNFα* or (b) *MRC-1* expression levels were measured by qRT-PCR using primer pairs from Qiagen-GeneGlobe program. Data are calculated using the $2^{-\Delta\Delta Ct}$ method and GAPDH transcript as normalizer (Qiagen-GeneGlobe program). Bars represent mean ± sem of ≥3 independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus untreated cells by unpaired *t*-test. (c) Schematic representation of the response of primary microglia to polarity gradient protocol used as revised from [34].

pathway is the ubiquitin-editing enzyme A20, which has recognized anti-inflammatory functions. In particular, our previous work has highlighted that miR-125b by terminating A20 activity strengthens and prolongs the noxious activation of NF-κB induced by stimulation of P2X7, with deleterious consequences on motor neuron survival. Consistently, miR-125b inhibition by restoring A20 levels can protect motor neuron [19]. Overall, these results strengthen the impact of miRNAs in modulating inflammatory genes linked to ALS and identify miR-125b as a key mediator of microglia dynamics and pathogenic mechanisms in the disease.

5.1. P2X7- and miR-125b-Dependent Regulation of Proinflammatory and Anti-Inflammatory Mediators in ALS Microglia. Several dangerous signals and endogenous triggers, among which extracellular ATP acting on P2X7, are known to contribute to the pathogenic proinflammatory response of ALS microglia. In particular, P2X7 was found increased in activated microglia of ALS patients [58] and its selective activation by the agonist 2′-3′-O-(benzoyl-benzoyl) ATP (BzATP) in primary microglia isolated from SOD1-G93A mouse brain induces morphologic transition, enhances the production of proinflammatory mediators such as TNFα, and influences cyclooxygenase-2 (COX2), NOX2, Racl, ERK1/2, p38, and NF-kB activation [15, 16, 19]. In order to widen our knowledge about the role of P2X7 in ALS neuroinflammation and the suppressive role exerted by miR-125b inhibition on proinflammatory marker production and consequent improvement of motor neuron survival [18, 19], we analyzed the expression of *IL1β* gene transcription after BzATP challenge. The expression of IL1β mRNA that is time-dependent and maximally induced by BzATP in two hours (data not shown) is significantly reduced in the presence of miR-125b inhibitor (Figure 2(a)). These data are in line with our previous results on *TNFα* and *NOX2* expression showing that the maximal repressive effect of miR-125b inhibitor is obtained at the peak of induction of these same proinflammatory M1 markers [19]. *IL1β*, together with TNFα,

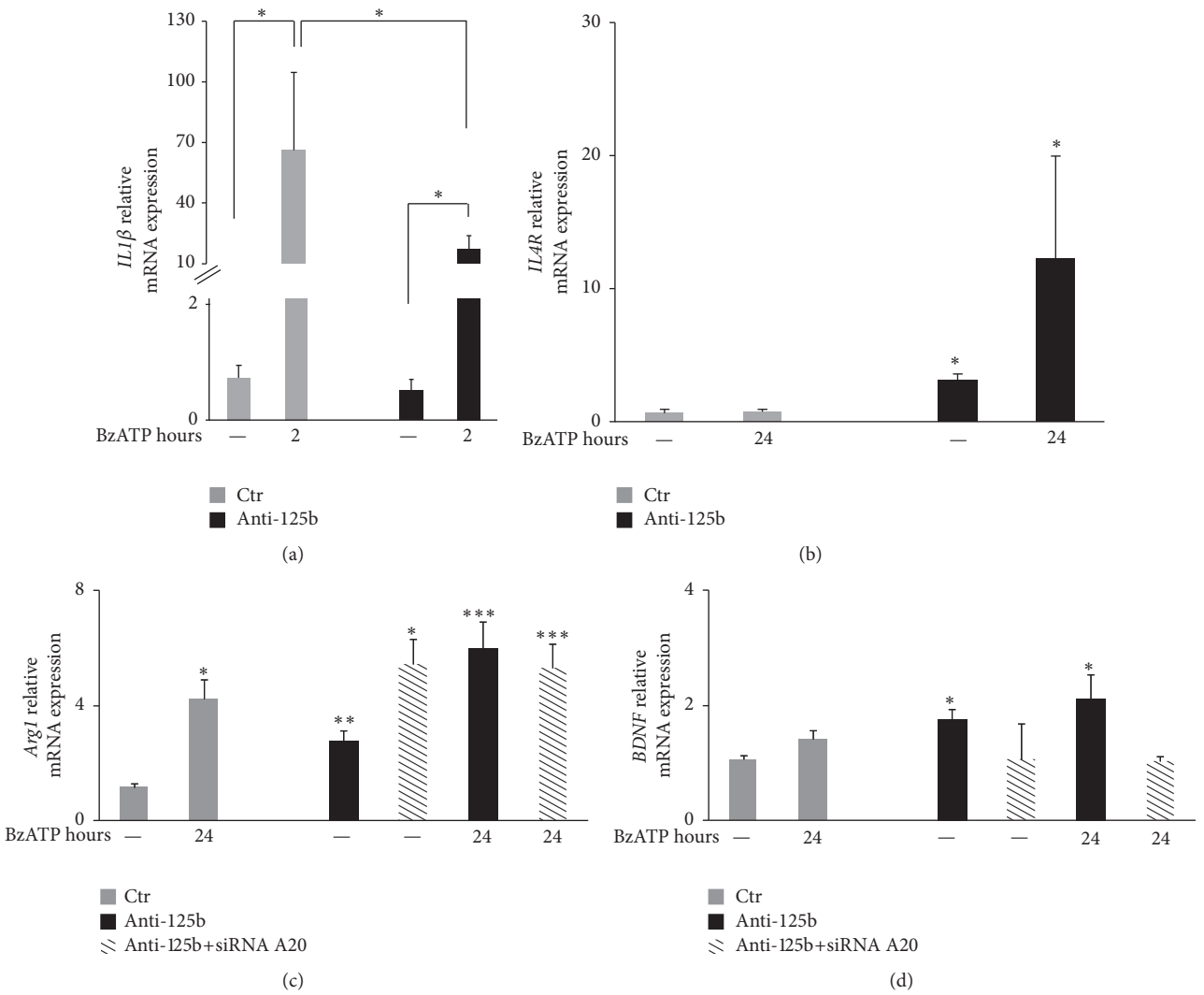

FIGURE 2: Regulation of proinflammatory and anti-inflammatory mediators by P2X7 activation and miR-125b inhibition in SOD1-G93A microglia. SOD1-G93A primary microglia obtained from brain cortex of P0/P1 B6.Cg-Tg(SOD1-G93A)1Gur/J mice were prepared as previously described [15]. Then, 5×10^5 cells/well were plated and transfected with 20 nM scramble miRIDIAN hairpin inhibitor (ctr) or miR-125b miRIDIAN hairpin inhibitor (anti-125b) for 48 h and exposed to BzATP 100 μM for the indicated times. Expression levels of *IL1β* (a) (primer pairs: F 5′-GCAACTGTTCCTGAACTCAACT-3′; R 5′-ATCTTTTGGGGTCCGTCAACT-3′), *IL4r* (b) (F 5′-CGAGTTCTCTGAAAACCTC-3′; R 5′-CCATCTGGTATCTGTCTG-3′), *Arg1* (c) (F 5′-CCACGGTCTGTGGGGAAAGCCAAT-3′; R 5′-CTGCCAGACTGTGGTCTCCACCCA-3′), and *BDNF* (d) (F 5′-CGGCGCCCATGAAAGAAGTA-3′; R 5′-AGACCTCTCGAACCTGCCCT-3′) were measured by qRT-PCR. Data are calculated using the $2^{-\Delta\Delta Ct}$ method and GAPDH transcript as normalizer (F 5′-CATGGCCTTCCGTGTTCCTA-3′; R 5′-CCTGCTTCACCACCTTCTTGAT-3′). Bars represent mean ± sem of ≥3 independent experiments $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus untreated cells, by unpaired t-test.

NOX2, and several other genes are known NF-kB responsive genes [59]. Therefore, as for TNFα, we do not exclude that regulation of *IL1β* expression by miR-125b might depend on the regulation of the NF-kB canonical pathway by miR-125b that was previously established, for instance, in microglia [19] and in lymphoma B-cells [60].

Due to the phenotypic complexity of microglia, the M1/M2 paradigm is simply assumed as a conventional generalized model to define functional phenotypes, however with the limitation that several markers classified as specific for characterizing M1 or M2 states can instead be found modulated by both M1 and M2 stimuli. For instance, despite the fact that the expressions of the cytokine IL-10 and interleukin-4 receptor (IL-4R) are considered a hallmark of microglia anti-inflammatory M2 phenotype, IL-10 and IL-4R can also be induced by proinflammatory stimuli such as LPS, perhaps as a feedback mechanism to restrain an exuberant immune response [36, 61]. In order to gain further insight into the microglia response to P2X7 activation and to deeper characterize the role of miR-125b on microglia activation, we evaluated the expression of M2 markers in SOD1-G93A microglia. *IL-4R* gene expression is known to be fundamental for microglia responsiveness to IL-4 and conversion toward a motor neuron protective/M2 phenotype

[62]. We thus measured IL-4R mRNA levels in the presence of BzATP and with or without miR-125b inhibition. Our results (Figure 2(b)) demonstrates that while the content of IL-4R mRNA is almost undetectable in SOD1-G93A microglia and this occurs independently from BzATP treatment, the presence of miR-125b inhibitor is able to up regulate both constitutive and BzATP-challenged IL-4R levels. Under these same conditions, we surprisingly show that BzATP enhances the expression of Arg1 mRNA levels after 24 h, while miR-125b inhibition again significantly upregulates Arg1 mRNA in the presence or absence of BzATP (Figure 2(c)). This is confirmed also in wild type cells (data not shown). Consistently with previous findings on the induction of IL-4R by LPS [61], we thus demonstrate that also a proinflammatory stimulus such as BzATP is able to induce the content of a typical M2 marker in ALS microglia. However, LPS and BzATP can apparently discriminate among different M2 mediators. Moreover, while BzATP fails in stimulating BDNF mRNA levels in SOD1-G93A microglia, miR-125b inhibition is able to significantly up regulate also BDNF, in the presence or absence of BzATP (Figure 2(d)). These results might further explain the beneficial effects exerted by miR-125b inhibition in SOD1-G93A microglia and motor neuron survival, thus suggesting that the neuroprotective effect exerted by miR-125b inhibition might depend not only on the suppression of toxic mediators among which IL-1β (Figure 2(a)), TNFα, and NOX2 [19], but also on the direct stimulation of M2 parameters such as IL4R, Arg1, and BDNF in ALS microglia.

6. Induction of BDNF by miR-125b Inhibition Is A20-Dependent

In the complexity of microglia responses, several transcription factors among which NF-kB are involved in both M1 and M2 regulation [63]. The NF-κB family of transcription factors consists of five proteins, p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), and p100/52 (NF-κB2) whose combination to form distinct transcriptionally active homo- and heterodimeric complexes creates an elaborate system to control the inflammatory response [64, 65]. While the p65/p50 dimer is recognized as a master activator of M1 genes, the accumulation of NFκB p50 and the formation of transcriptionally regulatory p50 homodimers appear to play an important role in the resolution of inflammation through the enhancement of M2 factors [66–71]. Moreover, while the role of p52/RelB dimer resulting from the activation of the so-called noncanonical way remains elusive, studies with RelB-deficient mice and the ability of p52 to bind the IL-10 promoter have also revealed an anti-inflammatory role for this pathway [57, 72, 73]. A20 protein is a master negative feedback regulator of canonical NF-kB signaling [74, 75] and it was recently highlighted as a broader regulator of NF-kB family, due to its positive action also on the noncanonical pathway [76]. As the BDNF promoter has functioning sites recognized for NF-kB family members [77] and miR-125b was reported to regulate A20 protein in SOD1-G93A microglia [19], we then asked if the mechanism by which miR-125b inhibition enhances M2 markers and particularly BDNF and Arg1 could be dependent on A20 acting on NF-kB components. As Figure 2(d) shows, in the absence of A20 we find that miR-125b inhibition fails to stimulate BDNF (but not Arg1) production, thus suggesting the existence of a potential miR-125b-A20-NF-kB-BDNF axis. By reinforcing this hypothesis, the silencing of A20 does not revert the overexpression of Arg1 by miR-125b inhibition (Figure 2(c)), perhaps because Arg1 transcription is known not to be regulated by NF-kB. Moreover, the lack of the A20 silencing effect on Arg1 induction would also suggest that overexpression of Arg1 is not related to the protective effect of microglial A20 toward motor neurons. Under this regards, a potential neuroprotective role of P2X7 would be perhaps not mediated by Arg1 expression.

7. Conclusions and Perspectives

Despite the fact that microglia are classified as resident macrophages of the CNS with whom they in part share shape and functions, several evidences prompt to discriminate them from peripheral macrophages, for instance, in terms of strength and plasticity in immune regulatory responses. Our work has shown here that brain microglia in primary culture undergo a preferential and irreversible M1 or M2 imprinting, once challenged, respectively, with M1 or M2 inflammatory stimuli, and this feature contributes to distinguishing microglia from macrophages. Moreover we have demonstrated that activation of P2X7 receptor in SOD1-G93A microglia surprisingly non only stimulates common M1 markers but also increases the M2 parameter Arg1 [15, 16, 20]. This would confirm the hypothesis that indeed P2X7 receptor might be involved not only in ALS toxic actions, but also in microglia-dependent neuroprotection [17, 78, 79], thus sustaining the contribution of P2X7 during the resolution of inflammation in macrophages and not only its active part in the release of proinflammatory cytokines [78]. Therefore, we do believe that a better understanding of the molecules and pathways, as well as the timing, responsible for this functional imprinting of microglia might help to select more effective microglia-targeted therapies during diseases. For instance, there is still no effective and enduring treatment against ALS and efficient therapeutic options to prevent pathological ALS sequelae are urgently needed. On one hand, it is now clear that dysregulations of microglia as responses to neuronal damage strongly contribute to ALS pathology and that microglia are involved in the progression of the disease by exacerbating central inflammatory mechanisms among which activation of P2X7. On the other hand, in the last few years several miRNAs emerging as important tools in the fine-tuning regulation of cell behavior have been associated with ALS. Aberrant expression of miRNAs relevant for neuronal function has repeatedly been reported in the spinal fluid, frontal cortex, and serum of familial and sporadic ALS patients, and miRNA activity has been demonstrated to be essential for long-term survival of motor neurons as well as microglia responsiveness. Actually, miRNAs are now recognized as potential therapeutic targets and biomarkers for neurodegeneration and neuroinflammation in ALS, to the point that identifying miRNAs at work in this diseases is more important than ever. In particular, we have also demonstrated

here that miR-125b inhibitors stimulate the expression of M2 markers IL4R, Arg1, and BDNF, other than inhibiting M1 parameters as we have established in previous work [18, 19], thus reinforcing the proinflammatory action, for instance, of miR-125b in ALS.

Overall, we can conclude that a subtle equilibrium in the timing and power of proinflammatory versus anti-inflammatory agents can imprint microglia to tip the balance toward toxicity or protection, motor neuron survival, or cell death in ALS. Up to now we have established the active participation of P2X7 and miR-125b in the inflammatory reaction of microglia; further work will tell us exactly when and how to manipulate these same pathways in order to improve their therapeutic perspective against ALS.

Abbreviations

ALS: Amyotrophic lateral sclerosis
Arg1: Arginase-1
BDNF: Brain derived neurotrophic factor
BzATP: 2′-3′-O-(Benzoyl-benzoyl) ATP
IFNγ: Interferon-γ
IL-1β: Interleukin-1β
IL-4: Interleukin-4
IL-4R: Interleukin-4 receptor
IL-6: Interleukin-6
IL-10: Interleukin-10
NF-kB: Nuclear factor kappa B
LPS: Lipopolysaccharide
miRNA: MicroRNA
MRC-1: Mannose Receptor C, type 1
NOS2: Nitric oxide synthase 2
NOX2: NADPH oxidase 2
SOD1: Superoxide dismutase 1
TNFα: Tumor necrosis factor α.

Competing Interests

The authors declare no competing financial interests.

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References

[1] S. Vucic, J. D. Rothstein, and M. C. Kiernan, "Advances in treating amyotrophic lateral sclerosis: insights from pathophysiological studies," *Trends in Neurosciences*, vol. 37, no. 8, pp. 433–442, 2014.

[2] N. A. Goyal and T. Mozaffar, "Experimental trials in amyotrophic lateral sclerosis: a review of recently completed, ongoing and planned trials using existing and novel drugs," *Expert Opinion on Investigational Drugs*, vol. 23, no. 11, pp. 1541–1551, 2014.

[3] N. Riva, F. Agosta, C. Lunetta, M. Filippi, and A. Quattrini, "Recent advances in amyotrophic lateral sclerosis," *Journal of Neurology*, vol. 263, no. 6, pp. 1241–1254, 2016.

[4] D. R. Rosen, T. Siddique, D. Patterson et al., "Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis," *Nature*, vol. 362, no. 6415, pp. 59–62, 1993.

[5] P. M. Andersen, "Amyotrophic lateral sclerosis associated with mutations in the CuZn superoxide dismutase gene," *Current Neurology and Neuroscience Reports*, vol. 6, no. 1, pp. 37–46, 2006.

[6] A. E. Renton, A. Chiò, and B. J. Traynor, "State of play in amyotrophic lateral sclerosis genetics," *Nature Neuroscience*, vol. 17, no. 1, pp. 17–23, 2014.

[7] M. E. Gurney, H. Pu, A. Y. Chiu et al., "Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation," *Science*, vol. 264, no. 5166, pp. 1772–1774, 1994.

[8] O. M. Peters, M. Ghasemi, and R. H. Brown, "Emerging mechanisms of molecular pathology in ALS," *Journal of Clinical Investigation*, vol. 125, no. 5, pp. 1767–1779, 2015.

[9] F. Puentes, A. Malaspina, J. M. van Noort, and S. Amor, "Non-neuronal cells in ALS: role of glial, immune cells and blood-CNS barriers," *Brain Pathology*, vol. 26, no. 2, pp. 248–257, 2016.

[10] K. G. Hooten, D. R. Beers, W. Zhao, and S. H. Appel, "Protective and toxic neuroinflammation in amyotrophic lateral sclerosis," *Neurotherapeutics*, vol. 12, no. 2, pp. 364–375, 2015.

[11] C. Volonté, S. Apolloni, S. D. Skaper, and G. Burnstock, "P2X7 receptors: channels, pores and more," *CNS & Neurological Disorders—Drug Targets*, vol. 11, no. 6, pp. 705–721, 2012.

[12] C. Volonté, S. Apolloni, C. Parisi, and S. Amadio, "Purinergic contribution to amyotrophic lateral sclerosis," *Neuropharmacology*, vol. 104, pp. 180–193, 2016.

[13] C. Volonté, S. Apolloni, M. T. Carrì, and N. D'Ambrosi, "ALS: focus on purinergic signalling," *Pharmacology and Therapeutics*, vol. 132, no. 1, pp. 111–122, 2011.

[14] F. Di Virgilio and M. Vuerich, "Purinergic signaling in the immune system," *Autonomic Neuroscience: Basic and Clinical*, vol. 191, pp. 117–123, 2015.

[15] N. D'Ambrosi, P. Finocchi, S. Apolloni et al., "The proinflammatory action of microglial P2 receptors is enhanced in SOD1 models for amyotrophic lateral sclerosis," *The Journal of Immunology*, vol. 183, no. 7, pp. 4648–4656, 2009.

[16] S. Apolloni, C. Parisi, M. G. Pesaresi et al., "The NADPH oxidase pathway is dysregulated by the P2X7 receptor in the sod1-g93a microglia model of amyotrophic lateral sclerosis," *Journal of Immunology*, vol. 190, no. 10, pp. 5187–5195, 2013.

[17] S. Apolloni, S. Amadio, C. Montilli, C. Volonté, and N. D'ambrosi, "Ablation of p2X7 receptor exacerbates gliosis and motoneuron death in the SOD1-G93A mouse model of amyotrophic lateral sclerosis," *Human Molecular Genetics*, vol. 22, no. 20, pp. 4102–4116, 2013.

[18] C. Parisi, I. Arisi, N. D'Ambrosi et al., "Dysregulated microRNAs in amyotrophic lateral sclerosis microglia modulate genes linked to neuroinflammation," *Cell Death and Disease*, vol. 4, no. 12, article no. e959, 2013.

[19] C. Parisi, G. Napoli, S. Amadio et al., "MicroRNA-125b regulates microglia activation and motor neuron death in ALS," *Cell Death and Differentiation*, vol. 23, no. 3, pp. 531–541, 2016.

[20] S. Apolloni, S. Amadio, C. Parisi et al., "Spinal cord pathology is ameliorated by P2X7 antagonism in a SOD1-mutant mouse model of amyotrophic lateral sclerosis," *DMM Disease Models and Mechanisms*, vol. 7, no. 9, pp. 1101–1109, 2014.

[21] M.-È. Tremblay, C. Lecours, L. Samson, V. Sánchez-Zafra, and A. Sierra, "From the Cajal alumni Achúcarro and Río-Hortega to the rediscovery of never-resting microglia," *Frontiers in Neuroanatomy*, vol. 9, article 45, 2015.

[22] A. ElAli and S. Rivest, "Microglia ontology and signaling," *Frontiers in Cell and Developmental Biology*, vol. 4, article 72, 2016.

[23] U.-K. Hanisch and H. Kettenmann, "Microglia: active sensor and versatile effector cells in the normal and pathologic brain," *Nature Neuroscience*, vol. 10, no. 11, pp. 1387–1394, 2007.

[24] X. Hu, R. K. Leak, Y. Shi et al., "Microglial and macrophage polarization—new prospects for brain repair," *Nature Reviews Neurology*, vol. 11, no. 1, pp. 56–64, 2015.

[25] M. A. Ajmone-Cat, M. Mancini, R. De Simone, P. Cilli, and L. Minghetti, "Microglial polarization and plasticity: evidence from organotypic hippocampal slice cultures," *Glia*, vol. 61, no. 10, pp. 1698–1711, 2013.

[26] J. M. Crain, M. Nikodemova, and J. J. Watters, "Microglia express distinct M1 and M2 phenotypic markers in the postnatal and adult central nervous system in male and female mice," *Journal of Neuroscience Research*, vol. 91, no. 9, pp. 1143–1151, 2013.

[27] S. E. Fendrick, Q.-S. Xue, and W. J. Streit, "Formation of multinucleated giant cells and microglial degeneration in rats expressing a mutant Cu/Zn superoxide dismutase gene," *Journal of Neuroinflammation*, vol. 4, article no. 9, 2007.

[28] D. R. Beers, J. S. Henkel, Q. Xiao et al., "Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 43, pp. 16021–16026, 2006.

[29] D. Brites and A. R. Vaz, "Microglia centered pathogenesis in ALS: insights in cell interconnectivity," *Frontiers in Cellular Neuroscience*, vol. 8, article 117, 2014.

[30] B. Liao, W. Zhao, D. R. Beers, J. S. Henkel, and S. H. Appel, "Transformation from a neuroprotective to a neurotoxic microglial phenotype in a mouse model of ALS," *Experimental Neurology*, vol. 237, no. 1, pp. 147–152, 2012.

[31] J. D. Cherry, J. A. Olschowka, and M. K. O'Banion, "Neuroinflammation and M2 microglia: the good, the bad, and the inflamed," *Journal of Neuroinflammation*, vol. 11, article no. 98, 2014.

[32] R. Franco and D. Fernández-Suárez, "Alternatively activated microglia and macrophages in the central nervous system," *Progress in Neurobiology*, vol. 131, pp. 65–86, 2015.

[33] R. M. Ransohoff, "A polarizing question: do M1 and M2 microglia exist?" *Nature Neuroscience*, vol. 19, no. 8, pp. 987–991, 2016.

[34] P. Pelegrin and A. Surprenant, "Dynamics of macrophage polarization reveal new mechanism to inhibit IL-1β release through pyrophosphates," *The EMBO Journal*, vol. 28, no. 14, pp. 2114–2127, 2009.

[35] G. Lopez-Castejón, A. Baroja-Mazo, and P. Pelegrín, "Novel macrophage polarization model: from gene expression to identification of new anti-inflammatory molecules," *Cellular and Molecular Life Sciences*, vol. 68, no. 18, pp. 3095–3107, 2011.

[36] O. Butovsky, A. E. Talpalar, K. Ben-Yaakov, and M. Schwartz, "Activation of microglia by aggregated β-amyloid or lipopolysaccharide impairs MHC-II expression and renders them cytotoxic whereas IFN-γ and IL-4 render them protective," *Molecular and Cellular Neuroscience*, vol. 29, no. 3, pp. 381–393, 2005.

[37] V. Chhor, T. Le Charpentier, S. Lebon et al., "Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia *in vitro*," *Brain, Behavior, and Immunity*, vol. 32, pp. 70–85, 2013.

[38] A. Mehta and D. Baltimore, "MicroRNAs as regulatory elements in immune system logic," *Nature Reviews Immunology*, vol. 16, no. 5, pp. 279–294, 2016.

[39] A. Jovičić, R. Roshan, N. Moisoi et al., "Comprehensive expression analyses of neural cell-type-specific miRNAs identify new determinants of the specification and maintenance of neuronal phenotypes," *Journal of Neuroscience*, vol. 33, no. 12, pp. 5127–5137, 2013.

[40] O. Butovsky, M. P. Jedrychowski, C. S. Moore et al., "Identification of a unique TGF-β-dependent molecular and functional signature in microglia," *Nature Neuroscience*, vol. 17, no. 1, pp. 131–143, 2014.

[41] A. Karthikeyan, R. Patnala, S. Jadhav, L. Eng-Ang, and S. Dheen, "MicroRNAs: key players in microglia and astrocyte mediated inflammation in CNS pathologies," *Current Medicinal Chemistry*, vol. 23, no. 30, pp. 3528–3546, 2016.

[42] C. Volonté, S. Apolloni, and C. Parisi, "MicroRNAs: newcomers into the ALS picture," *CNS and Neurological Disorders—Drug Targets*, vol. 14, no. 2, pp. 194–207, 2015.

[43] S. Bicker and G. Schratt, "MicroRNAs in ALS: small pieces to the puzzle," *The EMBO Journal*, vol. 34, no. 21, pp. 2601–2603, 2015.

[44] M. Benigni, C. Ricci, A. R. Jones, F. Giannini, A. Al-Chalabi, and S. Battistini, "Identification of miRNAs as potential biomarkers in cerebrospinal fluid from amyotrophic lateral sclerosis patients," *NeuroMolecular Medicine*, vol. 18, no. 4, pp. 551–560, 2016.

[45] I. Takahashi, Y. Hama, M. Matsushima et al., "Identification of plasma microRNAs as a biomarker of sporadic Amyotrophic Lateral Sclerosis," *Molecular Brain*, vol. 8, no. 1, article 67, 2015.

[46] E. Tasca, V. Pegoraro, A. Merico, and C. Angelini, "Circulating microRNAs as biomarkers of muscle differentiation and atrophy in ALS," *Clinical Neuropathology*, vol. 35, no. 1, pp. 22–30, 2016.

[47] A. Freischmidt, K. Müller, L. Zondler et al., "Serum microRNAs in sporadic amyotrophic lateral sclerosis," *Neurobiology of Aging*, vol. 36, no. 9, pp. 2660.e15–2660.e20, 2015.

[48] F. Cloutier, A. Marrero, C. O'Connell, and P. Morin, "MicroRNAs as potential circulating biomarkers for amyotrophic lateral sclerosis," *Journal of Molecular Neuroscience*, vol. 56, no. 1, pp. 102–112, 2015.

[49] J. M. Toivonen, R. Manzano, S. Oliván, P. Zaragoza, A. García-Redondo, and R. Osta, "MicroRNA-206: a potential circulating biomarker candidate for amyotrophic lateral sclerosis," *PLoS ONE*, vol. 9, no. 2, Article ID e89065, 2014.

[50] Z. Zhang, S. Almeida, Y. Lu et al., "Downregulation of microRNA-9 in iPSC-derived neurons of FTD/ALS patients with

TDP-43 mutations," *PLoS ONE*, vol. 8, no. 10, Article ID e76055, 2013.

[51] B. De Felice, M. Guida, M. Guida, C. Coppola, G. De Mieri, and R. Cotrufo, "A miRNA signature in leukocytes from sporadic amyotrophic lateral sclerosis," *Gene*, vol. 508, no. 1, pp. 35–40, 2012.

[52] O. Butovsky, S. Siddiqui, G. Gabriely et al., "Modulating inflammatory monocytes with a unique microRNA gene signature ameliorates murine ALS," *Journal of Clinical Investigation*, vol. 122, no. 9, pp. 3063–3087, 2012.

[53] S. Marcuzzo, D. Kapetis, R. Mantegazza et al., "Altered miRNA expression is associated with neuronal fate in G93A-SOD1 ependymal stem progenitor cells," *Experimental Neurology*, vol. 253, pp. 91–101, 2014.

[54] A. H. Williams, G. Valdez, V. Moresi et al., "MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice," *Science*, vol. 326, no. 5959, pp. 1549–1554, 2009.

[55] O. Butovsky, M. P. Jedrychowski, R. Cialic et al., "Targeting miR-155 restores abnormal microglia and attenuates disease in SOD1 mice," *Annals of Neurology*, vol. 77, no. 1, pp. 75–99, 2015.

[56] K. Nolan, M. R. Mitchem, E. M. Jimenez-Mateos, D. C. Henshall, C. G. Concannon, and J. H. M. Prehn, "Increased expression of microRNA-29a in ALS mice: functional analysis of its inhibition," *Journal of Molecular Neuroscience*, vol. 53, no. 2, pp. 231–241, 2014.

[57] A. E. Frakes, L. Ferraiuolo, A. M. Haidet-Phillips et al., "Microglia induce motor neuron death via the classical NF-κB pathway in amyotrophic lateral sclerosis," *Neuron*, vol. 81, no. 5, pp. 1009–1023, 2014.

[58] Y. Yiangou, P. Facer, P. Durrenberger et al., "COX-2, CB2 and P2X7-immunoreactivities are increased in activated microglial cells/macrophages of multiple sclerosis and amyotrophic lateral sclerosis spinal cord," *BMC Neurology*, vol. 6, article no. 12, 2006.

[59] N. Kopitar-Jeraia, "Innate immune response in brain, NF-kappa B signaling and cystatins," *Frontiers in Molecular Neuroscience*, vol. 8, article 73, 2015.

[60] S.-W. Kim, K. Ramasamy, H. Bouamar, A.-P. Lin, D. Jiang, and R. C. T. Aguiar, "MicroRNAs miR-125a and miR-125b constitutively activate the NF-κB pathway by targeting the tumor necrosis factor alpha-induced protein 3 (TNFAIP3, A20)," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 20, pp. 7865–7870, 2012.

[61] A. M. Fenn, C. J. Henry, Y. Huang, A. Dugan, and J. P. Godbout, "Lipopolysaccharide-induced interleukin (IL)-4 receptor-α expression and corresponding sensitivity to the M2 promoting effects of IL-4 are impaired in microglia of aged mice," *Brain, Behavior, and Immunity*, vol. 26, no. 5, pp. 766–777, 2012.

[62] W. Zhao, W. Xie, Q. Xiao, D. R. Beers, and S. H. Appel, "Protective effects of an anti-inflammatory cytokine, interleukin-4, on motoneuron toxicity induced by activated microglia," *Journal of Neurochemistry*, vol. 99, no. 4, pp. 1176–1187, 2006.

[63] T. Lawrence, "The nuclear factor NF-κB pathway in inflammation," *Cold Spring Harbor Perspectives in Biology*, vol. 1, no. 6, Article ID a001651, 2009.

[64] V. Y.-F. Wang, W. Huang, M. Asagiri et al., "The transcriptional specificity of NF-κB dimers is coded within the κB DNA response elements," *Cell Reports*, vol. 2, no. 4, pp. 824–839, 2012.

[65] S. T. Smale, "Dimer-specific regulatory mechanisms within the NF-κB family of transcription factors," *Immunological Reviews*, vol. 246, no. 1, pp. 193–204, 2012.

[66] J. Wessells, M. Baer, H. A. Young et al., "BCL-3 and NF-κB p50 attenuate lipopolysaccharide-induced inflammatory responses in macrophages," *Journal of Biological Chemistry*, vol. 279, no. 48, pp. 49995–50003, 2004.

[67] C. Porta, M. Rimoldi, G. Raes et al., "Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor κB," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 35, pp. 14978–14983, 2009.

[68] F. Oakley, J. Mann, S. Nailard et al., "Nuclear factor-κB1 (p50) limits the inflammatory and fibrogenic responses to chronic injury," *American Journal of Pathology*, vol. 166, no. 3, pp. 695–708, 2005.

[69] S. Cao, X. Zhang, J. P. Edwards, and D. M. Mosser, "NF-κB1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages," *The Journal of Biological Chemistry*, vol. 281, no. 36, pp. 26041–26050, 2006.

[70] T. Rolova, L. Puli, J. Magga et al., "Complex regulation of acute and chronic neuroinflammatory responses in mouse models deficient for nuclear factor kappa B p50 subunit," *Neurobiology of Disease*, vol. 64, pp. 16–29, 2014.

[71] T. Taetzsch, S. Levesque, C. Mcgraw et al., "Redox regulation of NF-κB p50 and M1 polarization in microglia," *GLIA*, vol. 63, no. 3, pp. 423–440, 2015.

[72] F. Weih, D. Carrasco, S. K. Durham et al., "Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-κB/Rel family," *Cell*, vol. 80, no. 2, pp. 331–340, 1995.

[73] C. Gasparini, B. M. Foxwell, and M. Feldmann, "RelB/p50 regulates TNF production in LPS-stimulated dendritic cells and macrophages," *Cytokine*, vol. 61, no. 3, pp. 736–740, 2013.

[74] A. Krikos, C. D. Laherty, and V. M. Dixit, "Transcriptional activation of the tumor necrosis factor α-inducible zinc finger protein, A20, is mediated by κB elements," *Journal of Biological Chemistry*, vol. 267, no. 25, pp. 17971–17976, 1992.

[75] K. Heyninck and R. Beyaert, "A20 inhibits NF-κB activation by dual ubiquitin-editing functions," *Trends in Biochemical Sciences*, vol. 30, no. 1, pp. 1–4, 2005.

[76] N. Yamaguchi, M. Oyama, H. Kozuka-Hata, and J.-I. Inoue, "Involvement of A20 in the molecular switch that activates the non-canonical NF-κB pathway," *Scientific Reports*, vol. 3, article 2568, 2013.

[77] M. Kairisalo, L. Korhonen, M. Sepp et al., "NF-κB-dependent regulation of brain-derived neurotrophic factor in hippocampal neurons by X-linked inhibitor of apoptosis protein," *European Journal of Neuroscience*, vol. 30, no. 6, pp. 958–966, 2009.

[78] C. de Torre-Minguela, M. Barberà-Cremades, A. I. Gómez, F. Martín-Sánchez, and P. Pelegrín, "Macrophage activation and polarization modify P2X7 receptor secretome influencing the inflammatory process," *Scientific Reports*, vol. 6, Article ID 22586, 2016.

[79] S. Apolloni, P. Fabbrizio, S. Amadio, and C. Volonté, "Actions of the antihistaminergic clemastine on presymptomatic SOD1-G93A mice ameliorate ALS disease progression," *Journal of Neuroinflammation*, vol. 13, no. 1, article 191, 2016.

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Research Article

Genetic Ablation of Soluble TNF Does Not Affect Lesion Size and Functional Recovery after Moderate Spinal Cord Injury in Mice

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Traumatic spinal cord injury (SCI) is followed by an instant increase in expression of the microglial-derived proinflammatory cytokine tumor necrosis factor (TNF) within the lesioned cord. TNF exists both as membrane-anchored TNF (mTNF) and as cleaved soluble TNF (solTNF). We previously demonstrated that epidural administration of a dominant-negative inhibitor of solTNF, XPro1595, to the contused spinal cord resulted in changes in Iba1 protein expression in microglia/macrophages, decreased lesion volume, and improved locomotor function. Here, we extend our studies using mice expressing mTNF, but no solTNF ($mTNF^{\Delta/\Delta}$), to study the effect of genetic ablation of solTNF on SCI. We demonstrate that TNF levels were significantly decreased within the lesioned spinal cord 3 days after SCI in $mTNF^{\Delta/\Delta}$ mice compared to littermates. This decrease did, however, not translate into significant changes in other pro- and anti-inflammatory cytokines (IL-10, IL-1β, IL-6, IL-5, IL-2, CXCL1, CCL2, or CCL5), despite a tendency towards increased IL-10 and decreased IL-1β, TNFR1, and TNFR2 levels in $mTNF^{\Delta/\Delta}$ mice. In addition, microglial and leukocyte infiltration, activation state (Iba1, CD11b, CD11c, CD45, and MHCII), lesion size, and functional outcome after moderate SCI were comparable between genotypes. Collectively, our data demonstrate that genetic ablation of solTNF does not significantly modulate postlesion outcome after SCI.

1. Introduction

Traumatic spinal cord injury (SCI) is most often caused by a sudden contusion of the spinal cord, where the initial tissue damage is followed by a second phase of cell death, inflammation, and degeneration that occurs over weeks and months after the initial trauma.

The microglial-derived cytokine tumor necrosis factor (TNF) increases at the lesion site within the first 1-2 hours after SCI in rodents [1–4] and spinal cord injured individuals display elevated TNF serum concentrations [5, 6], suggesting that TNF plays a major part in the development of secondary tissue damage [7].

TNF exists in two biologically active forms, a membrane-anchored form (mTNF) and a soluble form (solTNF), which is shed from the membrane by the metalloproteinase TNF-α converting enzyme (TACE/ADAM17). The biological effects of solTNF and mTNF are mediated through binding of TNF

receptor 1 (TNFR1) and TNFR2, which differ in expression, ligand affinity, cytoplasmic tail structure, and downstream signaling pathways (reviewed in Probert [8]).

In experimental focal cerebral ischemia, an acute CNS injury, microglial-derived mTNF has been shown to be neuroprotective through binding to TNFR1 [9–12]. However, in SCI the data are conflicting. Kim and colleagues found $TNFR1^{-/-}$ mice to have increased lesion size and worse functional outcome compared to controls [13], suggesting a protective role for TNFR1, whereas Genovese et al. [14] demonstrated reduced tissue damage and improved motor function in mice treated with the nonselective TNF inhibitor infliximab as well as in $TNFR1^{-/-}$ mice, indicating a detrimental role for TNFR1. Surprisingly, germ-line ablation of TNF in $TNF^{-/-}$ mice did not result in any differences in lesion size and functional outcome following SCI compared to controls [15]. We recently demonstrated that epidural administration of the dominant-negative solTNF inhibitor XPro1595 reduced lesion size and improved functional outcome following SCI, whereas etanercept, inhibitor of both mTNF and solTNF, had no effect [16]. Importantly, systemic administration of either compound was ineffective [16], in line with other studies showing that systemic administration of etanercept following SCI in mice does not reduce inflammation and tissue injury or infiltration of neutrophils nor improves the functional outcome [17]. Late blockage of peripheral TNF with etanercept was also ineffective in improving locomotor function in mice with SCI [18], while in rats it reduced tissue damage, improved hindlimb function, and facilitated myelin regeneration [19]. It should also be mentioned that a case report of a spinal cord injured patient treated chronically with etanercept for ankylosing spondylitis demonstrated reduced inflammation, reduced perilesional area, and improved motor recovery [20].

Even though the studies into the role of microglial-derived TNF following SCI are inconclusive, they clearly demonstrate that the TNF-TNFR signaling cascade plays an important part in tissue inflammation, although the contribution of solTNF versus mTNF to tissue damage and functional recovery remains to be elucidated. In this study, we investigated the effect of solTNF and mTNF in SCI using genetically modified $mTNF^{\Delta/\Delta}$ mice that express only mTNF [21]. We show that absence of solTNF in $mTNF^{\Delta/\Delta}$ mice does not affect lesion size and functional outcome 35 days after SCI. However TNF levels are significantly decreased within the lesioned spinal cord 3 days after SCI compared to littermate control mice ($mTNF^{wt/wt}$). These findings suggest that genetic ablation of solTNF does not affect lesion size and functional outcome after SCI.

2. Materials and Methods

2.1. Mice. Homozygous $mTNF^{\Delta/\Delta}$ and $mTNF^{wt/wt}$ littermates were obtained by crossing heterozygous $mTNF^{\Delta/wt}$ mice at the Biomedical Laboratory, University of Southern Denmark (SDU) [12, 21]. These mice were originally generated by replacing the endogenous TNF allele with Δ1–9, K11E TNF allele [21]. This resulted in loss of TACE-mediated cleavage preventing shedding of solTNF [21, 22] but maintenance of normal cell-surface expression of mTNF [21]. All experiments were performed blinded on age-matched (8–12 weeks) female $mTNF^{\Delta/\Delta}$ and $mTNF^{wt/wt}$ littermates. Animals were housed in ventilated cages with 1–3 cage-mates at a 12 h light/dark cycle, under controlled temperature and humidity, and with free access to food and water.

Mice were cared for in accordance with the protocols and guidelines approved by The Danish Animal Inspectorate under the Ministry of Food and Agriculture (J. numbers 2008-561-1523 and 2013-15-2934-00924); experiments are reported in accordance with the ARRIVE guidelines, and all efforts were made to minimize pain and distress.

2.2. Genotyping. DNA was extracted from tail biopsies from 3-4-week-old mice using a NucleoSpin Tissue kit (Macherey-Nagel) according to the manufacturer's instructions. DNA was amplified by PCR under the following conditions: 50°C for 2 minutes and 95°C for 10 minutes followed by 39 cycles of 95°C for 15 sec, 62°C for 1 minute, and 72°C for 1 minute and the following per PCR reaction: 12.5 μL Master Mix (Thermo Scientific), 1 μL forward primer (5′-GCGTCCAGCTGACTAAA), 1 μL reverse primer (3′-ACCACTAGTTGGTTGCTTTGAGAT), and 10 μL dH_2O. Both primers were from DNA Technology A/S and in working concentrations of 10 pmol/μL. The PCR products were visualized using the FlashGel system (Lonza). Four-microliter PCR products mixed with FlashGel loading buffer (Lonza) were loaded on FlashGel DNA cassette 1.2% agarose gel with 16 + 1 double wells (Lonza) and the gels were allowed to run for 25–30 minutes at 70 volts. In all gels, FlashGel system DNA marker (Lonza) and samples with known genotypes (homozygote, heterozygote, and wild type) were included.

2.3. Behavioral Analysis. We have previously performed a thorough behavioral assessment of $mTNF^{\Delta/\Delta}$ in naïve conditions and found no differences in behavioral phenotype compared to $mTNF^{wt/wt}$ littermates [12].

Elevated Plus Maze. To further examine anxiety-like behavior and locomotion, which could affect locomotor function after SCI, naïve mice were subjected to the elevated plus maze (EPM). The elevated plus maze apparatus consisted of two open arms and two closed arms (30 cm × 5 cm). The entire maze was elevated around 40 cm from the floor. Each mouse was placed in the center of the maze with the head facing towards the open arm. During a 5 min test, the time spent in the closed and open arms and the total distance moved were recorded using the SMART video tracking software (Panlab).

2.4. Contusive Spinal Cord Injury. Mice were anaesthetized using a ketamine (100 mg/kg, VEDCO Inc)/xylazine (10 mg/kg, VEDCO Inc) cocktail, laminectomized between vertebrae T8 and T10 and injured with an Infinite Horizon-0400 SCI Contusion Devise (Precision Systems and Instrumentation, LLC) by lowering the impactor onto the exposed cord at an impact of 75 Kdynes resulting in approximately 500 μm displacement (moderate injury) [16].

Mice were then sutured, injected with saline to prevent dehydration, and given buprenorphine hydrochloride (0.001 mg/20 g body weight Temgesic) four times at 8-hour intervals for postsurgical analgesia. Mice were housed separately in a recovery room and monitored for a 24- to 48-hour recovery period. Thereafter, mice were observed twice daily for activity level, respiratory rate, and general physical condition. Manual bladder expression was performed twice a day until bladder function was regained. Body weight was monitored weekly. In addition, mice received s.c. prophylactic injections of antibiotic gentamicin (40 mg/kg) for 7 days to prevent urinary tract infections. The persons performing the SCI have attended the SCI Research Training Program at the Ohio State University. In total, 2 $mTNF^{\Delta/\Delta}$ mice died during surgery, while 1 $mTNF^{wt/wt}$ and 1 $mTNF^{\Delta/\Delta}$ mouse were euthanized on day 8 and day 24, respectively, due to poor general health status.

2.5. Assessment of Functional Outcome after SCI

Basso Mouse Scale. Functional recovery of hind limb function after SCI was determined by scoring of the locomotor hindlimb performance in the open field using the Basso Mouse Scale (BMS) system, a 0 to 9 rating system designed specifically for the mouse [16, 23]. Under observer-blinded conditions, mice were evaluated over a 4-min period 1 and 3 days after SCI and weekly thereafter. Only mice with a score below 2, representing a successful lesion, on day 1 were included in the study. Before surgery, mice were handled and pretrained in the open field to prevent fear and/or stress behaviors that could bias the locomotor assessment.

Thermal Hyperalgesia. Thermal hyperalgesia (hind paw withdrawal from a normally innocuous heat source) was tested with a Hargreaves' heat source using the Plantar Test apparatus (Ugo Basile) [16]. Each paw was tested 5 times with at least 2 min break in between. The lowest and highest reflex latency scores of each paw were discarded and the bilateral mean was calculated and plotted. The behavioral test was performed weekly when the mice reached a BMS score of 5 and were capable of frequent or consistent stepping and thereby plantar placement of the paws, typically around 3 weeks after SCI.

Rung Walk. In order to test stepping, interlimb coordination, and balance, mice were tested on the rung walk also when they reached a BMS score of 5. The rung walk consisted of two plates of transparent polymer, approximately 110 cm × 20 cm, with a 2.5 cm space between them. The apparatus was placed on two cages with the home cage at one end, making the mice automatically walk in that direction. To avoid stopping or turning during trials, animals were pretrained 5 times prior to surgery with the final test serving as baseline. Following SCI, eligible mice were tested at 3, 4, and 5 weeks using a handheld GoPro HD camera with 48 fps. Data were evaluated frame by frame using QuickTime. Left and right scores were calculated as follows: 6, complete miss; 5, touching rung but sliding off and losing balance; 4, touch, miss but no loss of balance; 3, replacement, mouse placed paw on rung but quickly moves it; 2, recorrection, aims for a rung but changes direction; 1, anterior or posterior placement; 0, perfect step. The total number of mistakes was plotted for analysis as previously described [16].

Open Field Test. The open field test was performed with a nontransparent squared plastic box (45 × 45 × 45 cm) over a period of 10 min 35 days after SCI [16]. Movements were tracked using the SMART video tracking software (Panlab) connected to a video camera (SSC-D378P, Biosite). The distance travelled (m), speed (cm/sec), and entries into the three zones (wall, interperiphery, and center of the box) were recorded automatically. A center/perimeter ratio was calculated based on the number of entries. Rearing, grooming, jumping, digging, urination, and droppings were recorded manually and presented as number (n) of events [24].

2.6. Tissue Processing

2.6.1. Histopathology and Immunohistochemistry. For paraffin histopathology and immunohistochemical analyses, mice were deeply anaesthetized using an overdose of pentobarbital (200 mg/mL) containing lidocaine (20 mg/mL) and perfused through the left ventricle with ice-cold 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The spinal cords were quickly removed and tissue segments containing the lesion area (1 cm centered on the lesion) were paraffin-embedded and cut into 10 parallel series of 15 μm thick microtome sections. Sections were stored at room temperature until further processing.

2.6.2. Klüver-Barrera Luxol Fast Blue Staining for Myelinated Fibers. For evaluation of lesion pathology, 1 series of sections from each animal was stained in Luxol Fast Blue (LFB) (0.1% LFB in 95% ethanol (EtOH) and 0.05% acetic acid) at 60°C over night. Next day, sections were rinsed in 96% EtOH and distilled H_2O, immersed briefly in lithium carbonate (0.05% Li_2CO_3 in distilled water), and differentiated in 70% EtOH. Next, sections were rinsed thoroughly in distilled H_2O and immersed in 0.05% lithium carbonate to stop further differentiation. Sections were then placed in hematoxylin, rinsed in running tap water, and immersed briefly in eosin solution. Finally, sections were rinsed in 70% EtOH, followed by 3x 99% EtOH, placed in 3x xylene prior to mounting with Depex. Prior to staining, paraffin embedded sections were deparaffinized 3x 3 min in xylene, 3x 2 min in 99% EtOH, and 2x 2 min in 96% EtOH.

2.6.3. Immunohistochemical Staining for CD45, F4/80, and MBP. Heat-Induced Antigen Retrieval was done on paraffin embedded sections by boiling the sections in citrate buffer + 0.05% Tween, pH 6.0 (MBP), Tris-EGTA buffer, pH 9.0 (CD45), or TRS buffer (Target Retrieval Solution, Dako) (F4/80) first 15 min at 900 W and then 9 min at 440 W. The sections were allowed to cool in the buffer before they were blocked for endogenous peroxidase and biotin activity. Sections were then incubated with anti-CD45 IgG (30-F11 (Ly 5); BD Pharmingen) diluted 1 : 100, anti-F4/80 IgG (AbD Serotec) diluted 1 : 100, or anti-MBP IgG (Biolegend) diluted

1 : 150 and detected using biotin-labeled anti-rat (Dako), diluted 1 : 200, or anti-mouse, diluted 1 : 100, antibodies, followed by ready-to-use anti-rabbit horse-radish perioxidase- (HRP-) labeled polymer (EnVision+ System, Dako) with diaminobenzidine (DAB) as chromogen (Dako). Nuclei were counterstained using Mayer's haemalum w/4.5% chloralhydrate. As negative controls, staining specificity was tested on parallel sections of spinal cord tissue by omitting the primary antibody or substitution with a primary antibody with a similar concentration of a mouse IgG1 isotype control (Dako) (for MBP) in order to check for any unspecific reaction from the detection system. As positive control for antibody specificity, a mouse multiblock containing several different tissues, including lymphatic organs, was included. Activation patterns were investigated in 5 sections (representing 750 μm spinal cord) centered on the lesion epicenter from each animal.

2.6.4. Immunofluorescent Staining for Glial Fibrillary Acidic Protein (GFAP) and Ionized Calcium Binding Adaptor Molecule (Iba1). One series of sections from each animal was deparaffinized and rehydrated by placing the sections 3x 3 minutes in xylene, 3x 2 minutes in 99% EtOH, 2x 2 minutes in 96% EtOH, 2 minutes in 70% EtOH, and finally 5 minutes in running tap water. The sections were then demasked with TEG-buffer by placing the sections in warm TEG-buffer in a steamer for 15 minutes and then letting them cool for 15 minutes at room temperature before rinsing them for 15 minutes in running tap water. The sections were then rinsed 3x 15 minutes in Tris-buffered saline (TBS) before they were preincubated with 10% fetal bovine serum (FBS) in TBS with 0.5% Triton X-100 for 30 minutes. Hereafter the sections were incubated with Alexa Fluor® 488-conjugated anti-GFAP IgG (mouse monoclonal, clone 131-17719, Thermo Fischer Scientific) diluted 1 : 400 or anti-Iba1 IgG (Rabbit, Dako) diluted 1 : 400 1 hour at room temperature and hereafter over night at 4°C.

At day 2, sections were placed at room temperature for 30 minutes before they were rinsed in TBS for 10 minutes and then in TBS with 0.1% Triton X-100 for 10 minutes. Sections were then either stained with NeuroTrace® 530/615 Red Fluorescent Nissl Stain (Thermo Fischer Scientific) for 20 minutes (GFAP) or incubated with Alexa Fluor 488-conjugated chicken anti-rabbit IgG (Thermo Fischer Scientific) 1 : 500 for 2 hours and then rinsed 2 × 10 minutes in TBS before the nuclei were immersed in a TBS solution containing 10 μM diamidino-2-phenylindole (DAPI) for 10 minutes. The sections were shortly rinsed in distilled water before they were mounted with ProLong Diamond. Activation patterns were investigated in 5 sections (representing 750 μm spinal cord) centered on the lesion epicenter from each animal. Control reactions were performed by omitting the primary antibody or by substituting the primary antibody with Alexa Fluor 488 conjugated mouse $IgG1_{kappa}$ (Thermo Fischer Scientific) or substituting the primary antibody with rabbit serum (Dako) diluted to the same concentration as the primary antibody. Sections were devoid of staining in the FITC imaging filter.

2.7. Lesion Volume Estimation. The volume of the lesion was determined from the area of every 10th LFB- or GFAP-stained section sampled by systematic uniform random sampling. The area of the lesion site was estimated in LFB-stained sections as previously described [16] using the VisioMorph software (Visiopharm) and the Cavalieri principle for volume estimation. For estimation of the lesion area in GFAP-stained sections, photomicrographs were acquired using an Olympus BX51 microscope with an Olympus DP73 camera connected to a PC set up with the Olympus CellSens software. Lesion size was then estimated using ImageJ analysis software (NIH) as per directions of the ImageJ developers (http://rsb.info.nih.gov/ij/). Analysis performed on digital images was carried out on unmanipulated pictures. On the presented pictures the contrast and curves have been adjusted to allow readers to appreciate the details on small-scale figures.

2.8. Estimation of White Matter. The area of intact white matter, based on MBP staining, was estimated as a percentage of the total spinal cord tissue area. Estimations were based on 5 sections centered on the epicenter and 5 sections located 300 μm rostral to the lesion (n = 10 sections from 2-3 animals/group).

2.9. Flow Cytometry

2.9.1. Isolation of Cells for Flow Cytometry. Mice were perfused with PBS as described above, the spinal cords were quickly removed, and tissue segments containing the lesion area (2.5 cm centered on the lesion) and perilesion area (0.5 cm distal to and 0.5 cm proximal to the lesion were pooled to represent perilesion tissue) were placed in cold Hank's Buffered Salt Solution (HBSS). Tissue from individual mice was processed separately. Single-cell suspensions were obtained by homogenization in HBSS using 70-μm nylon cell strainers (BD Falcon). Cells suspensions were centrifuged at 300 ×g for 10 minutes at 4°C. The pellet was resuspended in PBS containing 0.5% FBS and Myelin Removal Beads II (Miltenyi Biotec) were added and the suspensions were incubated for 15 minutes at 4°C. Thereafter PBS containing 0.5% FBS was added and the suspensions were centrifuged at 300 ×g for 10 minutes at 4°C, after which the pellet was resuspended in PBS containing 0.5% FBS. The LS column (Miltenyi Biotec) was placed in the magnetic field of the MACS separator (Miltenyi Biotec) and columns prepared by washing with PBS containing 0.5% FBS. Next, the cell suspension was added and the flow-through was collected and centrifuged at 300 ×g for 10 minutes at 4°C. The pellet was washed by resuspending in PBS and centrifuged at 300 ×g for 10 minutes at 4°C. To lyse the red blood cells the pellet was resuspended in 0.83% ammonium chloride for 10 minutes after which the suspension was centrifuged at 300 ×g for 10 minutes at 4°C. The pellet was washed by resuspending in PBS and centrifuged at 300 ×g for 10 minutes at 4°C. Finally the pellet was resuspended in PBS and the cell suspension was stained for live/dead cells using Fixable Viability Dye eFlouro 506 (eBioscience) for 20 minutes at 4°C in the dark. The pellet was washed by resuspending in PBS and centrifuged at 300 ×g

for 10 minutes at 4°C. Cells were fixed in Cytofix/Cytoperm (BD Pharmingen) for 15 minutes at 4°C and centrifuged at 300 ×g for 10 minutes at 4°C. The pellet was washed twice in FACS staining buffer (HBSS containing 2% FBS and 0.1% sodium azide) and centrifuged at 300 ×g for 10 minutes at 4°C in between. Finally the pellet was resuspended in FACS staining buffer and kept in the dark at 4°C until staining for flow cytometry.

2.9.2. Flow Cytometry. The cells were blocked in 10% rat serum for 30 minutes on ice before they were washed with PBS and centrifuged at 600 ×g for 5 minutes at 4°C. The cells were resuspended in FACS staining buffer and stained with combinations of PE-CD11b (BD Biosciences, clone M1/70), PerCPCy5.5-CD45 (BD Biosciences, clone 30-F11), APC-CD3 (BD Biosciences, clone 145-2C11), PE-Cy7-Gr1 (Biolegend, clone RB6-8C5), PE-Cy7-CD11c (BD Biosciences, clone HL3), and 647-MHC class II (BD Biosciences, clone M5/114.15.2) antibodies for 30 minutes on ice. Finally, the cells were washed in PBS, centrifuged at 600 ×g for 5 minutes at 4°C, and resuspended in FACS buffer before they were run on a FACSverse flow cytometer, and 10^6 events were acquired per sample using forward scatter (FSC) and side scatter (SSC). The analysis was performed using the FACSuite software [25]. Positive staining was determined based on the respective isotype controls and the respective fluorescent minus one (FMO) controls. The mean fluorescent intensity (MFI) was calculated as the geometric mean of each population in the CD45 and CD11b gates, respectively [25].

2.10. Multiplex and Western Blotting Analyses

Protein Purification. Whole spinal cord protein samples from $mTNF^{\Delta/\Delta}$ and $mTNF^{wt/wt}$ mice exposed to SCI and allowed 3-day survival in addition to naïve $mTNF^{\Delta/\Delta}$ and $mTNF^{wt/wt}$ mice were obtained and protein extractions were prepared as previously described [11, 12].

Multiplex Analysis. To measure cytokine, chemokine, and TNFR protein levels by the MSD Mouse Proinflammatory V-Plex Plus Kit (IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, CXCL1, and TNF; K15012C, Mesoscale) and mouse TNF-RI, TNF-RII, RANTES, and MCP-1 Ultra-Sensitive Kits (Mesoscale), we used a SECTOR Imager 6000 (Mesoscale Discovery) Plate Reader according to the manufacturer's instructions. Samples were diluted twofold in Diluent 41 prior to measurement and samples were run in duplicate [11]. Data was analyzed using MSD Discovery Workbench software.

Western Blotting Analysis. Western blot analysis for Iba1 (1 : 500, Wako) was performed using 18 μg protein extract separated on 4–12% SDS-PAGE gels (Invitrogen) using MOPS SDS (Invitrogen) containing 0.25% antioxidant (Invitrogen) essentially as previously described [11]. α-Actin (1 : 100,000, Millipore) was used as loading control. SeeBlue prestained standard (Invitrogen) was used as a molecular weight marker. Bands were quantified using Image Lab Software (Bio-Rad). Analysis was performed with $n = 4$ mice/group and data were normalized to α-actin and presented as percentages relative to $mTNF^{wt/wt}$ mice.

As a control, 2 independent gels were prepared where the primary antibody was omitted in the protocol. Development of the membrane showed the absence of a 17 kDa band, corresponding to the size of Iba1.

2.11. Statistical Analysis. Comparisons were performed using repeated measures (RM) two-way ANOVA followed by multiple t-test analysis (BMS, rung walk, Hargreaves' tests, and weight change), Mann–Whitney, or Wilcoxon matched-pairs signed rank tests. Analyses were performed using Prism 4.0b software for Macintosh (GraphPad Software). Statistical significance was established for $p \leq 0.05$.

3. Results

3.1. Genetic Ablation of solTNF Does Not Alter Locomotor Function or Anxiety-Related Behavior. Using the open field, rotarod, and Y-maze tests, we previously demonstrated that $mTNF^{\Delta/\Delta}$ mice display no obvious behavioral abnormalities [12]. This was further validated in the present study using the EPM test. We found that the time spent in the closed (Figure 1(a)) and open arms (Figure 1(b)) and the total distance travelled in the EPM were comparable between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice (Figure 1(c)). Together our findings demonstrate that locomotor function following SCI will not be affected by innate changes in the behavioral phenotype between naïve $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice.

3.2. Genetic Ablation of solTNF Does Not Affect Lesion Size or Functional Outcome after SCI. On the basis of our previous findings of a neuroprotective effect of mTNF [12, 16], we investigated whether genetic ablation of solTNF in $mTNF^{\Delta/\Delta}$ mice could also improve functional recovery and reduce tissue damage following traumatic SCI. In this study, $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice were subjected to SCI and locomotor performance in the open field was recorded on days 1 and 3 and then weekly for 5 weeks and scored with the BMS. We found that there were no differences in BMS scores between the two genotypes at any time point after SCI (final BMS ± SEM; $mTNF^{wt/wt}$: 5.3 ± 1.0; $mTNF^{\Delta/\Delta}$: 5.3 ± 1.5) (Figure 2(a)). Both genotypes improved their BMS scores significantly over time. Mice were also evaluated using rung walk analysis when they reached a BMS score of 5 and weekly thereafter. Even though both genotypes displayed significant changes over time, no differences were observed between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice (Figure 2(b)). Thermal hyperalgesia was tested using Hargreaves' test. Despite findings of significant changes in nociception over time in both $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice, no difference between genotypes was observed (Figure 2(c)). Finally in order to investigate the effect of genetic ablation of solTNF on general activity and anxiety-related behavior after SCI, $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice were subjected to the open field test 35 days after SCI (Supplemental Figure 1, Supplementary Material available online at http://dx.doi.org/10.1155/2016/2684098). We found

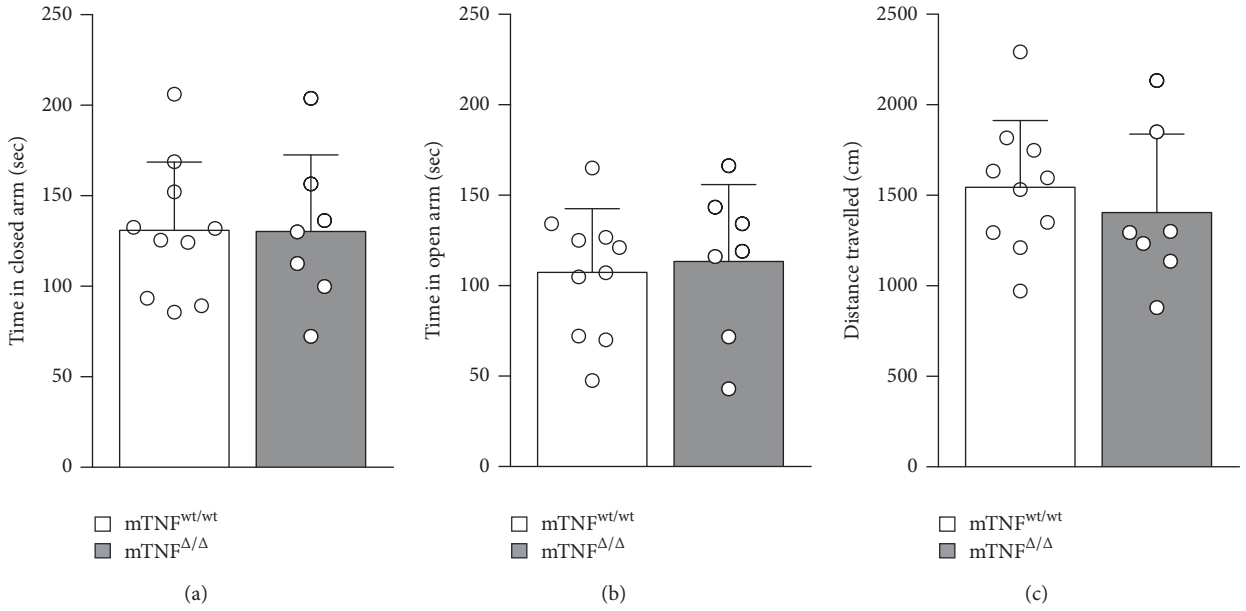


Figure 1: Elevated plus maze analysis in naïve $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice. (a–c) $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice spent comparable times in the closed arm (a) and the open arm (b) and travelled a similar distance (c) in the elevated plus maze test. Mann–Whitney test, $n = 7$–10 mice/group. Results are presented as mean ± SD.

no differences in the total distance travelled (Supplemental Figure 1A), in the speed at which the mice moved (Supplemental Figure 1B), in number of rearings (Supplemental Figure 1C), nor in number of zone changes ($p < 0.05$, $mTNF^{wt/wt}$: 45.0 ± 33.9; $mTNF^{\Delta/\Delta}$: 37.5 ± 31.2), demonstrating that mTNF is sufficient to sustain locomotor function. Also, grooming (Supplemental Figure 1D) and urination (Supplemental Figure 1E) and the center/perimeter ratio ($p < 0.05$, ratios: $mTNF^{wt/wt}$: 0.08 ± 0.09%; $mTNF^{\Delta/\Delta}$: 0.06% ± 0.10) were comparable between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice, and only the number of droppings differed significantly between the two genotypes (Supplemental Figure 1F). In line with these data, we observed no difference in lesion size (Figures 2(d)–2(f)) or the percentage of MBP^+ white matter area ($mTNF^{wt/wt}$: 48.6% ± 10.3%; $mTNF^{\Delta/\Delta}$: 49.4% ± 12.0%) between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice 35 days after SCI. Intense GFAP immunoreactivity was detected both in genotypes around the lesion and in the surrounding white matter, indicating formation of a glial scar (Figure 2(g)).

Body weight was affected over time by SCI, but there was no difference between genotypes (Supplemental Figure 1G).

3.3. Genetic Ablation of solTNF Reduces TNF Levels after SCI. We previously showed that TNF is decreased in $mTNF^{\Delta/\Delta}$ mice compared to $mTNF^{wt/wt}$ controls after experimental stroke [12]. Following SCI, the genetic ablation of solTNF also affected TNF in the spinal cord significantly (Figure 3(a)). TNF increased significantly in $mTNF^{wt/wt}$ mice compared to naïve conditions and was significantly increased compared to $mTNF^{\Delta/\Delta}$ mice 3 days after SCI, whereas TNF stayed at baseline levels after SCI in $mTNF^{\Delta/\Delta}$ mice (Figure 3(a)).

TNFR1 (Figure 3(b)) and TNFR2 (Figure 3(c)) were also significantly affected in the spinal cords of $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice. We found that, in both $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice, TNFR1 and TNFR2 increased significantly in the lesioned spinal cord 3 days after SCI compared to naïve conditions but with no significant difference between genotypes (Figures 3(b) and 3(c)). However, for both TNFR1 and TNFR2 there was a tendency towards reduced TNFR1 ($p = 0.06$) and TNFR2 ($p = 0.06$) levels in $mTNF^{\Delta/\Delta}$ compared to $mTNF^{wt/wt}$ mice 3 days after SCI.

3.4. Genetic Ablation of mTNF Does Not Affect Neuroinflammation in the Lesioned Spinal Cord 3 Days after SCI. IL-10 (Figure 3(d)), IL-1β (Figure 3(e)), and IL-6 (Figure 3(f)) were significantly increased in $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice 3 days after SCI compared to naïve conditions, but with no significant difference between genotypes. However, there was a tendency towards increased IL-10 ($p = 0.09$) and decreased IL-1β ($p = 0.09$) in $mTNF^{\Delta/\Delta}$ compared $mTNF^{wt/wt}$ mice 3 days after SCI. IL-5 (Figure 3(g)) was significantly increased at 3 days compared to naïve conditions only in $mTNF^{wt/wt}$ mice (Figure 3(h)), whereas IL-2 only increased significantly in $mTNF^{\Delta/\Delta}$ mice (Figure 3(h)). IFNγ and IL-4 protein levels were not affected 3 days after SCI (not shown).

CXCL1 (Figure 3(i)) and CCL2 (Figure 3(j)) were significantly upregulated in the lesioned spinal cord of $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice 3 days after SCI compared to naïve conditions, whereas the increase in CCL5 (Figure 3(k)) did not reach statistical significance. No differences in CXCL1, CCL2, and CCL5 were observed between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice (Figures 3(i)–3(k)).

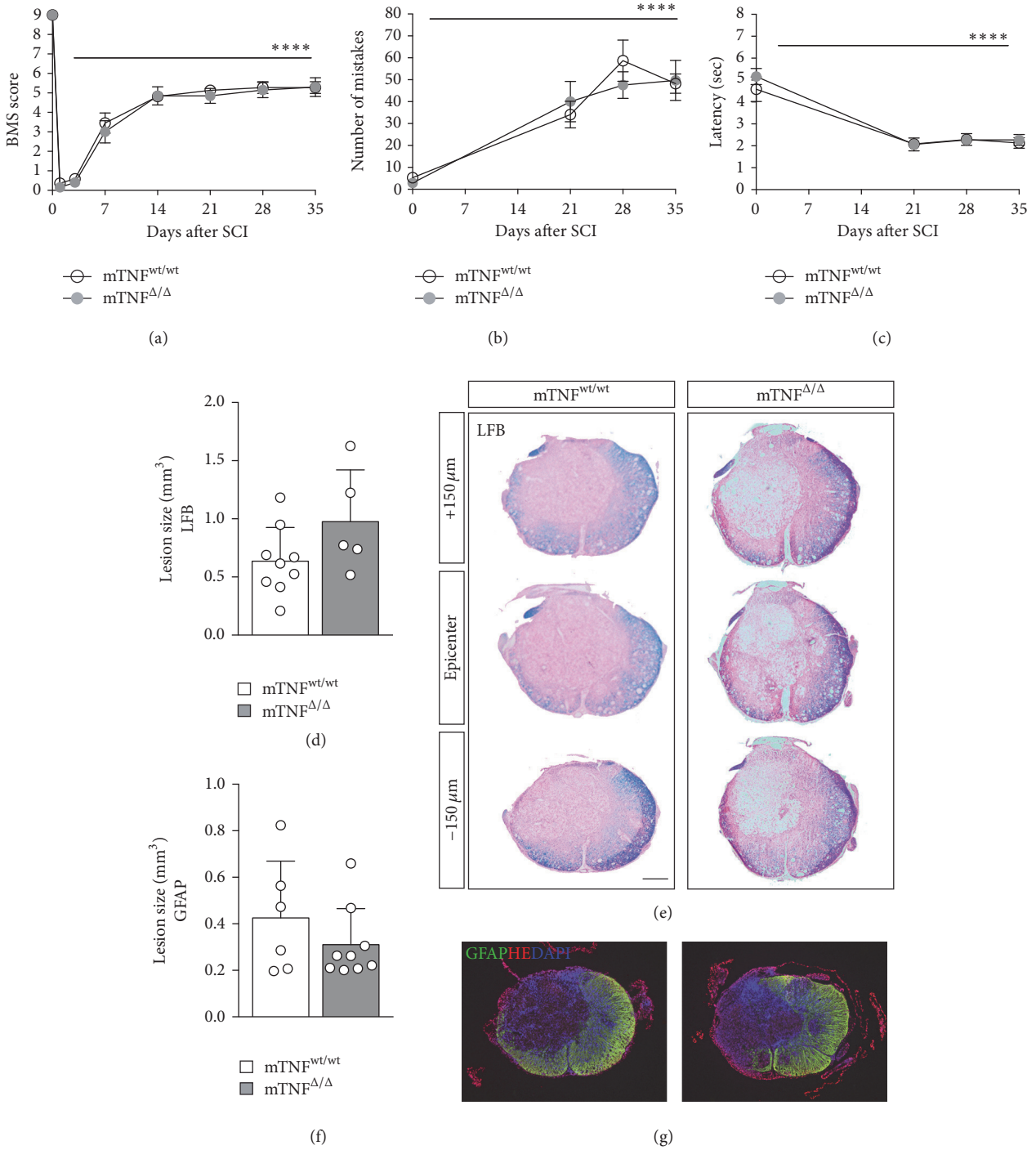
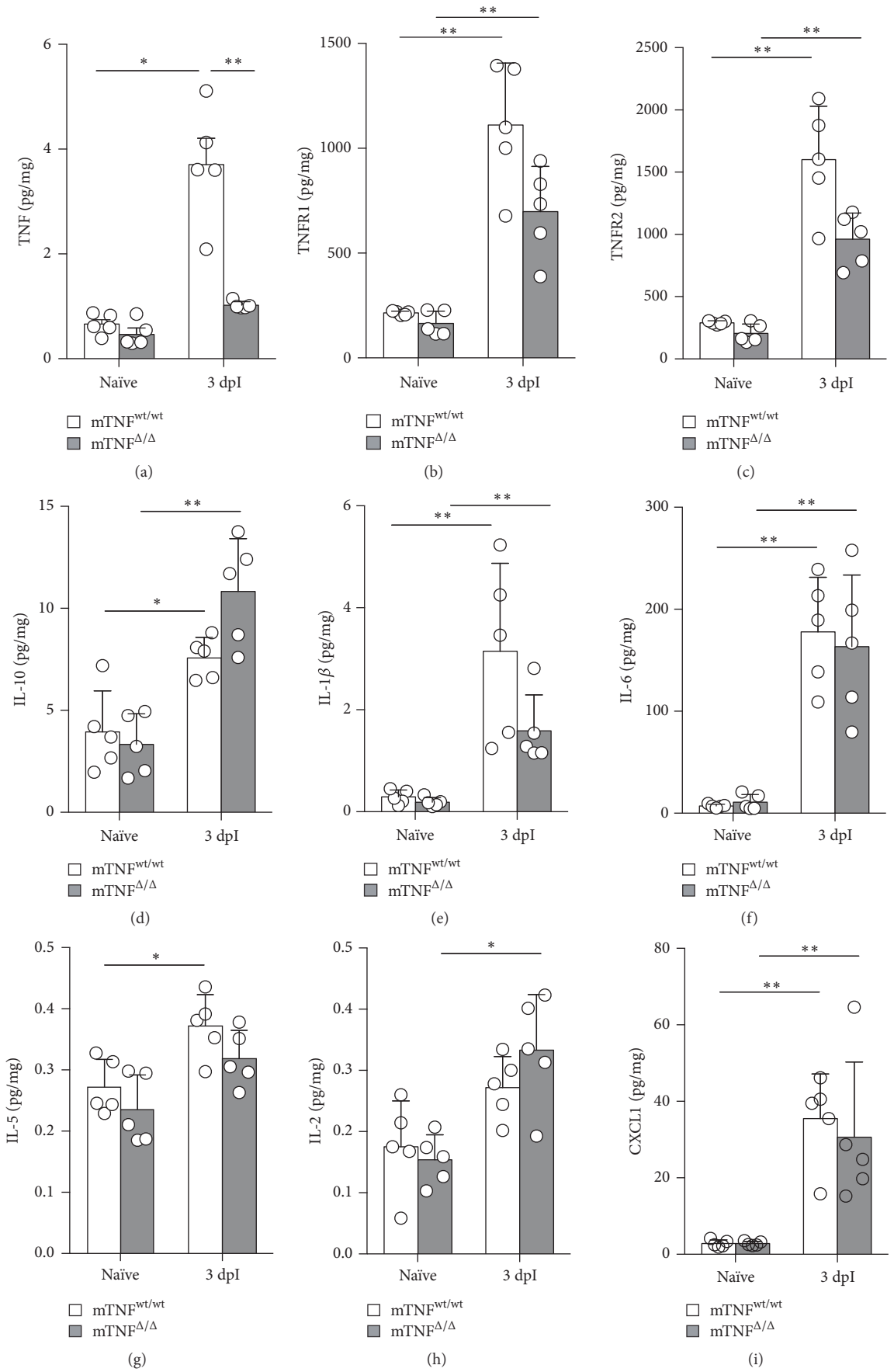


Figure 2: Genetic ablation of solTNF does not affect functional outcome or lesion size after SCI. (a) Analysis of BMS scores in $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice showed that genetic ablation of solTNF did not affect BMS scores after SCI. Both groups of mice improved their BMS score over time (two-way RM ANOVA; time $^{****}p < 0.0001$, $F_{3,133} = 295.1$), $n = 10$-11 mice/group. (b) Rung walk analysis showed that both groups of mice increased their number of mistakes after SCI (two-way RM ANOVA; time $^{****}p < 0.0001$, $F_{3,51} = 27.8$, Tukey's *post hoc* $^{****}p < 0.0001$), $n = 9$-10 mice/group, and no differences between genotypes were observed. (c) Thermal stimulation using Hargreaves' test showed no differences in latency time to withdraw paws between genotypes. Both groups decreased latency to remove their paws over time after SCI (two-way RM ANOVA; time $^{****}p < 0.0001$, $F_{3,51} = 14.72$, Tukey's *post hoc* $^{****}p < 0.0001$), $n = 9$-10 mice/group. (d) Analysis of lesion volumes in Luxol Fast Blue (LFB) stained sections 35 days after SCI showed that lesion size was comparable between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice (Mann–Whitney), $n = 5$–9 mice/group. (e) Representative LFB-stained thoracic spinal cord sections from $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice allowed 35-day survival after SCI. Scale bar: 100 μm. (f) Analysis of lesion volumes in GFAP-stained sections 35 days after SCI showed that lesion size was comparable between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice (Mann–Whitney), $n = 5$–9 mice/group. (g) GFAP labeling (green), fluorescent Nissl (red), and DAPI (blue) staining of representative spinal cord sections from $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice allowed 35-day survival after SCI. Results are presented as mean ± SD.



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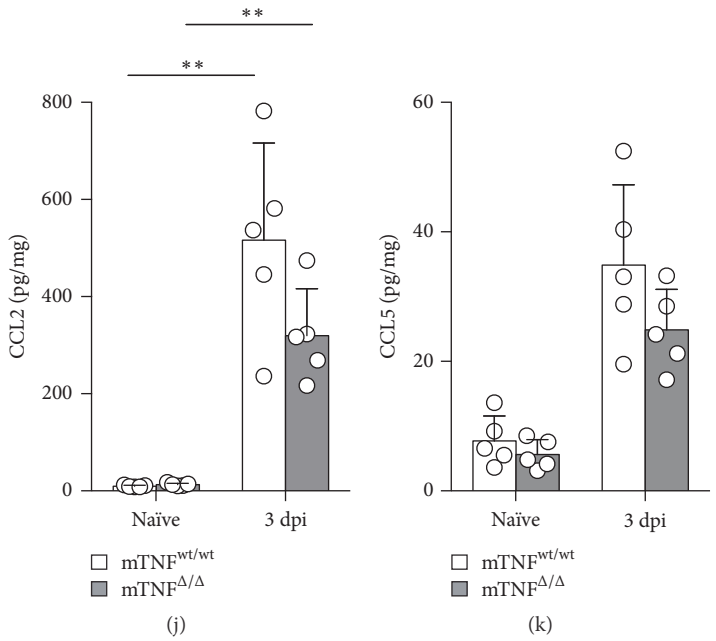


Figure 3: Cytokine and TNFR expression profiling after SCI. (a–k) TNF (a), TNFR1 (b), TNFR2 (c), IL-10 (d), IL-1β (e), IL-6 (f), IL-5 (g), IL-2 (h), CXCL1 (i), CCL2 (j), and CCL5 (k) protein levels were quantified by multiplex technology in naïve mice and 3 days after SCI in $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice. For each protein, results are expressed as mean ± SD, $n = 5$ mice/group. $^{*}p < 0.05$ and $^{**}p < 0.01$, Mann–Whitney test.

3.5. Genetic Ablation of solTNF Does Not Affect Monocyte/Macrophage Infiltration into the Lesioned Spinal Cord 3 Days after SCI. We have previously shown that monocyte/macrophage infiltration into the ischemic brain is decreased in $mTNF^{\Delta/\Delta}$ mice 24 hours after focal cerebral ischemia compared to $mTNF^{wt/wt}$ mice [12]. Based on this, we evaluated microglia ($CD11b^{+}CD45^{dim}$ cells), macrophage ($CD11b^{+}CD45^{high}Gr1^{-}$), granulocyte ($CD11b^{+}CD45^{high}Gr1^{+}$), and T cell ($CD45^{+}CD3^{+}$) populations 3 days after SCI using flow cytometry (Figures 4(a) and 4(b)) and found no differences in total numbers (Figure 4(c)) and percentages (Figure 4(d)) between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice. As CD45 MFI values in microglia have been shown to be affected by XPro1595 and etanercept treatment following experimental stroke, indicating increased activation by these cells [25], we also investigated CD11b and CD45 MFI levels in microglia, macrophages, and granulocytes 3 days after SCI (Figures 4(e) and 4(f)). However, no difference between genotypes was observed in MFI levels for either CD11b (Figure 4(e)) or CD45 (Figure 4(f)) in any of the cell populations investigated.

3.6. Genetic Ablation of solTNF Does Not Affect the Activation State of Microglia and Leukocytes 3 and 35 Days after SCI. In order to further investigate whether genetic ablation of solTNF affected the activation state of microglia and infiltrating cells, we performed flow cytometry and looked for changes in cell populations and MFI of MHCII and CD11c (Figure 5) in the lesion and perilesion areas (proximal and distal to the lesion). The finding of comparable numbers (Figure 5(a)) and percentages (Figure 5(b)) of $CD11b^{+}CD45^{dim}$ microglia and $CD11b^{+}CD45^{high}$ cells supported the finding that solTNF does not affect microglial and leukocyte recruitment following SCI. In order to investigate whether genetic ablation of solTNF affected activation state, $CD11b^{+}CD45^{dim}$ and $CD11b^{+}CD45^{high}$ populations were subgated to investigate MHCII (Figures 5(d)–5(f)) and CD11c (Figures 5(g)–5(i)) expression. Neither CD11c nor MHCII was found on the microglia population at this time point (Figures 5(d) and 5(g)). In contrast, MHCII (Figure 5(d)) and CD11c (Figure 5(g)) expression were increased on $CD11b^{+}CD45^{high}$ cells with comparable numbers (Figures 5(e) and 5(h)) and expression levels (Figures 5(f) and 5(i)) between genotypes. The number of $MHCII^{+}$ and $CD11c^{+}$ cells and the expression levels were significantly increased in the lesion area compared to the perilesion area. CD11c were only found on MHCII expressing $CD11b^{+}CD45^{high}$ cells (Figure 5(j)). In support of these data, the percentages of $MHCII^{+}$, $CD11c^{+}$, and $MHCII^{+}CD11c^{+}$ cells in the lesion area were comparable between genotypes (Figure 5(k)). Finally, Iba1 protein expression was investigated 3 days after SCI using Western blotting analysis (Figure 5(l)). However, we found no difference in the expression levels between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice supporting the flow cytometric analysis, demonstrating that genetic ablation of solTNF does not affect the activation state of microglia and infiltrating cells 3 days after SCI.

We also evaluated microglia/macrophage activation 35 days after SCI by immunostaining of F4/80, CD45, and Iba1. We observed no difference in the distribution or number of $F4/80^{+}$ (Figure 6(a)), $CD45^{+}$ (Figure 6(b)), or $Iba1^{+}$ (Figure 6(c)) cells between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice.

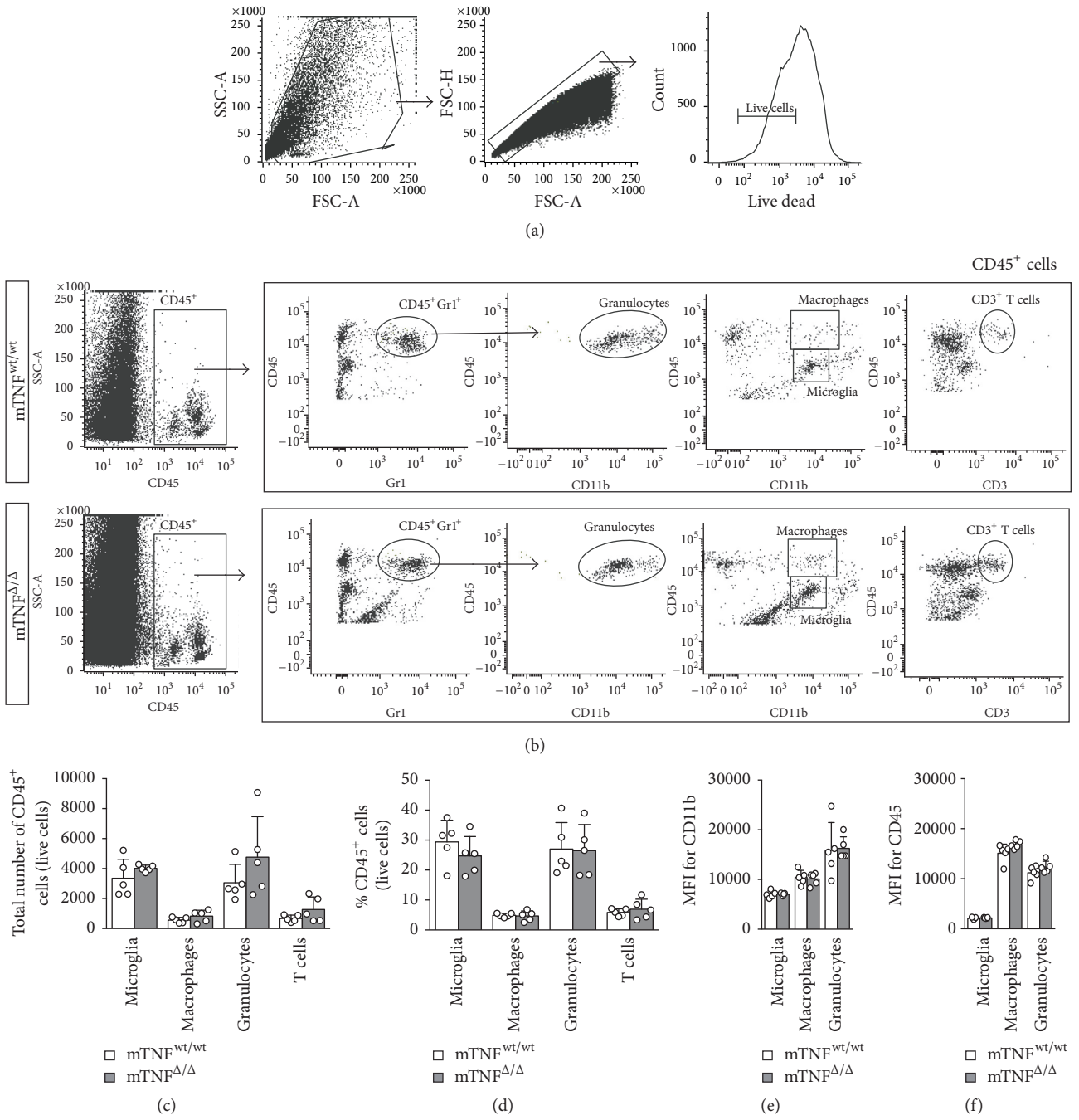
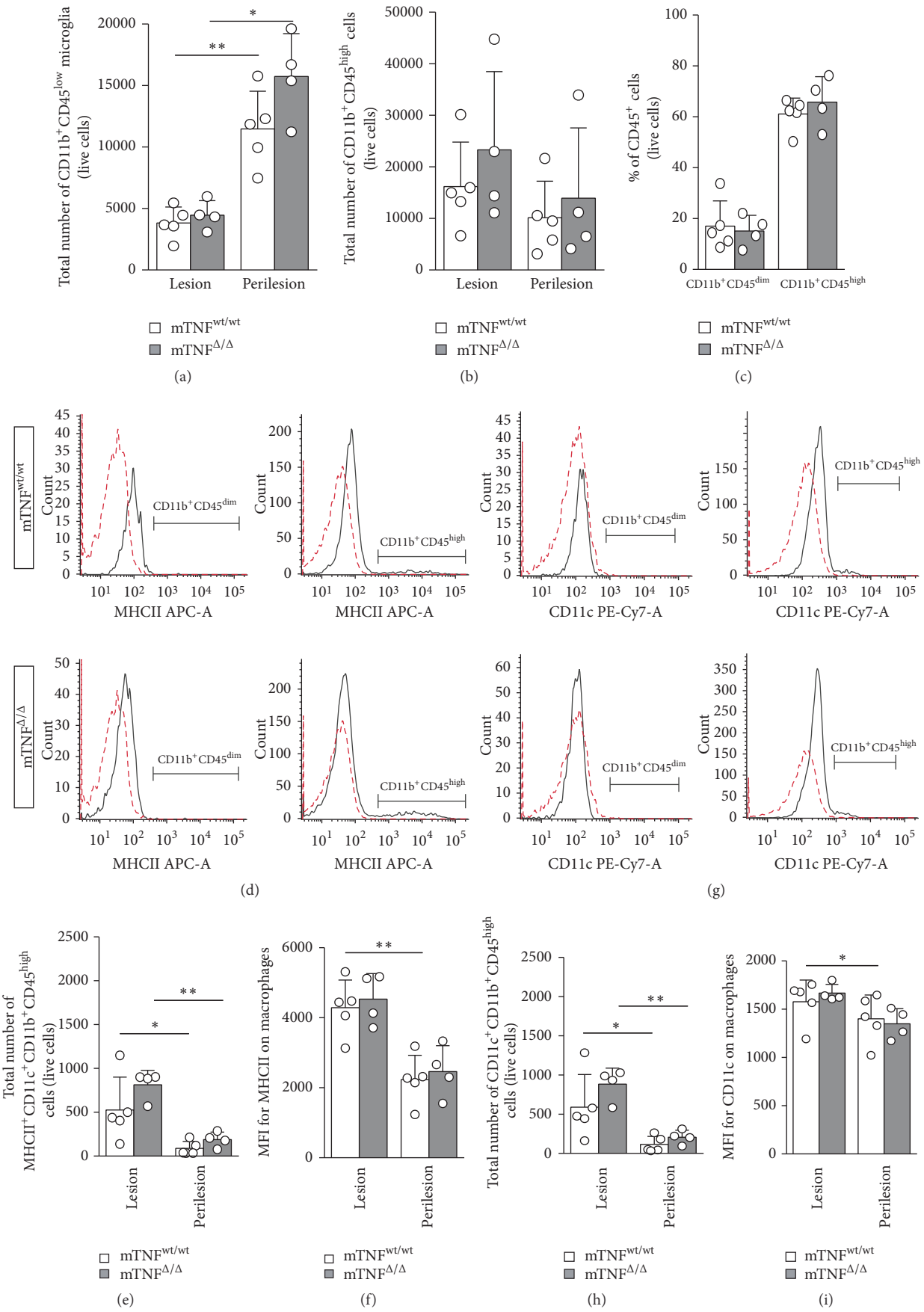


Figure 4: Flow cytometry of microglia and infiltrating leukocytes 3 days after SCI. (a) Gating strategy: FSC/SSC was used to define cell populations. Singlet cells were identified using FSC-A/FSC-H, and only live cells were included. (b) Representative flow cytometry plots comparing macrophage, granulocyte, and T cell infiltration in $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice. (c, d) Number (c) and percentage (d) of microglia ($CD11b^{+}CD45^{dim}$), macrophages ($CD11b^{+}CD45^{dim}Gr1^{-}$), granulocytes ($CD11b^{+}CD45^{dim}Gr1^{+}$), and T cells ($CD45^{+}CD3^{+}$) in $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice 3 days after SCI. Results are expressed as mean ± SD, $n = 5$ mice/group, Mann–Whitney test.

4. Discussion

In the present study, we found that genetic ablation of solTNF, but sustained expression of mTNF, does not affect functional outcome and lesion size after SCI. These findings are in line with previous published papers demonstrating that genetic ablation of TNF [15] and systemic administration of anti-TNF antagonists, such as XPro1595 [16] and etanercept [16, 17], do not affect lesion volume or improve functional outcome after SCI. However, the findings are also in contrast to our own recent studies demonstrating that selective central inhibition of solTNF is neuroprotective, as mice treated epidurally



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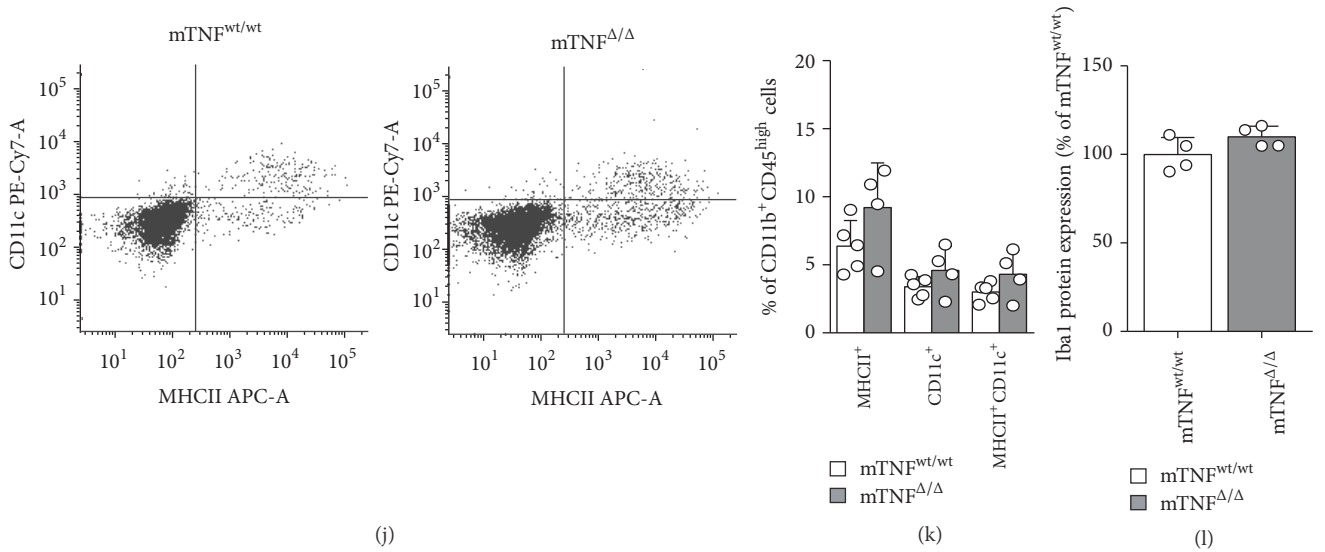

FIGURE 5: Flow cytometric analysis of the activation state of microglia and infiltrating cells 3 days after SCI. (a–c) Number of $CD11b^+CD45^{dim}$ microglia (a) and $CD11b^+CD45^{high}$ cells (b) and percentages of microglia and infiltrating cells (c) within the lesion and in the perilesion area 3 days after SCI. (d) Representative histograms of MHCII expression on $CD11b^+CD45^{low}$ microglia and $CD11b^+CD45^{high}$ cells located in the lesions of $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice 3 days after SCI. (e, f) The number of $MHCII^+CD11b^+CD45^{high}$ cells (e) and MFI for MHCII (f) were comparable between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice. (g) Representative histograms of CD11c expression on microglia and infiltrating cells located in the lesions of $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice 3 days after SCI. (h, i) The number of $CD11c^+CD11b^+CD45^{high}$ cells (h) and MFI for CD11c expression (i) were comparable between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice. (j) Representative dot blots comparing CD11c expressions on $MHCII^+CD11b^+CD45^{high}$ cells in $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice 3 days after SCI. (k) Percentages of $MHCII^+$, $CD11c^+$, and $MHCII^+CD11c^+$ cells in the lesion area. n = 4-5 mice/group. (l) Western blot analysis for Iba1 protein expression 3 days after SCI. Data are normalized to α-actin protein expression (n = 4/group). Results are presented as mean ± SD, $^*p < 0.05$, $^{**}p < 0.01$, Mann–Whitney and Wilcoxon matched-pairs signed rank tests.

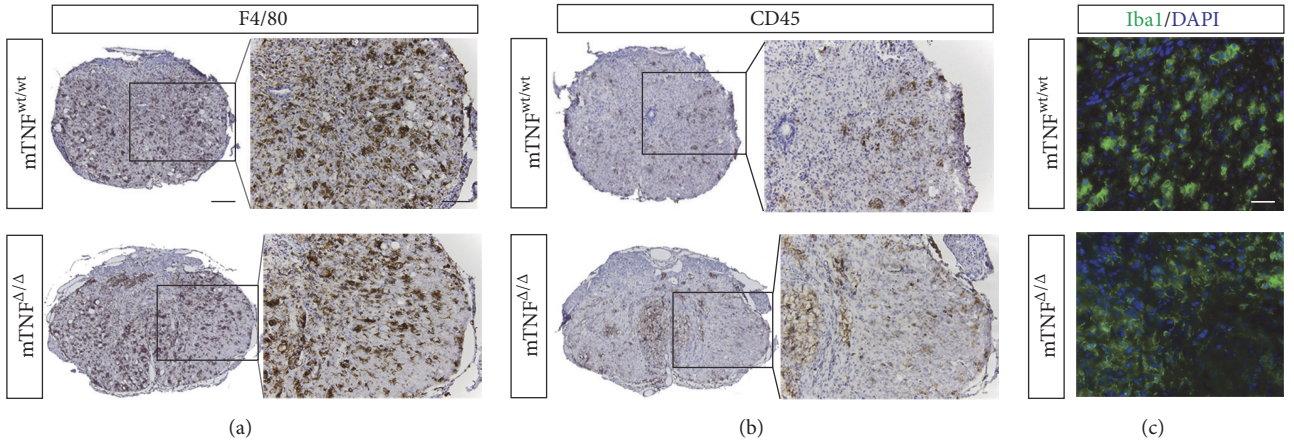

FIGURE 6: Microglial and leukocyte immunoreactivity 35 days after SCI. (a–c) Immunohistochemical staining for F4/80 (a) and CD45 (b) and immunofluorescent staining for Iba1 (c) were comparable between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice 35 days after SCI. Analysis was based on 5 sections from each animal, n = 4 mice/group. Scale bars: (a, b) low magnification = 200 μm and high magnification = 100 μm. (c) 40 μm.

with XPro1595 for 3 consecutive days displayed improved functional outcome, reduced lesion size, and altered neuroinflammation after moderate SCI [16]. As TNF reaches peak levels within the lesioned spinal cord within the first 1-2 hours after SCI [1–4] and elevation of solTNF is a hallmark of acute neuroinflammation (reviewed in [26]) it is possible that, by inhibiting solTNF, using XPro1595 for only 3 consecutive days is neuroprotective, whereas inhibiting solTNF for a sustained period of time (every 3 days for 35 days or as in $TNF^{-/-}$ and $mTNF^{\Delta/\Delta}$ mice) is not. This is supported by recent hypotheses that under normal physiological conditions solTNF signaling is important for synaptic scaling (reviewed in [26]) and therefore possibly also for neuroregeneration. In the present study, we observed a

tendency towards reduced TNFR1 and TNFR2 expression in the spinal cord of $mTNF^{\Delta/\Delta}$ mice 3 days after SCI, probably as a consequence of reduced solTNF levels. As pharmacological blockage of solTNF, using XPro1595 resulted in sustained TNFR2 and increased TLR4 expression in the lesioned cord, when administered epidurally [16], it is possible that, in order to obtain improved functional recovery and reduced injury following SCI, sustained or increased TNFR2 expression locally is a prerequisite. This is supported by studies in mice with experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, where mTNF has been associated with repair and remyelination via oligodendroglial TNFR2 [27], whereas the detrimental effects of solTNF have been associated with TNFR1 signaling [8, 28]. Finally, it is also possible that genetic modifications of TNF expression, as in $TNF^{-/-}$ and $mTNF^{\Delta/\Delta}$ mice, result in unknown phenotypical modifications of the mice that may not be apparent at first glance. Even though the behavioral phenotype did not appear to be altered in $mTNF^{\Delta/\Delta}$ mice this is the case in $TNF^{-/-}$ mice [29, 30] and discrepancies in study outcomes between genetically modified TNF models and studies using anti-TNF therapies have been encountered in other CNS models, including models for focal cerebral ischemia and multiple sclerosis (reviewed in [8, 31]). The explanation for these discrepancies so far remains unknown and requires further research into the different functional roles of solTNF and mTNF and the importance of signaling through TNFR1 versus TNFR2.

We also observed a tendency towards a reduction in CCL2 expression in $mTNF^{\Delta/\Delta}$ mice 3 days after SCI. CCL2 is a monocyte chemotactic protein and an important regulator of macrophage responses [32]. Its expression is upregulated in rat spinal cord tissue within the first 24 hours after SCI [33], as well as in serum samples from SCI patients [34]. Despite our previous findings of a reduced infiltration of monocytes/macrophages into the ischemic brain in our $mTNF^{\Delta/\Delta}$ mice, we did not observe a difference in infiltrating macrophages 3 days after SCI between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice. We also did not observe any differences in the activation state of microglia or infiltrating cells, as MFI levels for CD11b, CD45, MHCII, and CD11c and Iba1 protein expression were comparable between genotypes 3 days after SCI. Macrophage infiltration and microglial activation are known to peak around day 7 after SCI [35]; hence possible changes in microglia/macrophages are believed to take place at a later time after SCI, which may explain why we in the present study did not observe any change in the number of infiltrating macrophages. The finding that CXCL1, a neutrophil chemoattractant chemokine, did not differ between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice is in line with previous data showing that neutrophils, which are known to infiltrate early (peaking 1 day after injury) [35], were comparable between $mTNF^{wt/wt}$ and $mTNF^{\Delta/\Delta}$ mice.

The expression of IL-1β, a proinflammatory cytokine, increases within the first couple of hours after SCI [4] before the appearance of infiltrating lymphocytes and leukocytes [36], and direct injection of IL-1β into the spinal cord enhances vascular permeability and lymphocyte recruitment [37]. In the present study, there was a tendency towards reduced IL-1β expression in $mTNF^{\Delta/\Delta}$ mice 3 days after SCI. In IL-1$\alpha/\beta^{-/-}$ mice, locomotor activity and lesion area improved significantly after SCI, a process believed to be mediated by reduction of inflammatory responses, including a decrease in TNF expression [38]. We have previously shown that IL-1β protein levels are decreased in $mTNF^{\Delta/\Delta}$ mice following experimental stroke, which was accompanied by reduced infarct volumes [12], and that, by increasing the expression of the naturally occurring IL-1 receptor antagonist (IL-1ra) in microglia, infarct volumes following experimental stroke can be reduced [39]. As the neurotoxic effect of IL-1β, and hence its deleterious effect on lesion volume, is dependent on the balance between IL-1 and IL-1ra after acute injury to the CNS [39], it is possible that in our conditions a sufficient increase in IL-1ra does not occur, and therefore the decrease in IL-1β expression in our $mTNF^{\Delta/\Delta}$ mice is not sufficient to improve functional recovery and reduce lesion size.

IL-10 is a potent anti-inflammatory cytokine, which has been shown to reduce the development of inflammation and tissue injury associated with SCI [40]. IL-10 has also been shown to reduce IL-1β [41] and TNF [42] in rat models of SCI, leading to reduced inflammation, reduced neuronal damage, and improved functional recovery. In our study, we observed a tendency towards reduced IL-10 levels, while TNF and IL-1β were decreased, with no improvement in functional outcome 35 days after SCI. As the studies performed by Bethea et al. [42] and Plunkett et al. [41] were in rats and ours was in mice, it is possible that species differences account for the discrepancy. Our studies are in line with the findings by Abraham et al. [43] that showed greater damage at early time points (1 and 7 days) after SCI in $IL\text{-}10^{-/-}$ mice but no differences at 14 days after SCI. The pronounced early damage observed in $IL\text{-}10^{-/-}$ mice was associated with an almost twofold increase in peripheral neutrophils [43], suggesting an altered innate immune response to injury. Despite the findings of increased IL-10 3 days after SCI, we did not see any difference in infiltrating macrophages and neutrophils. At 35 days after SCI we did not detect differences in $CD45^+$, $F4/80^+$, or $Iba1^+$ microglia/macrophages. However, it should be noted that these observations were based on qualitative immunohistochemistry and further quantitative analysis should be performed in order to conclude whether leukocyte infiltration is affected by genetic ablation of solTNF following SCI.

In summary, our study, using genetically modified mice expressing only the membrane-bound form of TNF, demonstrate that the absence of solTNF does not affect lesion size and functional outcome but suggests that mTNF promotes an anti-inflammatory environment in the lesioned spinal cord that may be more favorable to functional recovery.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Ditte Gry Ellman and Kate Lykke Lambertsen conceived the studies, designed experiments, performed statistical analysis, and wrote the paper. Ditte Gry Ellman, Matilda Degn, Minna Christiansen Lund, Bettina Hjelm Clausen, Hans Gram Novrup, Simon Bertram Flæng, Louise Helskov Jørgensen, Lujitha Suntharalingam, Åsa Fex Svenningsen, and Roberta Brambilla performed experiments and analyzed and interpreted data. All authors read and approved the final manuscript. Matilda Degn, Minna Christiansen Lund, and Bettina Hjelm Clausen contributed equally.

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References

[1] C. X. Wang, B. Nuttin, H. Heremans, R. Dom, and J. Gybels, "Production of tumor necrosis factor in spinal cord following traumatic injury in rats," *Journal of Neuroimmunology*, vol. 69, no. 1-2, pp. 151–156, 1996.

[2] W. J. Streit, S. L. Semple-Rowland, S. D. Hurley, R. C. Miller, P. G. Popovich, and B. T. Stokes, "Cytokine mRNA profiles in contused spinal cord and axotomized facial nucleus suggest a beneficial role for inflammation and gliosis," *Experimental Neurology*, vol. 152, no. 1, pp. 74–87, 1998.

[3] I. Pineau and S. Lacroix, "Proinflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression pattern and identification of the cell types involved," *Journal of Comparative Neurology*, vol. 500, no. 2, pp. 267–285, 2007.

[4] J. Z. Pan, L. Ni, A. Sodhi, A. Aguanno, W. Young, and R. P. Hart, "Cytokine activity contributes to induction of inflammatory cytokine mRNAs in spinal cord following contusion," *Journal of Neuroscience Research*, vol. 68, no. 3, pp. 315–322, 2002.

[5] A. L. Davies, K. C. Hayes, and G. A. Dekaban, "Clinical correlates of elevated serum concentrations of cytokines and autoantibodies in patients with spinal cord injury," *Archives of Physical Medicine and Rehabilitation*, vol. 88, no. 11, pp. 1384–1393, 2007.

[6] B. Biglari, T. Swing, C. Child et al., "A pilot study on temporal changes in IL-1β and TNF-α serum levels after spinal cord injury: the serum level of TNF-α in acute SCI patients as a possible marker for neurological remission," *Spinal Cord*, vol. 53, no. 7, pp. 510–514, 2015.

[7] E. Esposito and S. Cuzzocrea, "Anti-TNF therapy in the injured spinal cord," *Trends in Pharmacological Sciences*, vol. 32, no. 2, pp. 107–115, 2011.

[8] L. Probert, "TNF and its receptors in the CNS: the essential, the desirable and the deleterious effects," *Neuroscience*, vol. 302, pp. 2–22, 2015.

[9] E. Taoufik, E. Petit, D. Divoux et al., "TNF receptor I sensitizes neurons to erythropoietin- and VEGF-mediated neuroprotection after ischemic and excitotoxic injury," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 16, pp. 6185–6190, 2008.

[10] K. L. Lambertsen, B. H. Clausen, A. A. Babcock et al., "Microglia protect neurons against ischemia by synthesis of tumor necrosis factor," *The Journal of Neuroscience*, vol. 29, no. 5, pp. 1319–1330, 2009.

[11] B. H. Clausen, M. Degn, M. Sivasaravanaparan et al., "Conditional ablation of myeloid TNF increases lesion volume after experimental stroke in mice, possibly via altered ERK1/2 signaling," *Scientific Reports*, vol. 6, p. 29291, 2016.

[12] P. M. Madsen, B. H. Clausen, M. Degn et al., "Genetic ablation of soluble tumor necrosis factor with preservation of membrane tumor necrosis factor is associated with neuroprotection after focal cerebral ischemia," *Journal of Cerebral Blood Flow & Metabolism*, vol. 36, no. 9, pp. 1553–1569, 2016.

[13] G.-M. Kim, J. Xu, J. Xu et al., "Tumor necrosis factor receptor deletion reduces nuclear factor-kappaB activation, cellular inhibitor of apoptosis protein 2 expression, and functional recovery after traumatic spinal cord injury," *The Journal of Neuroscience*, vol. 21, no. 17, pp. 6617–6625, 2001.

[14] T. Genovese, E. Mazzon, C. Crisafulli et al., "TNF-α blockage in a mouse model of SCI: evidence for improved outcome," *Shock*, vol. 29, no. 1, pp. 32–41, 2008.

[15] M. Farooque, J. Isaksson, and Y. Olsson, "Improved recovery after spinal cord injury in neuronal nitric oxide synthase-deficient mice but not in TNF-α-deficient mice," *Journal of Neurotrauma*, vol. 18, no. 1, pp. 105–114, 2001.

[16] H. G. Novrup, V. Bracchi-Ricard, D. G. Ellman et al., "Central but not systemic administration of XPro1595 is therapeutic following moderate spinal cord injury in mice," *Journal of Neuroinflammation*, vol. 11, no. 1, article 159, 2014.

[17] T. Genovese, E. Mazzon, C. Crisafulli et al., "Combination of dexamethasone and etanercept reduces secondary damage in experimental spinal cord trauma," *Neuroscience*, vol. 150, no. 1, pp. 168–181, 2007.

[18] P. M. Vidal, E. Lemmens, L. Geboes, T. Vangansewinkel, S. Nelissen, and S. Hendrix, "Late blocking of peripheral TNF-α is ineffective after spinal cord injury in mice," *Immunobiology*, vol. 218, no. 2, pp. 281–284, 2013.

[19] K.-B. Chen, K. Uchida, H. Nakajima et al., "Tumor necrosis factor-α antagonist reduces apoptosis of neurons and oligodendroglia in rat spinal cord injury," *Spine*, vol. 36, no. 17, pp. 1350–1358, 2011.

[20] M. Dinomais, L. Stana, G. Egon, I. Richard, and P. Menei, "Significant recovery of motor function in a patient with complete T7 paraplegia receiving etanercept," *Journal of Rehabilitation Medicine*, vol. 41, no. 4, pp. 286–288, 2009.

[21] S. R. Ruuls, R. M. Hoek, V. N. Ngo et al., "Membrane-bound TNF supports secondary lymphoid organ structure but is subservient to secreted TNF in driving autoimmune inflammation," *Immunity*, vol. 15, no. 4, pp. 533–543, 2001.

[22] D. Torres, L. Janot, V. F. J. Quesniaux et al., "Membrane tumor necrosis factor confers partial protection to Listeria infection," *American Journal of Pathology*, vol. 167, no. 6, pp. 1677–1687, 2005.

[23] D. M. Basso, L. C. Fisher, A. J. Anderson, L. B. Jakeman, D. M. McTigue, and P. G. Popovich, "Basso mouse scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains," *Journal of Neurotrauma*, vol. 23, no. 5, pp. 635–659, 2006.

[24] K. L. Lambertsen, J. B. Gramsbergen, M. Sivasaravanaparan et al., "Genetic KCa3.1-deficiency produces locomotor hyperactivity and alterations in cerebral monoamine levels," *PLoS ONE*, vol. 7, no. 10, Article ID e47744, 2012.

[25] B. H. Clausen, M. Degn, N. A. Martin et al., "Systemically administered anti-TNF therapy ameliorates functional outcomes after focal cerebral ischemia," *Journal of Neuroinflammation*, vol. 11, no. 1, article 203, 2014.

[26] M. K. McCoy and M. G. Tansey, "TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease," *Journal of Neuroinflammation*, vol. 5, article 45, 2008.

[27] P. M. Madsen, D. Motti, S. Karmally et al., "Oligodendroglial TNFR2 mediates membrane TNF-dependent repair in experimental autoimmune encephalomyelitis by promoting oligodendrocyte differentiation and remyelination," *Journal of Neuroscience*, vol. 36, no. 18, pp. 5128–5143, 2016.

[28] R. Brambilla, J. J. Ashbaugh, R. Magliozzi et al., "Inhibition of soluble tumour necrosis factor is therapeutic in experimental autoimmune encephalomyelitis and promotes axon preservation and remyelination," *Brain*, vol. 134, no. 9, pp. 2736–2754, 2011.

[29] B. T. Baune, F. Wiede, A. Braun, J. Golledge, V. Arolt, and H. Koerner, "Cognitive dysfunction in mice deficient for TNF- and its receptors," *American Journal of Medical Genetics, Part B: Neuropsychiatric Genetics*, vol. 147, no. 7, pp. 1056–1064, 2008.

[30] H. Golan, T. Levav, and M. Huleihel, "Distinct expression and distribution of vesicular proteins in the hippocampus of TNFa-deficient mice during development," *Synapse*, vol. 53, no. 1, pp. 6–10, 2004.

[31] K. L. Lambertsen, K. Biber, and B. Finsen, "Inflammatory cytokines in experimental and human stroke," *Journal of Cerebral Blood Flow & Metabolism*, vol. 32, no. 9, pp. 1677–1698, 2012.

[32] F. E. Perrin, S. Lacroix, M. Avilés-Trieueros, and S. David, "Involvement of monocyte chemoattractant protein-1, macrophage inflammatory protein-1α and interleukin-1β Wallerian degeneration," *Brain*, vol. 128, no. 4, pp. 854–866, 2005.

[33] D. M. McTigue, M. Tani, K. Krivacic et al., "Selective chemokine mRNA accumulation in the rat spinal cord after contusion injury," *Journal of Neuroscience Research*, vol. 53, no. 3, pp. 368–376, 1998.

[34] S. Q. Liu, Y. G. Ma, H. Peng, and L. Fan, "Monocyte chemoattractant protein-1 level in serum of patients with acute spinal cord injury," *Chinese Journal of Traumatology*, vol. 8, no. 4, pp. 216–219, 2005.

[35] K. D. Beck, H. X. Nguyen, M. D. Galvan, D. L. Salazar, T. M. Woodruff, and A. J. Anderson, "Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment," *Brain*, vol. 133, no. 2, pp. 433–447, 2010.

[36] D. Bartholdi and M. E. Schwab, "Expression of pro-inflammatory cytokine and chemokine mRNA upon experimental spinal cord injury in mouse: an in situ hybridization study," *European Journal of Neuroscience*, vol. 9, no. 7, pp. 1422–1438, 1997.

[37] L. Schnell, S. Fearn, M. E. Schwab, V. H. Perry, and D. C. Anthony, "Cytokine-induced acute inflammation in the brain and spinal cord," *Journal of Neuropathology & Experimental Neurology*, vol. 58, no. 3, pp. 245–254, 1999.

[38] A. Sato, H. Ohtaki, T. Tsumuraya et al., "Interleukin-1 participates in the classical and alternative activation of microglia/macrophages after spinal cord injury," *Journal of Neuroinflammation*, vol. 9, article 65, 2012.

[39] B. H. Clausen, K. L. Lambertsen, F. Dagnæs-Hansen et al., "Cell therapy centered on IL-1Ra is neuroprotective in experimental stroke," *Acta Neuropathologica*, vol. 131, no. 5, pp. 775–791, 2016.

[40] T. Genovese, E. Esposito, E. Mazzon et al., "Absence of endogenous interleukin-10 enhances secondary inflammatory process after spinal cord compression injury in mice," *Journal of Neurochemistry*, vol. 108, no. 6, pp. 1360–1372, 2009.

[41] J. A. Plunkett, C.-G. Yu, J. M. Easton, J. R. Bethea, and R. P. Yezierski, "Effects of interleukin-10 (IL-10) on pain behavior and gene expression following excitotoxic spinal cord injury in the rat," *Experimental Neurology*, vol. 168, no. 1, pp. 144–154, 2001.

[42] J. R. Bethea, H. Nagashima, M. C. Acosta et al., "Systemically administered interleukin-10 reduces tumor necrosis factor-alpha production and significantly improves functional recovery following traumatic spinal cord injury in rats," *Journal of Neurotrauma*, vol. 16, no. 10, pp. 851–863, 1999.

[43] K. E. Abraham, D. McMillen, and K. L. Brewer, "The effects of endogenous interleukin-10 on gray matter damage and the development of pain behaviors following excitotoxic spinal cord injury in the mouse," *Neuroscience*, vol. 124, no. 4, pp. 945–952, 2004.