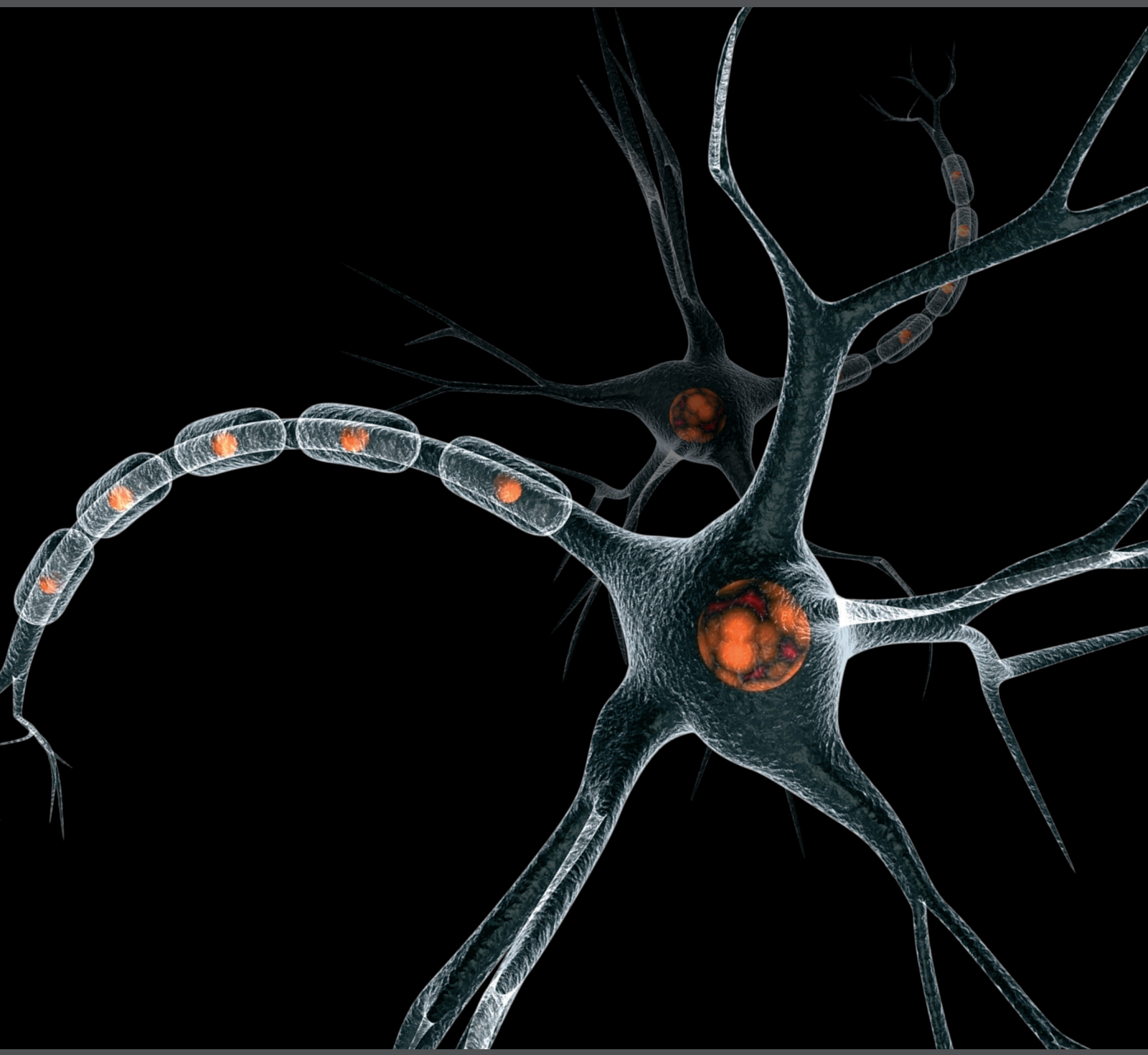
Neural Plasticity

Microglia in Neuronal Circuits

Guest Editors: Long-Jun Wu, Beth Stevens, Shumin Duan, and Brian A. MacVicar



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Editorial Board

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Editorial

Microglia in Neuronal Circuits

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1. Introduction

Microglia comprise a unique subset of glial cells as the principal brain immune cells and are actively engaged in physiological and pathological brain functions. Unlike other resident neural cells that are of neuroectodermal origin, microglia are of mesodermal origin and invade the neuroepithelium at early embryonic stages. As resident immune response cells, microglia are extremely sensitive to almost any brain disturbance. Therefore, microglia are traditionally recognized for their immune functions during acute brain injury, such as bacterial meningitis, ischemic stroke, and spinal cord injury, as well as chronic neurological disorders, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and neuropathic pain. Recently, the role of microglia in neurodevelopment and neural plasticity in the healthy brains has gained tremendous attention. These exciting results raise an intriguing possibility that microglia can integrate into the neuronal circuits in the healthy and diseased brain.

In support of this notion, it is emerging that microglia have remarkably dynamic processes and are frequently interacting with neurons and synaptic elements. Through these interactions, microglia may monitor neuronal/synaptic activities and thus survey the microenvironment in the brain. Indeed, recent studies have apparently shown that microglia function in neuronal circuits by playing diverse roles in neural development, behavior, and pathology in the brain. Therefore, microglia research has changed the way we think about neuronal network/plasticity and increased our understanding of brain diseases associated with abnormal microglia. Contributions to this special issue provide a snapshot of microglial function in the healthy and diseased brain and propose a fundamental role of microglia in neuronal circuits.

2. Microglia in the Healthy Brain

The vivid observation of microglia in the healthy brain through *in vivo* imaging in 2005 was a breakthrough in microglia research. For the first time, researchers witnessed that microglia are extremely motile and their processes are constantly monitoring the microenvironment without any pathological insults. Subsequently, studies were booming to focus on the potential role of microglia in the healthy brain, including synaptic pruning in the development and regulation of synaptic transmission/plasticity. On the other hand, several lines of evidence have also indicated the neuronal control of microglial activities under physiological conditions. In this special issue, U. B. Eyo and L. J. Wu highlight recent findings on this bidirectional interaction between neurons and microglia. The review summarized how microglia signal to neurons through direct physical contact or signaling molecules such as fractalkine, compliment, and DAP12, as well as how microglial activity is modulated by neuronal signals including classic neurotransmitters and chemotactic signals. In addition, the authors discussed studies of microglial depletion as an approach to understand microglial importance in neuronal development, function, and maintenance. This review on bi-directional microglial-neuronal communication provides an overview of how microglia are integrated into neuronal circuits in the healthy brain.

Recent studies have revealed a surprising role of microglia in the structural remodeling of neuronal circuits by using their immune abilities in the healthy brain. For example, microglia were demonstrated to eliminate neuronal precursors, synaptic elements, and newborn cells during adult neurogenesis. In this special issue, Z. Šišková and M.-E. Tremblay further zoom in on the microglial function in the neuronal circuits and review recent studies on the microglia-synapse interactions in the mature healthy brain. The focused review discusses the emerging roles of activity-dependent microglial elimination of synaptic elements (dendritic spines and axon terminals) notably by phagocytosis. This microglia-synapse interaction enables synaptic pruning and thus might be crucial for the experience-dependent remodeling of neuronal circuits in the mature brain as well as during normal aging.

In addition to structural remodeling, microglia are able to modulate synaptic activities and plasticity. Evidence from imaging, cellular, and electrophysiological approaches indicates that microglia affect synaptic maturation during development as well as the acute and dynamic regulation of neuronal activity in the mature healthy brain. In this special issue, S. E. Tsirka and colleagues review the recent studies on microglia as an active player in the regulation of synaptic activities and suggest that microglia are an important contributor to the potential quad-partite synapse. The review summarized some interesting mechanisms underlying microglial regulation of synaptic activities and synaptic numbers: the proteases secreted from microglia to remodel extracellular matrix, the release of microvesicles (shed vesicles or ectosomes) derived from microglia, and connexins and large pore channels as a way by which microglia interact directly with neurons. A plethora of potential messengers mediate the communication between microglia and neurons, including cytokines, purines, glutamate, prostaglandins, and nitric oxide. In this special issue, F. Ferrini and Y. De Koninck particularly discuss a unique microglial signaling molecule, brain-derived neurotrophic factor (BDNF), in controlling neuronal excitability in both physiological and pathological conditions.

3. Microglia in the Diseased Brain

Resting microglia rapidly transform into an activated state in most pathological processes, including host defense against infectious organisms, autoimmune inflammation, ischemia, trauma, chronic pain, and neurodegeneration. Activation of microglia is accompanied by changes in morphology, upregulation of immune surface antigens, production of cytotoxic or neurotrophic molecules, and phagocytosis of pathogens, degenerating cells, and inflammatory debris. Although microglial activation is well documented in a variety of neurological disorders, the definitive beneficial or detrimental roles of microglia in these diseases remain controversial. The consensus is that microglia play different roles based on the temporal and spatial context of brain diseases; the proinflammatory cytotoxic aspects of activated microglia might be important at an early stage while microglia's anti-inflammatory effects become more prominent later during tissue repair. Nevertheless, microglia evidently respond and even cause the abnormality of neuronal circuits under pathological conditions.

Neuronal cell death, loss of synapses, and neuroinflammation are hallmarks and emerged as a major correlate of cognitive decline in neurodegenerative disorders. In this special issue, Z. Šišková and M.-E. Tremblay extend the discussion of microglia-synapse interaction to the context of neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and prion diseases. Chronic microglial activation under these pathological conditions likely contributes to synaptic dysfunction and elimination, thereby exacerbating neurodegeneration. Richardson and Hossain specifically review recent studies on the role of microglia in Parkinson's disease. Activated microglia and subsequent neuroinflammation have been consistently associated with the pathogenesis of Parkinson's disease. Therefore, the authors discuss the potential of targeting microglia to reduce neuroinflammation, with particular focus on microglial ion channels as novel therapeutic targets for neuroprotection in Parkinson's disease.

The physiology of microglia in the spinal cord is less well studied; however, there is strong evidence of spinal cord microglia in the genesis of chronic pain. In this special issue, R.-R. Ji and colleagues discuss the microglial activation through the mitogen-activated kinase pathways, as well as microglial mediators (tumor necrosis factor-alpha, interleukin-1 beta, and BDNF) in regulating synaptic plasticity of pain circuits in the spinal cord in neuropathic pain. Ferrini and De Koninck focus specifically on microglial BDNF in multiple neurological conditions, including epilepsy, drug addiction, spinal cord injury, and neuropathic pain. In particular, microglial BDNF in the spinal cord is well established in neuronal disinhibition in neuropathic pain in the following signaling cascade: the BDNF activation of neuronal TrkB receptor, downregulation of the K^+-Cl^- cotransporter KCC2, disruption of Cl^- homeostasis, and hence the reduced strength of $GABA_A$- and glycine receptor-mediated inhibition. Spinal cord injury triggers inflammation with activation of innate immune responses, where both microglia and macrophages are activated and accumulated. In this special issue, Y. Ren and W. Young review the beneficial mechanisms of macrophages on spinal cord injury by inhibition of proinflammatory responses, stimulation of angiogenesis, secretion of neurotrophic factors, and clearance of myelin debris in the injured spinal cord, providing a rationale of macrophage-based therapies for spinal cord injury. Therefore, insights into the communication between microglia/microphages and neurons in the spinal cord will not only further our understanding of microglia function in neuronal network but may also lead to novel therapeutics for ameliorating a wide array of neural dysfunctions, including chronic pain and spinal cord injury.

4. Concluding Remarks

This special issue summarizes a broad range of topics on microglia in neuronal circuits in both the healthy and diseased brains, with particular emphasis on bidirectional microglia-neuron communication, microglial remodeling

of synapse, microglial regulation of synaptic activities, microglial BDNF signaling, microglia in neurodegeneration such as Parkinson's disease, spinal microglia in neuropathic pain, and macrophages in the spinal cord injury. In spite of the controversy, it is clear that microglia are important and in need of further study in the central nervous system. We hope that papers published in this special issue will serve to increase the scientific knowledge on microglial function in the brain and offer new perspectives on the potential therapeutics targeting microglia/macrophages in various neurological disorders. The past few years have witnessed many important discoveries in the microglia field; however, there is still a long road ahead for exploring the mechanisms underlying microglial function in neuronal circuits at both the molecular and system levels.

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Review Article

Microglia and Synapse: Interactions in Health and Neurodegeneration

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A series of discoveries spanning for the last few years has challenged our view of microglial function, the main form of immune defense in the brain. The surveillance of neuronal circuits executed by each microglial cell overseeing its territory occurs in the form of regular, dynamic interactions. Microglial contacts with individual neuronal compartments, such as dendritic spines and axonal terminals, ensure that redundant or dysfunctional elements are recognized and eliminated from the brain. Microglia take on a new shape that is large and amoeboid when a threat to brain integrity is detected. In this defensive form, they migrate to the endangered sites, where they help to minimize the extent of the brain insult. However, in neurodegenerative diseases that are associated with misfolding and aggregation of synaptic proteins, these vital defensive functions appear to be compromised. Many microglial functions, such as phagocytosis, might be overwhelmed during exposure to the abnormal levels of misfolded proteins in their proximity. This might prevent them from attending to their normal duties, such as the stripping of degenerating synaptic terminals, before neuronal function is irreparably impaired. In these conditions microglia become chronically activated and appear to take on new, destructive roles by direct or indirect inflammatory attack.

1. Physiological Conditions

Microglial cells derive from primitive myeloid progenitors (which originate from the yolk sac) invading the central nervous system (CNS) during embryonic development. As a consequence, they are the only immune cells that permanently reside in the CNS [1–3]. In the healthy CNS, microglia occupy minimally overlapping territories in which they continuously survey their environment by structurally remodeling their ramified processes on a time scale of minutes [4–8]. These surveillant microglia can respond rapidly to any pathological stimulus resulting from injury or disease by transforming their morphology and functional behavior [9–12]. Traditionally, these changes in the microglial phenotype are referred to as *microglial activation*. Activated microglia have the capacity to proliferate, migrate, and release reactive oxygen species, neurotoxins, and proinflammatory and anti-inflammatory cytokines. These activated microglia can secrete trophic factors, present antigens to T cells, and phagocytose pathogens, degenerating cells, and inflammatory debris [9–11, 13–15]. In addition, they can separate presynaptic terminals from the postsynaptic neuronal parts in a process known as *synaptic stripping* [16, 17]. It has long been thought that most of these vital functions can only be performed by activated microglia.

However, in recent years, several fundamental insights into the roles of microglia have been provided with new, noninvasive approaches that have allowed the study of their function while avoiding their activation [18–21]. Surprisingly, surveillant microglia were found to (i) eliminate neuronal precursors in the cortical proliferative zones and to (ii) regulate the density of dendritic spines in the hypothalamus, with consequences on the masculinization of adult copulatory

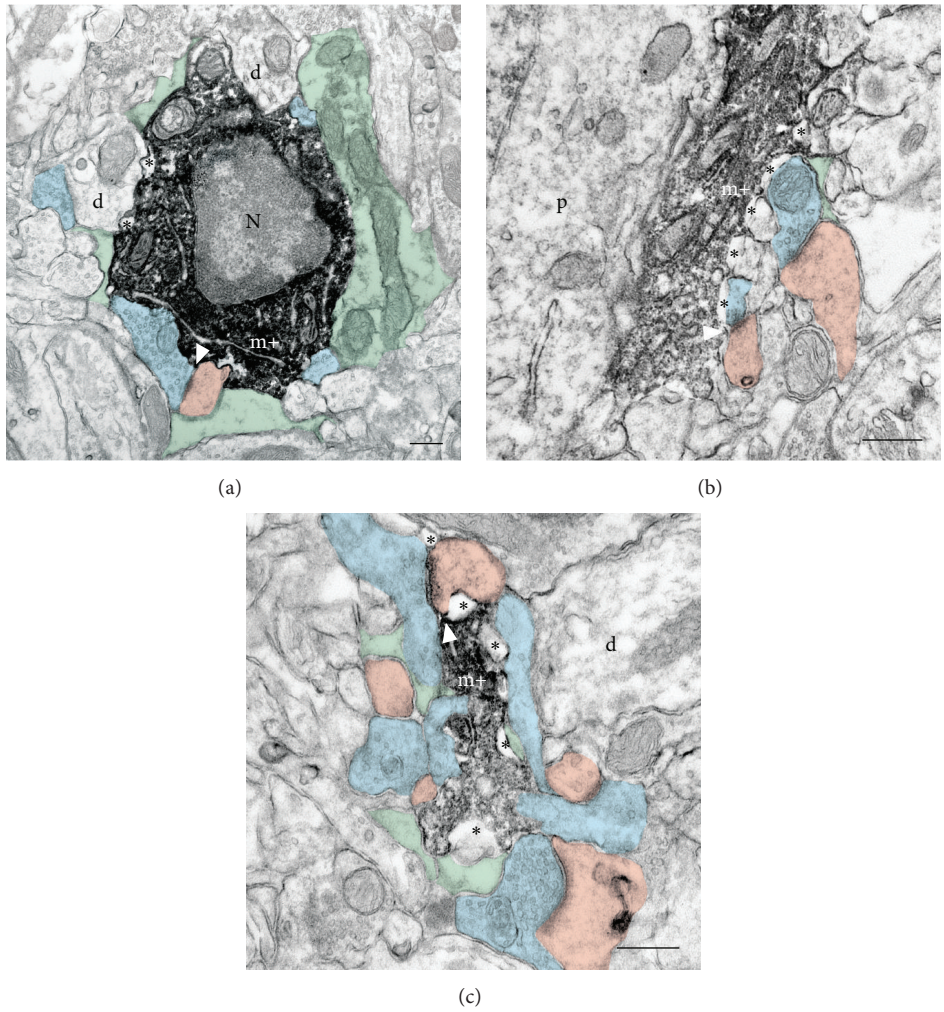


Figure 1: Ultrastructural relationships between microglia and synaptic elements in physiological conditions. Electron microscopy examples of Iba1-immunostained microglial (m+) cell body (a), as well as large (b) and small (c) processes, showing direct juxtaposition with axonal terminals (in blue), dendritic spines (in pink), astrocytic processes (in green), and synaptic cleft (arrow) in adolescent mouse visual cortex. d: dendrite; N: nucleus; p: perikaryon; asterisks: extracellular space. Scale bars: 250 nm. Reproduced from Tremblay et al. [30].

behavior; (iii) the functional maturation of glutamatergic receptors in the hippocampus; and (iv) the activity of tectal neurons in the zebrafish [22–25]. In the mature CNS, surveillant microglia were also found to (v) phagocytose newborn cells during adult hippocampal neurogenesis and (vi) regulate glutamatergic synaptic transmission in the hippocampus [26, 27].

With relation to synapses, it was also recently revealed that surveillant microglia directly contact synaptic elements and eliminate particular subsets of axonal terminals and dendritic spines, depending on changes in neuronal activity and sensory experience, both in the developing and mature brain [21, 28–32]. Microglial interactions with synaptic elements are prevalent [29–32], with almost all of the microglial processes (~94%) juxtaposing axonal terminals, dendritic spines, perisynaptic astrocytic processes, or synaptic clefts and ~68% of all microglial processes contacting more than one synaptic element simultaneously [30] (Figure 1). Morphological specializations resembling finger-like protrusions wrapping around dendritic spines were described based on electron microscopy with three-dimensional reconstruction (Figure 2). Clathrin-coated pits are also frequently encountered among microglial processes, synaptic structures, and perisynaptic astrocytic processes, suggesting direct exchanges of molecular signals between microglia and synapses by clathrin-mediated endocytosis of membrane-bound receptors and ligands [30]. Importantly, when microglial phagocytosis is compromised during early postnatal development, a sustained impairment of synaptic connectivity is present until adulthood [28]. These results imply that, in addition to the immune defense of the brain, microglia-specific activity plays a crucial role in the refinement of neuronal circuits.

Microglial involvement in pruning of synapses, that is, an activity-dependent process required for the maturation of neuronal circuits, is now well-established during postnatal development. Importantly, in the past years, synaptic pruning was found to be determined by the microglial chemokine

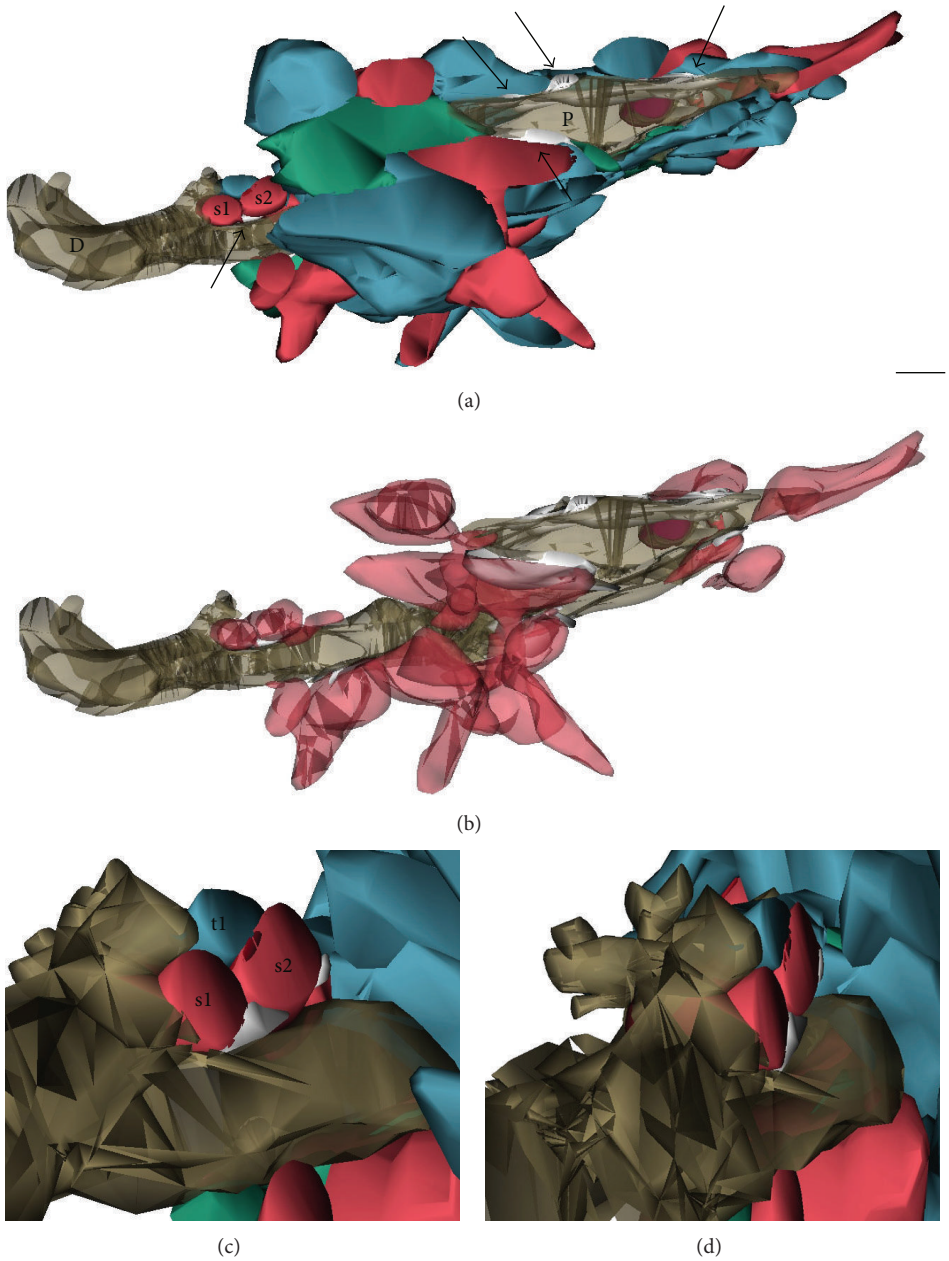
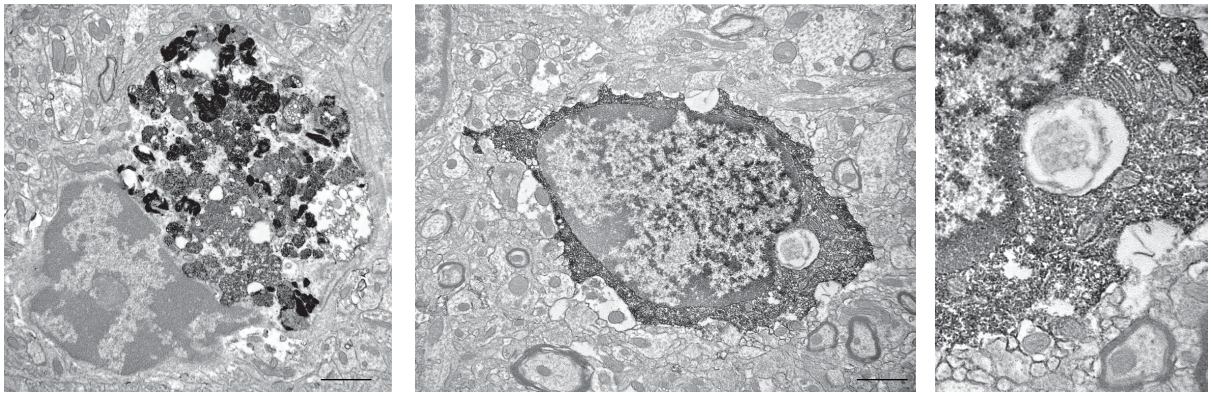


Figure 2: Three-dimensional reconstruction of microglial relationships with multiple synaptic elements at multiple synapses in physiological conditions. (a) Partial reconstruction of a microglial proximal process (P), cut in transverse, and a distal protrusion (D), cut longitudinally, in adolescent mouse visual cortex. The purple element indicates a phagocytic inclusion. The process and protrusion simultaneously contact multiple axonal terminals (in blue), dendritic spines (in red), and perisynaptic astrocytic processes (in green) and are distinctively surrounded by extracellular space pockets (arrows) of various sizes and shapes (in white). (b) Additional view showing only microglia, dendritic spines, and extracellular space. (c) and (d) Insets illustrating the three-dimensional relationships between the distal protrusion, one axonal terminal (t1), two dendritic spines (s1 and s2; postsynaptic density in dark red), and a pocket of extracellular space (in white), which are partially reconstructed. For clarity, an astrocytic process was removed from the scene. Scale bar: 250 nm. Reproduced from Tremblay et al. [30].

receptor CX_3CR1 and the classical complement cascade, including, most notably, signaling between the microglial complement receptor 3 (CR3) and the neuronal opsonin C3 [21, 28]. A similar role was recently proposed in the mature CNS, in the experience-dependent remodeling of neuronal circuits, but the molecular cues remain largely unknown, besides ATP signaling through purinoceptors [4, 6, 18]. In the mature brain, phagocytic inclusions showing ultrastructural features of axonal terminals and dendritic spines were frequently observed inside microglial cell bodies or processes, in both the visual and auditory cortices [31]. The engulfed synaptic elements displayed various signs of health, such as an



(a) (b)

Figure 3: Microglial accumulation of phagocytic inclusions during aging and age-associated loss of sensory function. Examples of Iba1-immunostained microglia containing (a) accumulation of cellular inclusions from the phagocytic elimination of neurons or glial cells and (b) a single cellular inclusion that resembles an axonal terminal with clearly visible synaptic vesicles (inset) in the visual cortex of a 20-month-old mouse with age-associated loss of visual function. Scale bars: 1000 nm. Reproduced from Tremblay et al. [31].

electron-lucent cytoplasm, intact organelles, and cytoskeletal elements, in contrast to the apoptotic elements that are phagocytosed during adult neurogenesis or in contexts of disease [26, 33–36]. Importantly, microglia-synapse interactions were also found to be regulated by neuronal activity, with the phagocytic inclusions becoming more prevalent during manipulations of visual experience [30, 31], including a period of light deprivation followed or not by reexposure to light, a paradigm associated with increased neuronal circuit remodeling and dendritic spine elimination [37–39]. Other mechanisms by which microglia could eliminate synaptic elements may notably include the release of proteases, such as cathepsins, matrix metalloproteinases (MMPs), and tissue-plasminogen activation, as these cells were found to be uniquely surrounded by pockets of extracellular spaces of various sizes and shapes, suggesting their ability to remodel the geometry of the extracellular space locally and thus the concentration of signaling molecules in the synaptic environment [30]. These proteases have been associated *in vitro* with dendritic spine growth and increases in synaptic strength and *in vivo* with dendritic spine motility and elimination, as well as experience-dependent plasticity [18].

During normal aging, the microglial population may be more heterogeneous, displaying variable morphology and different distribution within the brain parenchyma [31]. It has been revealed that ~20% of all microglia in the visual and auditory cortex are completely filled with cellular debris (including axonal terminals, dendritic spines, lysosomal vacuoles, and lipopigments) akin to fat granule cells or gitter cells (see Figure 3). Another distinct feature of the aging brain is the prevalence of microglial interactions with degenerating neurons and synapses, which are identified by their electron-dense, dark ultrastructural contents. The prevalence of phagocytic inclusions and microglial contacts with degenerating elements may be particularly exacerbated by the loss of visual or auditory function [31]. Furthermore, numerous microglial processes protruding into the synaptic cleft have been observed, suggestive of synaptic stripping [17, 31]. In addition, enlargement and thickening of the microglial cell body, increased granulation, impairment of remodeling, and retraction of microglial processes have been described in various brain regions [12, 31, 40–42]. These compromised microglial functions might cause impaired reaction to neuronal abnormalities, in addition to impairing synaptic plasticity, thereby exacerbating the cognitive decline associated with aging [43, 44].

2. Neurodegenerative Diseases

Age is the largest risk factor for the development and progression of neurodegenerative diseases. Several of them, including Alzheimer's and prion diseases, share a common element of pathology: the misfolding and aggregation of otherwise soluble proteins, which are normally mostly enriched at synapses [45]. Over the past two decades, there has been some progress in our understanding of these complex pathologies with respect to the mechanisms underlying neuronal dysfunction. While neuronal death is the final, irreversible outcome in such diseases, the loss of synapses has emerged as a major correlate of cognitive decline [46]. Within the scope of this review, the following section will examine the interactions of microglia with synapses in Alzheimer's (AD) and prion diseases.

2.1. Alzheimer's Disease. In AD, deposition of misfolded extracellular and intracellular proteins is correlated with neuronal dysfunction and loss leading to clinical symptoms of dementia. The principal structural unit of the extracellular deposits is a relatively small peptide, amyloid β-protein (Aβ), which is capable of forming long, insoluble amyloid fibrils that accumulate in deposits known as *senile plaques* during the evolution of the disease [46]. A number of studies have implicated the oligomeric Aβ forms alone as capable of impairing synaptic function, even in the absence of amyloid

fibrils or plaques [47, 48]. Furthermore, it appears that the synapses are the initial targets and their loss is the major correlate of cognitive impairment [49–51].

Following amyloid plaque formation, activated microglia accumulate in its vicinity [52]. Although their exact function remained elusive for some time, recent studies have implied a dual role for microglia in AD pathology. *In vivo* imaging showed that Aβ plaques can form surprisingly quickly (over 24 hours) and microglia might help to restrict their growth [53]. In agreement with this finding, microglial depletion has been linked to increased plaque load in the brain, indicating that microglia might be neuroprotective by removing Aβ [54]. Plaque removal is accomplished by secretion of proteolytic enzymes and via receptors, such as class A scavenger receptors, the receptor for advanced glycation end products (RAGE), and β1 integrins [55–58]. It has been suggested that Ccr2, a chemokine receptor expressed on microglia, might also facilitate the removal of Aβ in the early stages of AD [59]. Following immunization therapy, significant amounts of Aβ within the microglial cells of AD patients were observed during postmortem analysis [60]. Several other studies have also documented that Aβ clearance might be a crucial recovery-promoting mechanism [61–63]; however, in most cases, the precise identity of the phagocytic cells involved remains yet to be determined.

Interestingly, observations contradictory to the aforementioned findings were made by another study following ablation of microglia for up to 4 weeks. Despite a dramatic reduction in microglial numbers, no change in the amount or morphology of Aβ deposits was observed and neuronal damage appeared unaltered [64]. It is worth considering that longer periods of microglial depletion and earlier onset might be required to better understand their role in plaque removal [65]. Nevertheless, this might be challenging to accomplish, because a rapid, efficient repopulation resembling that of the endogenous microglial population occurs in the brain following chemical depletion of microglia [66].

On the other hand, microglia are activated by Aβ to produce cytokines, chemokines, and neurotoxins and may therefore exacerbate neuronal degeneration [57, 67, 68]. One of the examples is the chemokine receptor CX_3CR1 for fractalkine/CX_3CL1, a ligand expressed in neurons that is known for recruiting CX_3CR1-expressing microglia to injured neurons [69]. In a mouse model of AD, microglial CX_3CR1 knockout prevented neuronal loss, indicating that microglia might be involved in neuronal elimination during neurodegeneration [70]. In addition to extracellular protein deposition, intracellular neurofibrillary tangle formation is another major component of AD pathology associated with processes of microglial activation. In an animal model of tauopathy mimicking neurofibrillary tangle formation, microglial activation coincided with the elimination of synapses; however, no evidence of synaptic stripping was provided [71]. Although some studies have shown that microglia can ingest apoptotic neurons and neuritic blebs [72], the precise nature of the involvement of microglia in the events underlying synaptic degeneration and elimination remains to be determined. Taken together, microglia appear to be either beneficial by removing Aβ or detrimental through their proinflammatory activities, thereby likely worsening the disease outcome (Figure 4).

2.2. Prion Diseases. Prion diseases are a group of progressive neurodegenerative conditions affecting both humans and animals. The hallmark pathological features, which are associated with accumulation of a misfolded isoform of the cellular prion protein (PrP^{Sc}), are spongiform degeneration of the brain with extensive neuronal loss, dendritic and synaptic abnormalities, and astrogliosis [73–77]. The misfolded, protease-resistant protein was long implicated in the demise of neurons; however, recent evidence suggests that it might be the protein oligomers, as in AD, that precipitate the synaptic dysfunction [78]. In animal models, the appearance of behavioral abnormalities is associated with a loss of synapses in the hippocampus [79, 80] before the loss of neurons occurs [80, 81]. The presynaptic terminal has been postulated as the initiation site of synaptic demise, followed by dendritic spine degeneration [75, 79, 81, 82]. Similar to AD, microglia undergo a functional transformation associated with a typical change of their morphology [83–85]. However, their molecular fingerprint appears to be anti-inflammatory [86], akin to a macrophage involved in a phagocytic process [87]. At the present time, it is not known whether microglia become activated because of the PrP^{Sc} accumulation or because of the synaptic changes [88], nor it is known what leads to the anti-inflammatory phenotype they appear to adopt. Taken together, it seems likely that microglia with this functional profile might be involved in synaptic stripping. While the aforementioned remains elusive in AD, a series of recent discoveries have pointed to its absence in prion disease [89, 90]. So far, no stripping of either pre- or postsynaptic elements has been observed by electron microscopy; however, engulfment and digestion of neuronal corpses have not been excluded. The density of glutamatergic synapses in the hippocampus gradually decreases, while the remaining synapses hypertrophied, similar to AD [91–93]. A striking feature of the synaptic pathology is the progressive change in the curvature of the PSD, which, as the disease evolves, gradually envelopes the presynaptic element, which appears to be internalized by the dendritic spine. Surprisingly, the loss of synapses is not associated with microglia or astrocytes; instead, it appears to be a neuron-autonomous event (Figure 5 [89, 94]). The subsequent loss of spines has been associated with the development of varicosities on dendritic shafts. Interestingly, only the persistent spines are lost, while the transient spines appear to be unaffected [95].

However, it appears that synaptic degeneration is not a ubiquitous early event in prion disease and that the synaptic vulnerability to toxic protein depends prominently on the structure and function of the target neurons. Brain region-specific presynaptic and postsynaptic degenerative processes independent of microglia were described by a recent study; while synaptic pathology was present in the hippocampus, virtually no synapses had been lost in the cerebellum [90].

Taken together, while activated microglia are thought to exacerbate chronic neurodegenerative conditions such as prion diseases [96], their involvement in synaptic loss via

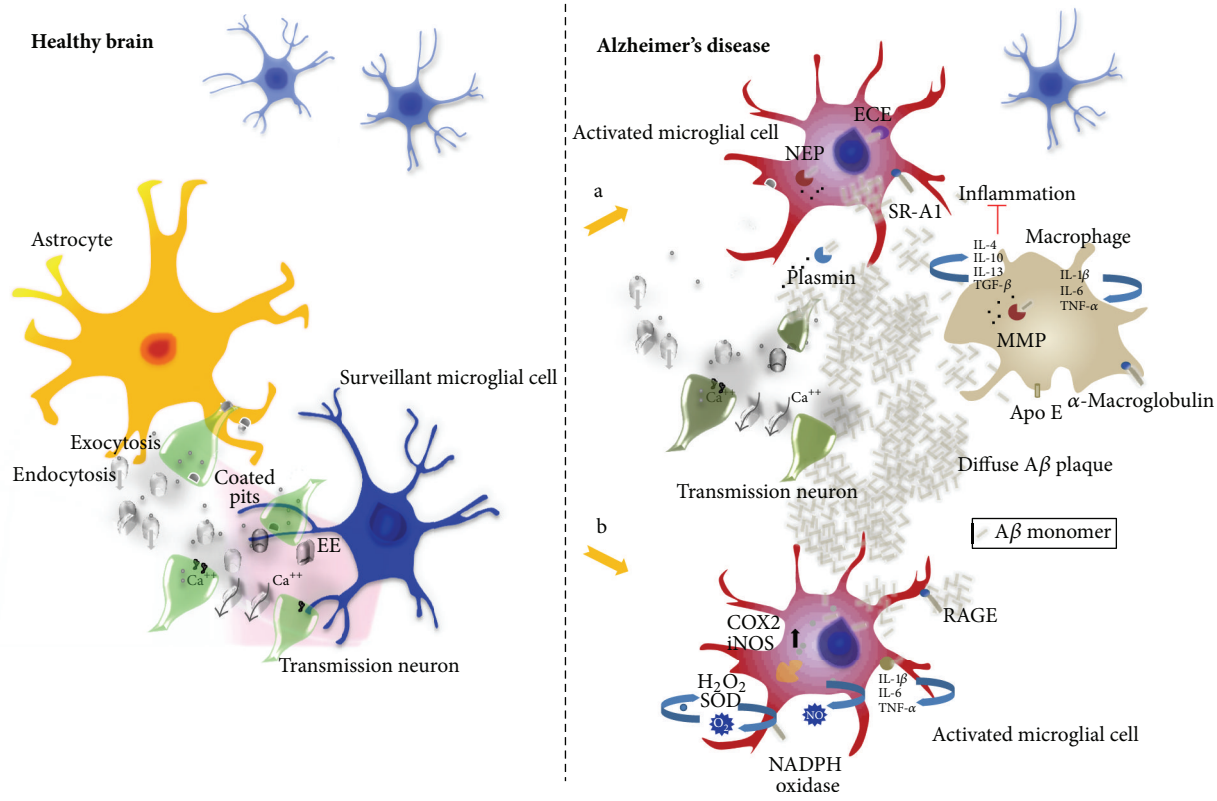


FIGURE 4: The role of microglial cells in Alzheimer's disease. Increased accumulation of Aβ peptides is thought to trigger a variety of pathological events, which subsequently compromise neuronal function. For example, Aβ molecules are known to interact with neurotransmitter receptors, disrupt synaptic and mitochondrial function, and promote neuronal proapoptotic signalling. Injured neurons release a variety of factors that together with Aβ accumulation trigger microglial activation. (a) Extracellular Aβ is taken up and degraded by microglia via receptors such as β-integrins, various enzymes including MMPs, and other uptake-mediating molecules, for example, ECE and NEP. Additionally, macrophages can degrade Aβ molecules and, together with microglia, help restrict amyloid plaque formation in the brain. These activities might represent an important recovery-promoting mechanism. (b) In response to the deposition of Aβ and the release of chemoattractants from injured neurons, activated microglia can release a range of proinflammatory mediators such as cytokines (IL-1β, IL-6, and TNF-α) and induce generation of reactive oxygen species. Although this initial response might be an attempt to protect the brain, these proinflammatory activities are believed to be a major detrimental factor, worsening the disease outcome.

synaptic stripping appears to be unlikely given the recent findings. However, the decision between synaptic elimination and its maintenance in the degenerating brain might be regulated by a more subtle, indirect mechanism, such as extracellular signaling, the nature of which remains to be clarified.

3. Conclusion and Perspectives

Together, these recent discoveries demonstrate that microglia preserve the health of neuronal circuitry by continuous surveillance and dynamic adaptation to changes in neuronal activity and sensory experience. Upon activation, microglia may intervene by eliminating particular subsets of synaptic structures (e.g., axonal terminals and dendritic spines) if a threat to neuronal integrity arises.

In neurodegenerative conditions associated with pathological accumulation of misfolded proteins, such as AD and prion diseases, chronic activation of microglia might exacerbate ongoing degenerative processes. In AD, the activation of microglia appears to have contradictory consequences. On the one hand, microglial activation appears to be beneficial by facilitating the removal of neuronal corpses, inflammatory debris, and Aβ plaques. However, the secretion of neurotoxins and proinflammatory activities associated with microglial activation may worsen the disease outcome. In prion diseases, microglial activation occurs relatively early during the disease process and it is one of the few correlates of synaptic and behavioral abnormalities. While chronic activation of microglia likely worsens the disease outcome, microglia were not observed to engage in synaptic removal via synaptic stripping.

Taken together, all essential cognitive functions, such as learning, memory, and language, rely on the experience-dependent remodeling of neuronal circuits, a process in which microglial interactions with synapses play a role of paramount importance. It is a pressing matter to understand

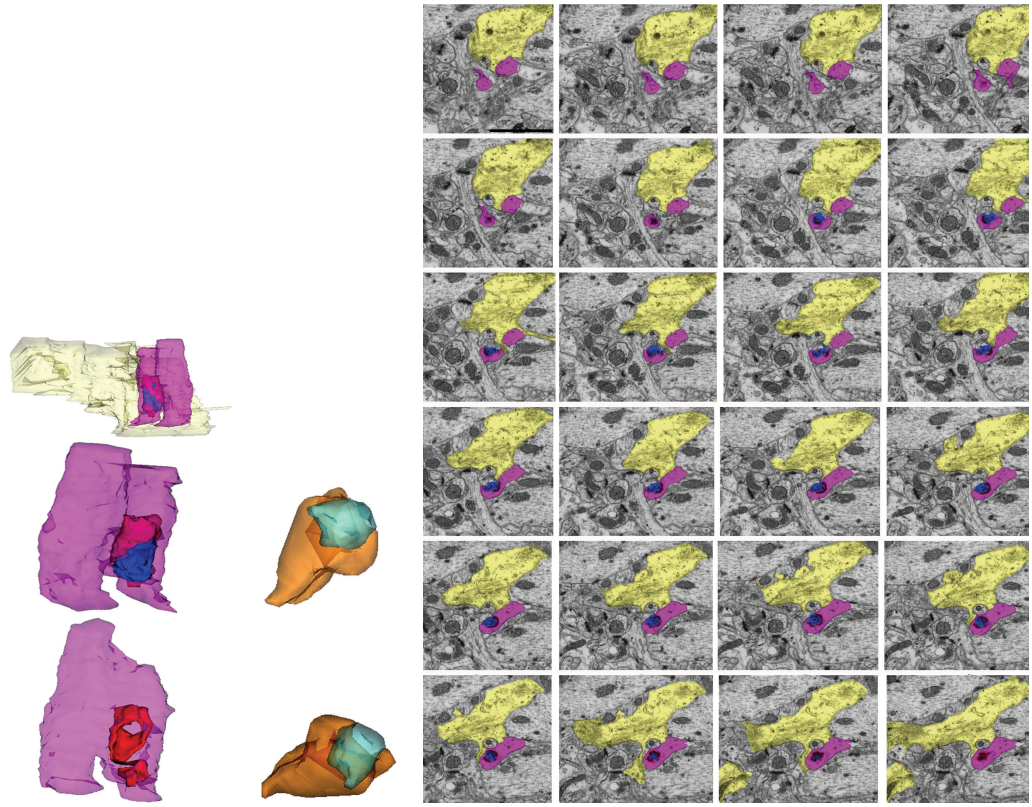


Figure 5: Three-dimensional reconstructions of degenerating synapses in the stratum radiatum of hippocampus in prion disease. (a) Rotations of a synaptic profile generated from 46 consecutive sections; the material originating from the presynaptic terminal (in blue) remains outside and inside (engulfed by the PSD (in red)) of the dendritic spine (in purple). Note the presence of one astrocytic process (in yellow) in proximity but not engaged with the degenerating terminal. The first 24 consecutive sections, from which the profile was generated, are illustrated in (c). (b) Rotations of a synaptic profile from 20 consecutive sections. The presynaptic element (in blue) appears internalized by the dendritic spine head (in orange); however, a fine strand of material originating from the presynaptic element remains in association with the extracellular space and is not within the encircling PSD of the spine. (c) Electron micrographs of serial sections illustrating a degenerating synaptic terminal in the stratum radiatum neuropil. The cytoplasm of the presynaptic element (in blue) is electron-dense in all sections; although the synaptic vesicles are still visible, the presynaptic element is disconnected from the projecting axonal terminal and remains arrested and almost completely engulfed by the PSD (in red) of the dendritic spine (in purple). A process of one astrocytic cell (in yellow) is in close proximity. Scale bar: 1000 nm. Reproduced from Šišková et al. [89].

the exact nature of these interactions, because in neurodegenerative conditions it is at the synapse where the fate of a neuron seems to be decided.

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Review Article

Microglia: An Active Player in the Regulation of Synaptic Activity

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Synaptic plasticity is critical for elaboration and adaptation in the developing and developed brain. It is well established that astrocytes play an important role in the maintenance of what has been dubbed "the tripartite synapse". Increasing evidence shows that a fourth cell type, microglia, is critical to this maintenance as well. Microglia are the resident macrophages of the central nervous system (CNS). Because of their well-characterized inflammatory functions, research has primarily focused on their innate immune properties. The role of microglia in the maintenance of synapses in development and in homeostasis is not as well defined. A number of significant findings have shed light on the critical role of microglia at the synapse. It is becoming increasingly clear that microglia play a seminal role in proper synaptic development and elimination.

1. Microglia

Microglia constitute approximately 10% of the cells in the CNS. They have been traditionally thought to function as the immunocompetent cells of the brain and spinal cord [1] and to be the sensors of injury and infection in the tissue [2, 3]. They derive from primitive c-kit(+) erythromyeloid precursors from the yolk sac [4–6], migrate into the brain during the period of early embryonic development prior to the formation of the blood-brain barrier (BBB), and remain there once the BBB is formed [7]. It is notable that this population is self-sustaining, and peripheral macrophages only contribute to this population in disease states, in which the BBB becomes compromised [8].

Within the brain, microglia have been described to have the ability to detect and mount an inflammatory response to various insults. Sensing neuronal injury has been ascribed primarily to purinergic and chemokine receptors on the surface of microglia, as they monitor the levels of extracellular ATP and secreted chemokines, respectively [9]. Their reaction to neuronal injury is accomplished as they undergo a process collectively called "activation." Activation consists of several biological events that include migration to the site of injury, local proliferation, a change in morphology and gene expression, antigen presentation, and phagocytosis of dead cells and cell debris [10, 11]. During activation, some of the changes in gene expression involve the secretion of cytokines and chemokines, which modulate the CNS environment and regulate the state of inflammation. Inflammation in turn affects the progression of neuronal death after CNS insult. Microglia can secrete both proinflammatory mediators, such as tumor-necrosis-factor- (TNF-) α [12, 13] or interleukin- (IL-) 1β, nitric oxide (NO) [14, 15], and glutamate [16], and anti-inflammatory effectors, such as IL-4 and IL-13, which can enhance neuronal survival [17, 18].

Depending on the predominance of factors secreted, microglia have classically been characterized, similarly to macrophages, as M1 (proinflammatory) or M2 (anti-inflammatory) cells [19]. The M1 and M2 distinctions serve to separate activated microglia into the two broad categories. It is argued, however, that no specific marker designates a microglial response as definitively M1 or M2. Moreover, microglia differentially express pro- and anti-inflammatory markers making the M1 and M2 phenotypes the extremes of the categories, respectively, with variable phenotypes seen in between the two [8]. With that in mind, M1 microglia

have been associated with neurotoxic and neurodegenerative outcomes, as they are observed in a variety of chronic neurodegenerative diseases, such as Alzheimer's [20], end-stage amyotrophic lateral sclerosis (ALS) [21], and multiple sclerosis (MS) [22]. Stroke [23] and traumatic brain injury [24] show a characteristic accumulation of M1 microglia as well. A shift to an M2 phenotype of activated microglia has been correlated with neuroprotection, recovery, and repair in various disease settings [25–27].

2. Microglia as Regulators of Neuronal Function and Plasticity

Until recently, initial studies to understand neuronal-microglial interactions had described that a variety of neuroactive substances, such as NO [29] and TNF-α [30], have potent effects on neuronal function, in particular, synaptic plasticity. However, the cellular origin of these molecules had not been attributed to microglia but, rather, to astrocytes. The focus was maintained on the effect that inflammatory processes have on synaptic plasticity. In neuroinflammatory diseases, such as experimental autoimmune encephalomyelitis (EAE), a rodent model of MS, alterations in synaptic plasticity have been noted. Specifically, in the hippocampus of diseased animals, there is greater induction of long-term potentiation (LTP), an electrophysiological measurement that relates to the connectivity and strength of synapses. This change in LTP was attributed to the secretion of IL1β from the accumulated microglial cells [31]. Bacterial lipopolysaccharide (LPS) strongly upregulates IL1β secretion by macrophages.

It is notable, however, that prolonged exposure to inflammatory cytokines can result in priming or sensitization of microglia so they more readily adopt an M2, rather than M1, phenotype in response to inflammation. This is quite the opposite response to that in acute exposure [32]. As such, chronic inflammation can be induced by LPS infusion and has been reported to attenuate LTP in the dentate gyrus (DG) of the hippocampus. This is accompanied by the loss of pyramidal neurons [33]. Similarly, using LPS infusion, Min et al. found that LTP, dependent on either NMDA receptors (NMDAR) or on voltage-dependent calcium channels, was impaired [34]. Further work is necessary to elucidate the specific mechanism causing these phenomena.

The cytokine, TNF-α, increases the surface expression of AMPA receptors in neuronal cultures, which is accompanied by the enhancement of synaptic strength [30]. In a model of neuropathic pain in the C fibers of the dorsal horn of the spinal cord, inhibition of microglial activation by minocycline resulted in the induction of long-term depression (LTD) rather than LTP. This change in plasticity was found to involve Src family kinases and to be mediated partially by TNF-α [35]. Some studies have found that microglial activation, when both genetically and pharmacologically induced, results in an increase of AMPAR/NMDAR ratio and an enhanced ratio of AMPAR- over NMDAR-mediated currents [36]. These studies demonstrated that when microglia were activated under pathological inflammatory conditions, they caused synaptic alterations via secretory mediators. The precise role of microglia on synaptic activity in the normal brain remained unclear.

Imaging studies have shown that microglia extend and retract their processes continuously to survey their local environment in the healthy brain [37, 38]. Moreover, interactions between microglia and neuronal synapses in the visual cortex have been directly visualized by electron microscopy (EM) and by in vivo two-photon microscopy. The availability of visual stimuli resulted in enhancement of the duration of these contacts and the preservation of the synapse [39]. These intriguing imaging observations first indicated the possibility that microglia could modulate neuronal functions by direct physical contacts. On the other hand, Wake et al. demonstrated that, under conditions of prolonged ischemia, contact time between microglial processes and synapses increased, associated with a greater chance for elimination of presynaptic boutons [40]. It is likely that the mechanism of synaptic pruning is modulated by a distinct molecular mechanism in each of these states and not simply by the longevity of contact.

3. Mechanisms Governing the Interactions of Neurons and Microglia

Paolicelli et al. [41] explored whether there is a functional role for microglial interactions with synaptic structures during the development of the postnatal brain, using imaging and electrophysiological approaches. They used a transgenic mouse line expressing GFP in microglia, under the control of the chemokine receptor CX3CR1 promoter. Thus, they were able to label and visualize microglia as well as manipulate them. The authors found that the number of synaptic elements and dendritic spines expressing the postsynaptic marker PSD95 in $Cx3cr1^{GFP/+}$ mice was about 3-fold higher than that in mice deficient in CX3CR1 ($Cx3cr1^{KO/KO}$). Their result provided some insight into potential roles of microglia in synapse maturation, along with the possibility that this may be a direct CX3CR1-mediated event [42]. These microglial properties, thus, extend beyond immune surveillance and indicate modulatory roles during normal brain development.

Schafer et al. [43] provided direct evidence, via confocal microscopy and electron microscopy, that microglia phagocytose synapses in the dorsal lateral geniculate nucleus (dLGN) as well. The authors proposed that the classical complement cascade, which includes members C1q and C3, was a potential molecular pathway of microglia-synapse interactions in postnatal brain development. According to the experimental data, the synapses that were tagged with C1q and C3 were phagocytosed by microglia that expressed complement receptor CR3. In mice deficient in the receptor or the ligand, higher numbers of synaptic inputs were observed. Moreover, these animals showed deficits in their ability to segregate the territories of each eye. Therefore, the microglia-mediated engulfment was important to drive synaptic stripping during normal development. Together,

these observations reveal that complement-mediated phagocytic activity of microglia is crucial in microglia-synapse interactions during normal brain development.

To further address whether microglia contribute to synaptic activity in the normal young adult brain, Ji et al. [28] used an electrophysiological approach in organotypic hippocampal brain slices and primary neuronal cultures. In this system, they manipulated the presence of microglia by either depleting them using clodronate, replenishing them in previously depleted cultures, or by using cocultures of neurons and microglia. The absence of microglia resulted in a robust increase of synaptic frequencies known as spontaneous and miniature excitatory postsynaptic currents (sEPSC and mEPSC) from the CA1 region of the hippocampus. This increase was subsequently reversed when microglia were replenished in the organotypic slices. In the complementary approach, the addition of microglia to neuronal cultures decreased the synaptic activity measured compared to cultures of neurons alone. The change in synaptic activity coincided with changes in synaptic numbers, which suggested that microglia could participate in the control of synaptic activity by regulating synaptic numbers (Figure 1). As shown in previous reports [43], they also observed that the phagocytic activity of microglia drove synapse elimination when microglia were coincubated with neurons. This could be one mechanism by which synapse numbers are regulated in the normal brain; however, it is still undefined whether synaptic pruning and phagocytic engulfment by microglia occur via a universal mechanism under normal and pathological conditions.

A proposed mechanism by which microglia could regulate synaptic activity was suggested by the same study [28]. Overall expression of synaptic adhesion molecules, such as protocadherin and SynCAM1, which determine synapse remodeling, stability, and synaptic activity, was decreased in neurons incubated with microglia compared to neurons alone (Figure 1). The decreased levels of the synaptic adhesion molecules were recovered to wild-type levels when neurons were incubated with microglia deficient in the serine protease tissue plasminogen activator (tPA) (Figure 2), potentially implicating serine proteolytic functions in the stability of these proteins.

Structural changes at the synapse are closely associated with synaptic stability. In particular, numerous synaptic adhesion molecules, such as classic cadherins (E-cadherin and N-cadherin), protocadherins, and NCAM, have been studied in modulating structural and functional synaptic plasticity. Hippocampal slices pretreated with antibodies against the extracellular domain of N- and E-cadherins or with antagonistic peptides that inhibit cadherin dimerization exhibit a significantly reduced LTP [44]. Moreover, expression of mutant N-cadherin or short hairpin RNA-mediated knockdown of N-cadherin prevents LTP-induced long-term stabilization of synapses [45]. Additionally, Yamagata et al. showed that blocking antibodies to protocadherins or NCAM in hippocampal slices diminished synaptic transmission and LTP induction [46].

Proteases in a synaptic microenvironment are important in the regulation of dynamic changes in the adhesion molecules associated with synaptic plasticity [47]. Proteases, such as matrix metalloproteinases (MMPs) and tPA, secreted from neurons, astrocytes, or microglia under basal or pathological conditions of the CNS have been associated with the targeted degradation or proteolytic processing of extracellular matrix (ECM) and cell adhesion molecules on the cell surface and at the synapse [48–55]. In particular, application of tPA or MMP-9 was shown to be involved specifically in the production of LTP and synaptic growth. Emerging evidence has shown that application of MMP-9 or tPA decreases the levels of N-cadherin and diminishes synaptic transmission [56]. Moreover, tPA regulates MMP activity [57], which leads to the regulation of synaptic plasticity. It is possible that proteases secreted from microglia could regulate synaptic activity by remodeling the ECM which is known to affect synaptic connectivity [58].

4. Connexins and Large Pore Channels

One way of communication among microglia is through connexins and large pore channels. Connexins (Cx) are proteins found in gap junctions, connecting adjacent cells. Each of the connected cells provides an array of Cx isoforms, which form oligomers containing 6 of these Cx proteins. This complex is called a connexon and constitutes a hemichannel [59]. The most common isoforms in mammals are Cx36, Cx43, and Cx45. Connexins are traditionally described as being expressed in astrocytes and in neurons. Cx36 and Cx43 have been reported to be expressed in microglia [60, 61], where they are thought to be involved in the local release of proinflammatory cytokines (TNF-α and IL1β) [62] and metabolites. During inflammatory events, the expression of Cx43 was shown to increase. This increase results in the formation of a functional syncytium among microglial cells, confirmed by the diffusion of the fluorescent dye, Lucifer yellow. However, the syncytium neither forms in nonactivated microglia [63], nor happens if the gap junction formation is inhibited by inhibitors of Cx43, indicating the involvement of Cx43 in the process. Cx36 remains active in resting microglia and does not become upregulated during microglial activation.

In disease settings, it has been reported that blocking Cx hemichannels resulted in the blockade of the microglial release of glutamate [64], which led to the subsequent exaggerated activation of neurons (excitotoxicity). In a model of spinal cord injury (partial cord transection), inhibition of Cx resulted in improved functional recovery [65].

Similar to Cx proteins, large pore channels are formed in microglia and consist primarily of pannexins and P2X channels. They are purinergic and activated by extracellular ATP. Among them, $P2X_4$ is the channel that becomes primarily upregulated in activated microglia [63]. In a recent report, Li et al. state that in the optic tectum of larval zebrafish, neuronal activity drives the activation of pannexin-1 hemichannels. These can then "steer" the processes of resting microglia and facilitate their contact with highly active neurons [66]. In turn, when resting microglia are in contact with neurons or neurites, a decrease in both spontaneous and visually evoked

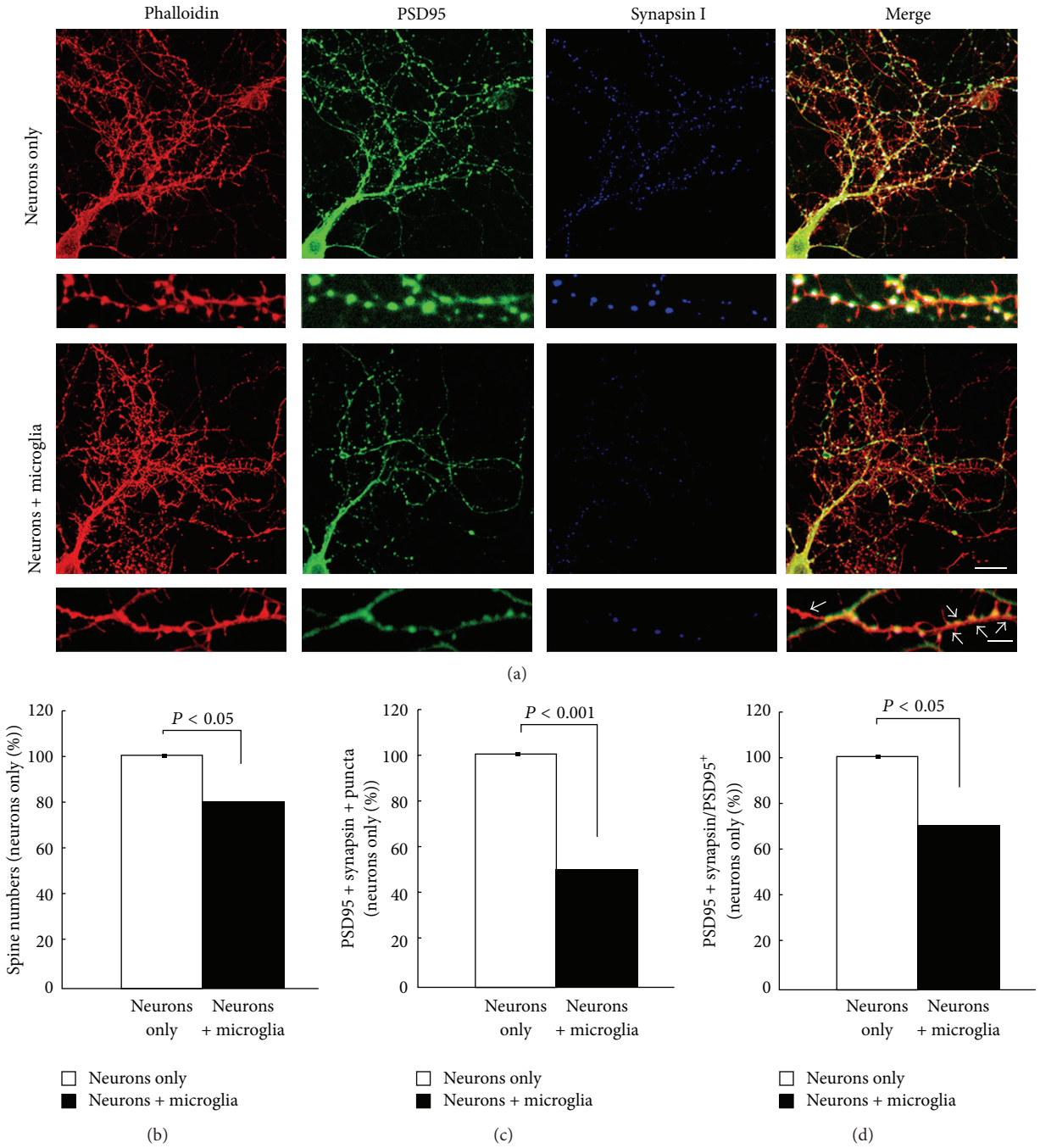

FIGURE 1: Microglia alter the synaptic density of hippocampal neurons. Hippocampal neurons with or without microglia were stained with PSD95 (green), synapsin I (blue), and phalloidin (red) (a). The smaller boxes show magnified images. Arrows depict $PSD95^+$ synapsin 1^- puncta. Scale bars: 20 μm (upper panel); 5 μm (lower panel). Quantification of spine numbers (b), $PSD95^+$synapsin 1^+ puncta (c), and $PSD95^+$ synapsin 1^+ puncta in total $PSD95^+$ puncta (d) in neurons cultured with or without microglia. Values are presented as mean ± SEM and expressed as a percent of the neurons-only control sample (adapted from [28]).

neuronal activities is observed, specifically for the neurons contacted.

These results indicate that connexins and large pore channels could constitute one way by which microglia interact directly with neurons, especially during neuronal insult and inflammation, and could directly affect neuronal activity and survival.

5. Direct Modulation of Neurotransmitter Release and Homeostasis

As mentioned above, microglia can generate neurotransmitters, primarily glutamate. They also respond to changes in neurotransmitters by changing morphology and the motility of their processes. Such responses have been documented

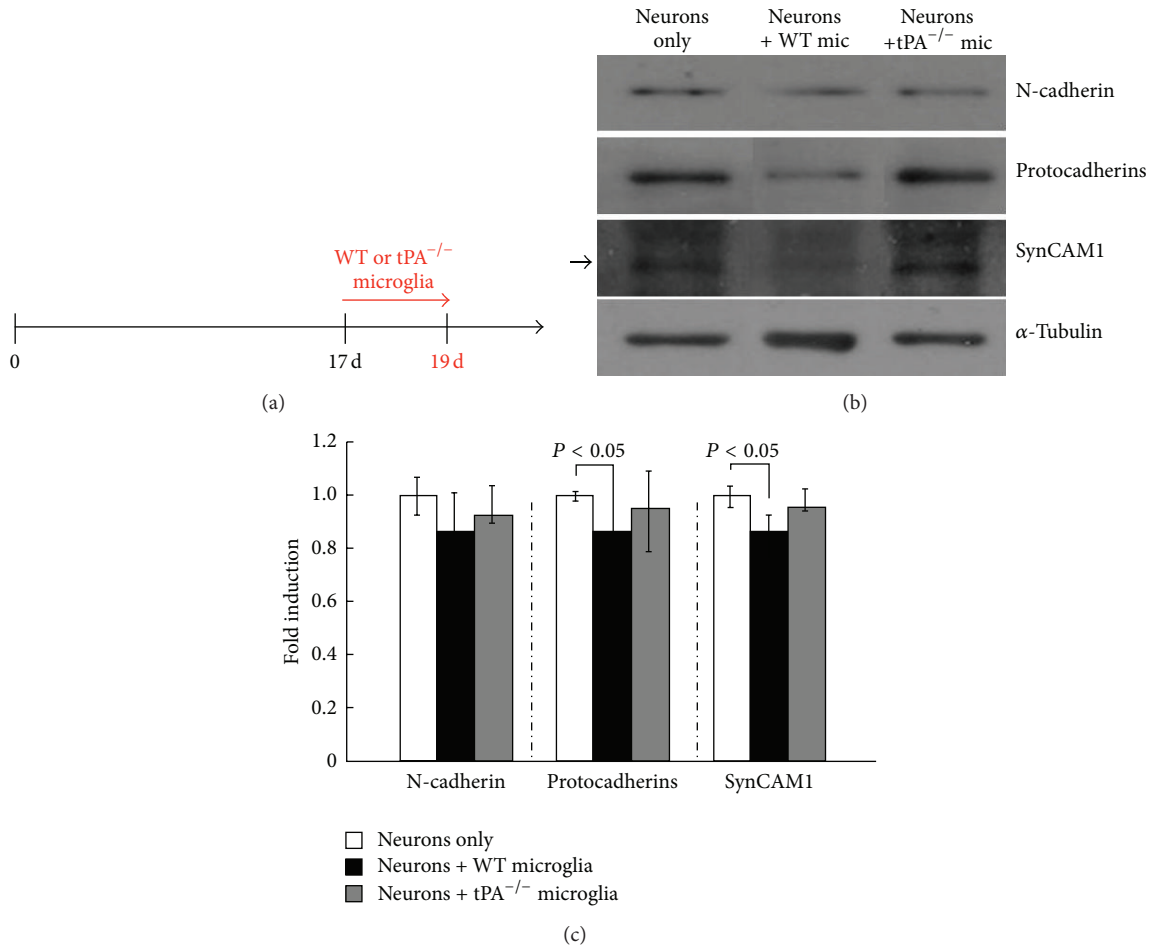

FIGURE 2: Microglial tPA deficiency preserves the levels of synaptic adhesion molecules. (a) Hippocampal neurons at 19 DIV were cocultured with microglia for 2 days. (b) The western blot analysis of the levels of N-cadherin, pan-γ-protocadherin, and SynCAM-1 in neurons in the absence or presence of microglia from wild-type (WT) or tPAKO(tPA$^{-/-}$) mice. α-Tubulin was used as a loading control. (c) Quantification was performed using the ImageJ software and normalized against α-tubulin ($n = 4$). $^{*}P < 0.05$ compared to neurons alone.

both for glutamatergic but also for GABAergic transmission [67]. Application of the glutamate receptor inhibitors NBQX and GYKI, as well as the GABAergic signaling inhibitor bicuculline, has been shown to decrease microglial process motility [68]. Although there is debate on whether microglia express glutamate receptors [68], the presence of $GABA_A$ receptors on the surface of human microglia has been documented [69]. However, there is no concrete evidence that microglia respond in an obvious way to direct application of agonists of glutamate or GABA receptors, that is, in a pure microglial cell culture. Rather, they seem to respond indirectly to such application, on a slice or tissue, suggesting that these agonists potentially have indirect effects on the cells. These indirect effects have been postulated to be mediated through ATP's effect on purinergic receptors since they are expressed on the surface of the cells [37]. Although the source of the ATP release is not entirely determined, the most likely mechanism involves release through neuronal pannexin channels [70].

In models of disease, specifically in the EAE model of MS, the presence and accumulation of activated microglia have been correlated with decreases in the Purkinje cell survival, connectivity in the cerebella of the EAE animals, and attenuation of GABAergic transmission [71]. This has also been observed in the EAE striatum [72] and hippocampus, where a decrease in GABAergic interneurons was also noted, accompanied by induction of LTP [31]. These results suggest that, in this context, microglia may be direct regulators of the numbers of GABAergic neurons and the subsequent attenuation of GABAergic inhibitory transmission.

6. Ectosome and Lipid Signaling

A new way of communication in the CNS has been described involving the release of microvesicles, also referred to as *shed vesicles* or *ectosomes*, from the plasma membrane [73]. These materials were originally thought to be inert but have been recently recognized as critical in mediating cell-to-cell communication. The vesicles contain lipids, cell surface proteins, and material from the cytoplasm or nucleus of the cell [74]. The vesicles are recognized by the recipient cell

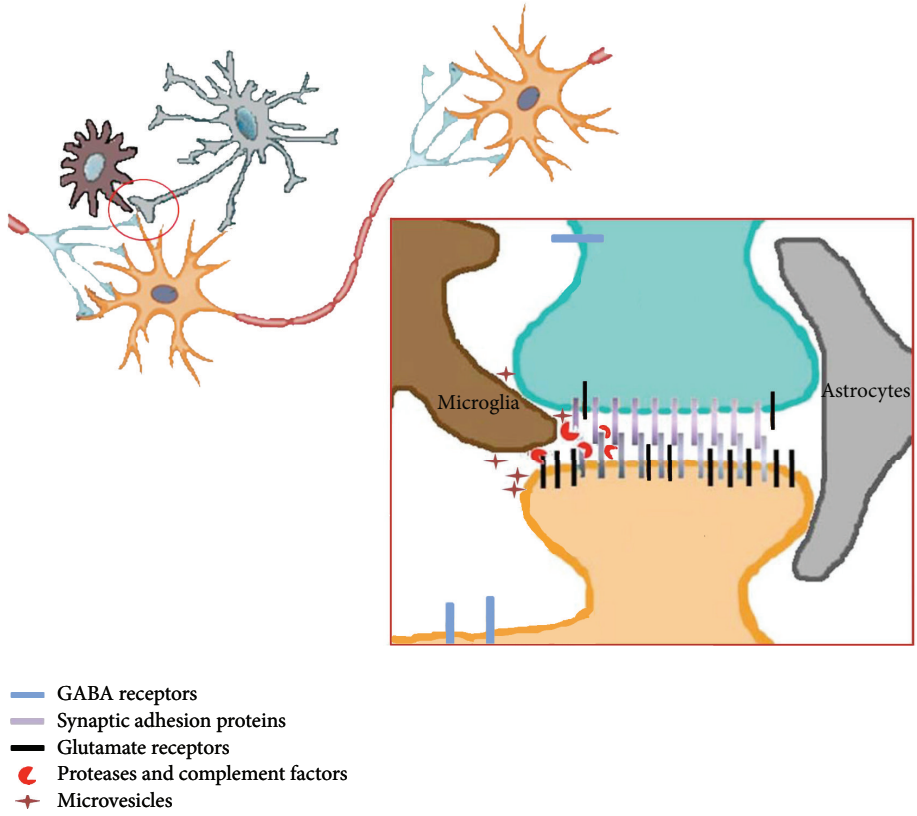

Figure 3: Schematic depicting some of the potential mechanisms through which microglia can affect neuronal activity. Potential interactions between neurons, astrocytes, and microglia at the synapse. The area depicted by the red circle is magnified in inset. Inset: microglial processes in proximity to neuronal synapses can modify neuronal activity via multiple potential pathways. They can secrete proteases to modulate the stability of synaptic adhesion molecules (which in turn influences synaptic transmission) or remove complement-tagged structures. They can release ectosomes that directly interact with the neuronal membranes and initiate signaling cascades. They can also affect directly glutamatergic or GABAergic transmission.

through the presence of phosphatidylserine on their surface [75] and interact with the relevant receptors. They can also directly fuse with the recipient cell.

On the surface of microglia, $P2X_7$ receptors, which respond to the release of ATP, mediate the shedding of ectosomes [76]. This process is triggered by the activity of acid sphingomyelinase and involves the activation of the effector protein p38. Although this is not a mechanism exclusive to microglia (as astrocytes also have been shown to express the $P2X_7$ receptors), microglia constitute a significant source of these shed vesicles.

Signaling through these microvesicles has been reported in different systems. One of the factors thought to contribute to such signaling is Annexin A2 [77], a protein expressed by microglia that affects their activation [78, 79]. Annexin A2 has been shown in different systems to affect neuronal ion channels and neuronal functioning [80, 81], either directly or through its interaction with p11 [82].

In a recent report, it was noted that microvesicles derived from microglia were able to increase the frequency and amplitude of EPSCs [74]. This effect required interaction between microglia and neuronal cells and did not involve secretion of cytokines. It did involve, however, an increase in the metabolism of sphingolipids in neuronal cells. This resulted in an acute increase in excitatory neurotransmitter release. Although in a more chronic exposure to these shed particles the release of cytokines as regulators of neuronal activity cannot be excluded, these data provide another possible pathway by which microglia affect neuronal activity.

7. Conclusions

Given the evidence from imaging, cellular, and electrophysiological approaches, the physical proximity between neurons and microglia seems to result in synaptic maturation and synaptic activity (Figure 3). Several different mechanisms, either involving direct contact and interaction between the two cell types, or mediated through chemical ligands and effectors [9], are described as potential regulators of these microglial functions. The findings indicate that microglia affect both the maturation of the CNS during development and the acute and dynamic regulation of neuronal activity in the mature, healthy, or unhealthy CNS and suggest that they are active contributors to a potential quad-partite synapse [83].

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Review Article

Managing Inflammation after Spinal Cord Injury through Manipulation of Macrophage Function

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Spinal cord injury (SCI) triggers inflammation with activation of innate immune responses that contribute to secondary injury including oligodendrocyte apoptosis, demyelination, axonal degeneration, and neuronal death. Macrophage activation, accumulation, and persistent inflammation occur in SCI. Macrophages are heterogeneous cells with extensive functional plasticity and have the capacity to switch phenotypes by factors present in the inflammatory microenvironment of the injured spinal cord. This review will discuss the role of different polarized macrophages and the potential effect of macrophage-based therapies for SCI.

1. Introduction

A large body of studies on rodents, primates, and humans has revealed that spinal cord injury (SCI) provokes an inflammatory response that causes further tissue damage and neurodegeneration [1]. Macrophages accumulate within the epicenter of the injured spinal cord and orchestrate this inflammatory response. Recent work indicates that macrophages display great plasticity and can alter their phenotypes and functions according to changes in the microenvironment. Understanding the mechanisms that promote anti-inflammatory properties of macrophages and control phenotype plasticity suggests novel therapeutic strategies for treating SCI and other related conditions. Several macrophage subsets with distinct functions have been reported, including M1 (classical activation), M2 (alternative activation), regulatory macrophages, tumor associated macrophages (TAM), and myeloid-derived suppressor cells (MDSC), and so forth [2].

Th1 cytokines and LPS-induced STAT1 signaling activate macrophages to express the classical M1 phenotype. M1 macrophages produce proinflammatory cytokines (TNF-α, IL-1), reactive oxygen species (ROS), and NO, contributing to tissue inflammation and damage. By contrast, M2 macrophages induced by Th2 cytokines produce anti-inflammatory factors (IL-10, TGF-β) and have a reduced capacity to produce proinflammatory molecules, thereby contributing to wound healing and tissue-remodeling. M2 macrophages can be separated into at least three different subgroups based on the type of stimulation and the subsequent expression of surface molecules and cytokines. IL-4 and IL-13 lead to M2a macrophages, immune complexes, and agonists of toll-like receptors (TLRs) which drive the M2b subtype, while IL-10, TGFβ, or glucocorticoids induce M2c macrophages [3].

Compared to M2 macrophages, M1 macrophages produced high levels of IL-1β, IL-6, IL-12, IL-23, CCL5, TNF-α, IFN-γ, and iNOS [4, 5], which have deleterious effects in SCI [6, 7]. In addition, a recent study showed that M1 macrophages also express higher levels of leukotriene B4 (LTB4) and prostaglandin than M2 [8]. Leukotrienes, the bioactive lipids metabolized via cyclooxygenase (COX) and 5-lipoxygenase (5-LOX), are not only potent mediators of inflammation and secondary injury within the injured spinal cord [9], but also contribute to pathological sensory abnormalities [10, 11]. Inhibition of leukotriene production by COX/5-LOX inhibitor licofelone enhances anti-inflammatory within the chronic lesion site, and reduces

mechanical hypersensitivity in rats several months after SCI [12]. This study suggests that inhibition of M1 activation or suppressing the expression of M1 inflammatory mediators, may represent a novel and promising approach in SCI treatment.

The M2 classification represents the extremes of macrophage activation states and does not represent the more complex heterogeneity *in vivo* environment, where macrophages may adopt one phenotype and then switch phenotypes and functions in response to different stimuli [13]. Detailed analysis of macrophage transcriptome revealed the heterogeneity in the gene expression pattern of different tissue-resident macrophages [14]. For example, macrophages stimulated by myelin debris or oxidized phospholipids may adopt a novel phenotype that differs from conventional M1 and M2 phenotypes [15, 16]. Moreover, platelet factor-4 (CXCL4) can induce a unique macrophage transcriptome generating a new macrophage subtype, characterized by reduced CD163 and other scavenger receptor expression and phagocytic capacity [17, 18]. Therefore, Mosser and Edwards proposed to classify macrophages according to their functions: host defense, wound healing, and immune regulation [13].

Abrogating the proinflammatory environment in the injured spinal cord has therefore become a major therapeutic target to reduce secondary cell death and promote neuronal regeneration. Therapeutic approaches have been designed to target macrophages specifically in many diseases including cancers, atherosclerosis, diabetes, and inflammatory diseases [19]. Therapeutic targeting of macrophages in SCI is now in progress. Currently, 20 ongoing clinical trials are testing treatments that may alter macrophages to have neuroprotective, regenerative, or cell transplantation/replacement effects on SCI [20]. The beneficial mechanisms of macrophages on SCI include inhibition of proinflammatory responses, stimulation of angiogenesis, providing neurotrophic factors, and triggering clearance of myelin debris and dangerous apoptotic cells such as neutrophils from the injured spinal cord.

Therapies targeting macrophages can be applied at several levels, that is, stopping inflammatory monocyte recruitment, inhibiting macrophage proliferation, blocking M1 activation pathway, reprogramming macrophages towards the M2 phenotype, and transplantation of beneficial macrophages. Although some of these approaches for SCI treatment were not originally designed as macrophage oriented, these therapies have the potential to affect macrophage activation and function [20].

2. Reducing Inflammatory Monocyte Recruitment

Monocyte recruitment is a key determinant sustaining macrophage numbers at inflammatory sites and contributes to pathogenesis of inflammation. Monocytes are divided into two subsets primarily by their expression of chemokine receptor and the presence of specific surface molecules [21]. $LY6C^{hi}CX3CR1^{lo}$ inflammatory monocytes (analogous to $CD14^{hi}CD16^{lo}$ human monocytes) give rise to proinflammatory macrophages and express high levels of CCR2, while $Ly6C^{lo}/CX3CR1^{hi}$, whereas noninflammatory monocytes are $CCR2^{lo}$ (analogous to $CD14^{lo}CD16^{hi}$ human monocytes), which are recruited to noninflamed tissues [22]. Recently, the International Union for Immunological Societies (IUIS) nomenclature cautioned against using terms "inflammatory" monocytes and "resident" monocytes to avoid confusion [23]. As per nomenclature proposed by IUIS and WHO, $CD14^{++}$ monocytes which form major blood monocyte population were termed "classical monocytes", while $CD16^{++}$ expressing monocytes which constituted around 10% monocytic population were termed "nonclassical" [23, 24] (Table 1).

Reducing inflammatory monocyte infiltration attenuates disease progression in mouse models of myocardial infarction, cancer, atherosclerosis, and pancreatic islet transplantation in diabetes [25]. The macrophage chemoattractant protein-1 (MCP-1/CCL2) is a potent chemokine that attracts monocytes to the injured nervous system [26–28]. CCR2, the receptor of CCL2, is the best-characterized and most widely implicated chemokine receptor in models of human disease [29]. *In vivo*, the CCL2/CCR2 interaction is associated with an M1 response [30] and mediates the recruitment of $CCR2^{+}$ leukocytes into the CNS in a nonredundant manner. $CCR2^{-/-}$ mice have decreased levels of inflammation in numerous disease models. $Ly6C^{hi}CCR2^{+}$ monocytes participate in CNS injury and degenerative diseases [27, 31]. Many CNS disease models, including SCI, involve circulating $Ly6C^{hi}/CX3CR1^{lo}/CCR2^{+}$ monocytes that migrate to CNS by crossing the blood-brain barrier (BBB) in response to CCL2, and upregulate inflammatory molecules and replenish the resident microglia/macrophage populations [32–36]. Therefore, therapeutic targeting of CCR2 could not only inhibit inflammatory monocyte recruitment selectively but also block M1 activation and thus dampen detrimental inflammation.

Leuschner et al. developed a lipid nanoparticle that encapsulated a short interfering RNA (siRNA) that targets Ccr2 mRNA (termed siCCR2) [25]. In the ischemia-reperfusion injury model, administration of siCCR2 resulted in reduced monocyte/macrophage accumulation in the heart and reduced the infarct size. In atherosclerosis mouse model, siCCR2 treatment reduced $LYC6^{hi}$ monocyte infiltration in the atherosclerotic plaques and reduced the lesion size. The advantage of this approach is that it only decreased CCR2 expression on $LY6C^{hi}$ monocytes, while noninflammatory monocytes were not affected. Therefore, this approach can be applied for treating SCI to prevent excessive inflammatory monocyte infiltration.

Ma et al. showed that depletion of CCR2 inhibited the recruitment of monocytes and the degradation of myelin at the injury site at 7 days following SCI [37]. However, in another study, Shechter et al. reported that IL-10 produced by infiltrating monocyte-derived macrophages located at the margins of the lesion site contribute to recovery following SCI [33]. Depleting $Ly6C^{+}CCR2^{+}$ monocytes in the blood with CCR2 antibody reduced recruitment of monocytes to the injured cord, preventing recovery and increasing larger lesion size compared to controls. The effects mediated by CCR2 following SCI are complex, reflecting heterogeneity of

Table 1: Inflammatory (classical) and resident (nonclassical) monocytes.

	Classical/inflammatory	Nonclassical/patrolling	References
Surface markers	Mouse: $CD115^+$, $Ly6C^{++}$, $CD43^+$, $CCR2^{hi}$, $CD62L^+$, $CX3CR1^{lo}$	Mouse: $CD115^+$, $Ly6C^-$, $CD43^{++}$, $CCR2^{lo}$, $CX3CR1^{hi}$	[22–24, 108–110]
	Human: $CD14^{++}$, $CD16^-$	$CD14^+$, $CD16^{++}$, MHCII	
Functions	Steady state precursor for $Ly6C^-$ Infiltrate into inflamed tissues	Patrolling and enter noninflamed tissue	[21, 111]

macrophage responses to chemokines and other intercellular signaling molecules after SCI.

3. Inhibiting Macrophage Proliferation, Differentiation, and Survival

In situ proliferation is crucial for macrophage accumulation in inflamed tissues [38–40]. Regulation of macrophage proliferation, differentiation, and survival controls the magnitude, duration, and characteristics of tissue immune and homeostatic responses [41]. SCI also causes extensive proliferation of microglia and resulting macrophages in injured spinal cords. As Kigerl et al. showed, the majority of macrophages accumulated in an injured spinal cord are M1 [42]. Limiting M1 macrophage proliferation within the lesion site is a potential approach to suppress inflammation in injured spinal cords.

Several growth factors influence myeloid differentiation. Macrophage colony-stimulating factor (M-CSF) signaling through its receptor (M-CSFR) acts specifically on bone marrow precursors committed to the monocyte/macrophage lineage to promote their proliferation and differentiation [43]. Blocking MCSF-MCSFR signaling stops macrophage proliferation [44]. IL-10, IL-4, and liver X receptor (LXR) agonists block M-CSF-induced macrophage proliferation [45–48]. These agents not only inhibit macrophage proliferation but also participate in activation of M2 macrophage phenotype [49, 50]. These treatments not only limit macrophage proliferation but at the same time reprogram M2 macrophage activation within inflammatory lesions and potentiate the role of these cells to resolve inflammation. However, blocking M-CSF signaling may also have an adverse effect on neuronprotection because M-CSF promotes neuroprotection in mouse models from nerve injury, stroke, and Alzheimer's disease [51–53].

4. Blocking M1 Activation Pathway

Promoting conversion of M1 to M2 macrophages decreases inflammatory responses in injured spinal cords. TNF-α contributes to M1 activation and reducing TNF-α activity may inhibit M1 macrophage polarization. Although the beneficial effects of TNF-α blocking after CNS injury are controversial depending on the animal models [54–58], many studies have implicated that TNF-α in the pathological process of SCI and blocking TNF-α activity by neutralizing antibodies and blockers improves spinal cord recovery [59, 60]. TNF-α levels increase shortly after SCI, and therefore, TNF-α activity must be blocked immediately after injury to reduce TNF-α-induced detrimental effects. A recent study showed that delayed peripheral TNF-α inhibition is not an effective therapeutic strategy after SCI [61].

Esposito and Cuzzocrea [59] summarized therapeutic strategies developed to reduce TNF-α activity, including antibodies, soluble receptors, recombinant TNF-binding proteins, TNF receptor fusion proteins, and nonspecific agents (e.g., thalidomide). Several are effective in animal SCI models [56, 62]. For example, infliximab, a monoclonal antibody against TNF-α, inhibited NF-κB activity, which associated with M1 macrophage polarizing pathway [19] and improved locomotor function in the rat acute spinal cord injury [63]. Infliximab, combined with methylprednisolone (MP), exhibited the synergistic effect [63]. Chen et al. reported that (TNF)-α antagonist (etanercept) reduces the associated tissue damage of spinal cord injury, improves hindlimb locomotor function, and facilitates myelin regeneration [64].

5. Reprogramming towards M2 Phenotype or Regulatory Macrophages *In Vivo*

Reprogramming M1 macrophages to adopt the M2 or regulatory phenotype may be helpful for controlling and resolving inflammation after SCI. Many mediators and mechanisms regulate macrophage phenotype, including the cytokines IL-4 and IL-13, immune complex, and TLR signaling [50]. Phenotypic conversion of macrophages from M1 to M2 has therapeutic potential in SCI. To reprogram macrophages directly in the injured spinal cord, the drugs should be able to cross the blood-brain barrier (BBB).

5.1. Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ). Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a ligand dependent nuclear receptor that plays a pivotal role in macrophage cholesterol homeostasis and inflammation. Activation of PPAR-γ by natural or synthetic ligands is a novel anti-inflammatory target for many inflammatory diseases, including stroke and neurodegenerative diseases [65]. The natural ligand of PPAR-γ, 15d-prostaglandin J2 (15d PGJ2) [66, 67], and potent exogenous agonists thiazolidinediones (TZDs) promotes polarization of M1 macrophages toward the M2 phenotype [68, 69]. Specific PPAR agonists, particularly rosiglitazone and pioglitazone, have great promise for CNS injury due to their ability to increase functional recovery and reduce lesion volumes following injury [70]. These PPAR-γ agonists are neuroprotective *in vitro* and *in vivo* in SCI and surgical trauma and some neurodegenerative diseases [71–78].

Another PPAR-γ agonist Atorvastatin (brand name: Lipitor) enhances the phagocytic activity *in vitro* and readily crosses the BBB. Atorvastatin inhibits inflammatory response, improves neuroprotective effect, and induces significant behavioral recovery in three SCI studies from two laboratories [79]. Although the therapeutic effects of these PPAR agonists are thought to be a direct result of PPAR activity, recent data suggest that some of the effects may involve other mechanisms [70].

5.2. Mesenchymal Stem Cells (MSC). Bone marrow mesenchymal stem cells (MSC) regulate immune response and induce anti-inflammatory effects. MSCs can home into wound sites to polarize M1 macrophages to M2 phenotypes and contributes to immunosuppression and tissue regeneration [80–82]. These properties of MSCs make them attractive candidate cell therapies for inflammatory diseases. In addition to the anti-inflammatory function, other advantages of MSC therapies include potential for neural differentiation, ability of MSCs to home into injury sites, absence of side effects, and availability of autologous and allogeneic MSCs.

Although MSCs can be isolated from a wide range of adult tissues including skeletal muscle, adipose tissue, lung, liver, and bone marrow [83], umbilical cord blood (UCB) has been proven to be a valuable source of MSCs. Human UCB-derived MSCs (hUCB-MSCs) have potent anti-inflammatory and immunosuppressive actions [84, 85]. Recent studies indicate that transplanted MSCs significantly improve functional recovery after SCI due to angiogenic stimulation and neuroprotection [86–89]. Acute transplantation of human MSC after SCI in rats increases axonal growth and improved locomotor function [90]. Grafted MSCs modified the inflammatory environment by shifting the macrophage phenotype from M1 to M2 and reduced TNF-α and IL-6, and increased IL-4 and IL-13. These studies suggest that MSCs are a promising and novel anti-inflammatory therapeutic strategy to improve functional recovery after SCI and other inflammatory CNS conditions.

5.3. Neural Stem/Progenitor Cells (NS/PCs). Nishimura et al. found that neural stem/progenitor cells (NS/PCs) promote functional recovery when transplanted during the subacute phase of SCI [91]. These cells altered the microenvironment to favor conversion of macrophages to M2, acting synergistically with other factors to promote axonal sprouting and functional recovery. Timing, however, appears to be a very important issue. For example, some work demonstrated that NS/PC cells transplanted into chronically injured SCI do not survive or have beneficial effects [91–93]. In the subacute phase, M2 macrophages, which have infiltrated into the injury site, may contribute to functional repair after NS/PC transplantation.

5.4. Blockade of IL-6 and Upregulation of IL-10. Blockade of IL-6 signaling promotes functional recovery by inhibiting M1 and promoting M2 macrophage activation after SCI [94]. Guo et al. showed that administration of G-CSF within the first 72 h after SCI can reduce early inflammation-reduced detrimental effect and promote anti-inflammatory response via inhibiting M1 activation and favoring the M2 polarization [95]. Jiang et al. reported that substance P, a neuropeptide that functions as a neurotransmitter and a neuromodular, can stimulate IL-10 expression and induce M2 macrophages [96]. Many other drugs alter these proinflammatory and anti-inflammatory cytokines in injured spinal cords, including methylprednisolone, a therapy that has been extensively used to treat human spinal cord injury [97].

6. Transplantation of Regulatory Macrophages

Transplanted macrophages improve functional recovery and morphological appearance in SCI models [98–100]. Transplantation of *ex vivo* manipulated macrophages should reduce injury and facilitate regeneration. However, adoptive transfer of M2-polarized macrophages injected into mice with SCI may have complex effects. Injury-derived factors in the injured spinal cords downregulated grafted M2a macrophages phenotypes, while inducing or maintaining M1 macrophages [42]. In unpublished studies, we found that transplantation of M2a macrophages induced by IL-4 into the injured spinal cord did not improve functional recovery.

Shechter et al. recently demonstrated that the injured spinal cord recruits M2 macrophages ($LY6C^{lo}CX3CR1^{hi}$) through remote blood-cerebrospinal fluid (CSF) barrier and brain ventricular choroid plexus [101]. Both CSF and choroid plexus maintained an M2 anti-inflammatory profile after SCI. Direct intracerebroventricular injection of naïve $CD115^{+}$ monocytes isolated from bone marrow reduced lesion size and promoted functional recovery. This study not only provides insight into the mechanism of M2 macrophage infiltration, but suggests new approaches for macrophage transplantation.

In contusion model of SCI, administration of recombinant human αB-crystallin (CRYAB), a small heat-shock protein HSPB5 modulates the inflammatory response in the injured spinal cord, causing increased infiltration of granulocytes while reduced recruitment of inflammatory macrophages, promoting greater motor recovery even on delayed treatment, that is, 6 h post SCI [102]. These immunomodulatory effects are credited to the ability of HSPB5 to induce IL10 expressing regulatory-like macrophages via TLR1/2 and endosomal/phagosomal coreceptor CD14 [103].

In another study, transplantation of IL-10-deficient monocyte-derived macrophages failed to promote recovery in contrast to the engraftment with wild type macrophages [33]. These studies suggested that the anti-inflammatory cytokine IL-10 is a critical factor determining the beneficial functional recovery after SCI. Therefore, transplantation of "beneficial" macrophages (anti-inflammatory macrophages with intact phagocytic capacity) can release large amounts of IL-10 directly in the injured spinal cord to promote functional recovery without provoking the inflammatory response macrophages. Regulatory macrophages may satisfy this requirement.

The hallmark of regulatory macrophages is their ability to produce high levels of IL-10 and little to no detectable IL-12 compared to other macrophage subsets in response to FcRγ ligation [104]. In addition to immune complex, other

factors such as prostaglandins, apoptotic cells, IL-10, and some ligands for G-protein coupled receptors, can stimulate differentiation of regulatory macrophages [13]. Compared to M2 macrophages, regulatory macrophages do not express M2 markers such as argenase-1, YM1, and RELMα, and signaling is STAT6 independent [105]. The major role of regulatory macrophages is to inhibit immune response and limit tissue damage in mouse models including experimental autoimmune encephalomyelitis (EAE) and septic shock in mouse models [104].

7. Conclusion

Many studies have clarified the concept of using macrophages as a cell-based therapeutic strategy for SCI. Macrophage targeting strategies to combat SCI should incorporate approaches focused on inhibiting inflammatory monocyte migration and polarizing macrophages towards M2 and other beneficial macrophage phenotypes. However, macrophage-based therapy for SCI treatment is still in its infancy. A better understanding of the mechanisms of macrophage activation and functions offers the possibility of new and practical therapies for patients with SCI. The type and number of macrophages in the injured spinal cord need to be carefully analyzed by studying more specific and better markers. Moreover, most studies of M1/M2 activation and function are in mice. Caution must be taken when translating mouse studies to human. Different mouse strains have very different immune and inflammatory responses that differ considerably from humans [106]. For example, Ym1, FIZZ1, and arginase 1 are markers for mouse M2 macrophages but not in human macrophages [107]. Moreover, the prolonged treatment by using M2 macrophages or regulatory macrophages may have unwanted side effects such as fibrosis, scarring, and tumor progression [2], in addition to their anti-inflammatory effect. Further studies are needed to understand the mechanisms that fine-tune the M2 macrophage activation to achieve regeneration without long-term side effects [30].

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Review Article

Microglial Ion Channels as Potential Targets for Neuroprotection in Parkinson's Disease

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Parkinson's disease (PD) is a chronic, degenerative neurological disorder that is estimated to affect at least 1 million individuals in the USA and over 10 million worldwide. It is thought that the loss of neurons and development of inclusion bodies occur gradually over decades until they progress to the point where ~60% of the dopamine neurons are lost and patients present with motor dysfunction. At present, it is not clear what causes this progression, and there are no current therapies that have been successful in preventing PD progression. Although there are many hypotheses regarding the mechanism of PD progression, neuroinflammation may be a major contributor to PD pathogenesis. Indeed, activated microglia and subsequent neuroinflammation have been consistently associated with the pathogenesis of PD. Thus, interference with this process could provide a means of neuroprotection in PD. This review will discuss the potential of targeting microglia to reduce neuroinflammation in PD. Further, we discuss the potential of microglial ion channels to serve as novel targets for neuroprotection in PD.

1. Introduction

Parkinson's disease (PD) is a disabling neurodegenerative disorder, estimated to affect over 10 million people worldwide and over 1 million people in the United States. With the number of Americans over 65 rapidly increasing, it is inevitable that there will be a drastic rise in PD cases over the next 20 years [1]. PD presents clinically as bradykinesia, muscular rigidity, a resting tremor, and postural instability, all of which are the direct result of degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc). Neuropathologically, PD is characterized by the loss of pigmented neurons in the SNc, the presence of Lewy bodies, and cytoplasmic inclusions containing ubiquitin and α-synuclein [2]. Dopaminergic cell bodies in the SNc provide dopaminergic innervation to the striatum, and degeneration of these neurons results in dopamine depletion in the striatum. In turn, dopamine depletion and the loss of dopamine neurons lead to the hallmark motor dysfunctions of PD, typically after a loss of ~80% of striatal dopamine. Unfortunately, there are limited treatment options currently available for PD, and these treat the symptoms not the disease itself. Therefore, there is a significant need to find therapeutics that target the disease process itself.

2. Neuroinflammation and Microglia in PD

Although the precise mechanism(s) for neurodegeneration in PD is unknown, there is extensive evidence to suggest that neuroinflammation contributes to the pathogenic process of PD. The midbrain, which encompasses the SNc, contains a higher proportion of microglia, the resident immune cells of the brain, than other brain regions [3]. Postmortem PD brains display evidence of inflammation and oxidative stress, including increased microglial activation and lipid peroxidation [4, 5]. The landmark study by McGeer and coworkers [6] first described increased number of microglia in the substantia nigra of post-mortem PD patients. In humans, persistent neuroinflammation and sustained microglial activation were observed in post-mortem brains of humans

who developed a Parkinsonian syndrome after accidentally injecting the neurotoxicant MPTP many years earlier [7]. Microglial activation also appears to be a contributing factor to dopaminergic neurodegeneration in animal models of PD, including those employing rotenone, MPTP (Figure 1), and paraquat [8–10]. Further, long-term increases in microglial activation following MPTP exposure were observed in non-human primates [11]. The finding of sustained microglial activation in postmortem samples and animal model has since been confirmed in living PD patients undergoing PET scans with the ligand PK11195 [12]. Thus, reducing or preventing sustained microglial activation may lead to reduction of neurodegeneration.

3. Clinical Trials Targeting Neuroinflammation in PD

Early studies demonstrating elevated oxidative damage in PD led to the idea that antioxidants might be effective neuroprotective agents in PD. The most notable test of this hypothesis was the DATATOP trial, which tested the ability of vitamin E (2000 IU per day) to delay disease progression. Unfortunately, vitamin E was ineffective, and the study was stopped because of hepatotoxicity [13]. Several epidemiological studies reported that regular use of nonsteroidal anti-inflammatory drugs, particularly Ibuprofen, is associated with a lower risk of PD [14–16]. These findings led to renewed hope that targeting neuroinflammation would lead to neuroprotection in PD.

There are currently several preclinical and clinical studies ongoing for neuroprotection in PD [17], including a prominent one based on the ability of the tetracycline antibiotic minocycline to reduce microglial activation. Early studies reported that minocycline reduced dopaminergic neurodegeneration in rodent models of PD through a reduction of microglial activation [18]. However, subsequent studies reported that minocycline exacerbated MPTP toxicity in both mice [19] and monkeys [20]. Yet another study reported that minocycline could indeed reduce microglial activation, based on morphological criteria, but did not prevent dopaminergic neurodegeneration following MPTP exposure, which was attributed to an inability to decrease release of TNFα [21]. There is also concern because a previous clinical trial for minocycline in amyotrophic lateral sclerosis had to be stopped because of disease acceleration [22] and that minocycline was ineffective in reducing clinical symptoms of multiple-system atrophy [23]. However, the ongoing clinical trial for minocycline in PD has yet to report results.

4. Targeting the Consequences of Activated Microglia

Microglia, often referred to as the resident macrophages of the brain, play a key role in dopaminergic neurodegeneration [24]. Microglia can be activated by a number of signals, including lipopolysaccharide, which interacts with the Toll-like receptor, and can contribute to dopamine neuron death *in vitro* and *in vivo* [25]. Likewise, damaged neurons also release factors, such as α-synuclein, neuromelanin, and calpain, which activate microglia [26, 27]. This activation is characterized by an increase in number, changes in morphology to an irregular and elongated body and short processes, and intense labeling with Iba-1 (see Figure 1). During ongoing neuroinflammation, activated microglia produce a variety of proinflammatory mediators including reactive oxygen species (ROS) and nitric oxide (NO), along with a variety of cytokines, including TNFα. This in turn can lead to dopamine neuron death. Thus, there exists a vicious cycle between microglial activation and dopaminergic neurodegeneration that may contribute to the pathophysiology and progression of PD.

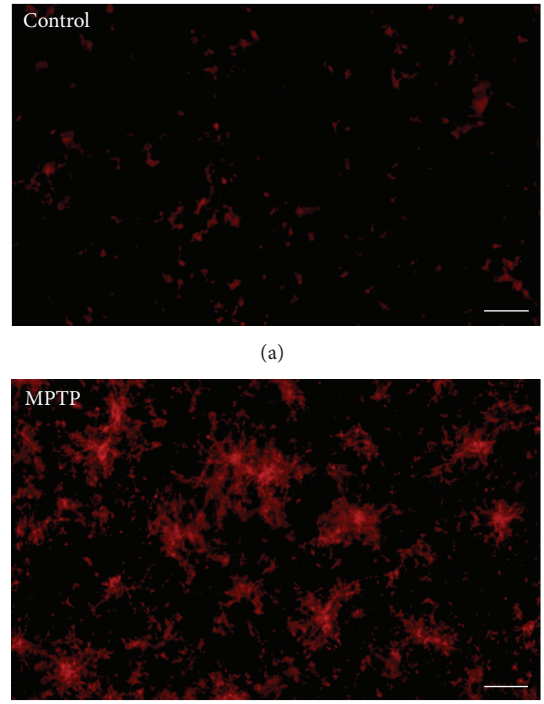

Figure 1: Acute MPTP-induced microglial activation in the striatum of adult mice. (a) Microglia in resting condition in control and (b) activated microglia in MPTP treated animals. MPTP was dissolved in physiological saline and administered subcutaneously (s.c.) a dose of 10 mg/kg every 2 hr for a total of 4 injections. Mice were killed 48 h after last injection and processed for immunofluorescence staining. Microglia were labeled with MAC-1 antibody. Scale bar = 400 μm.

For years, researchers used genetic or pharmacological means to target the untoward effects of microglial activation. Many of these studies reported neuroprotection in animal models of PD, but few have reached the point of clinical trials, and none have proven successful in the clinic to date. Here, we briefly review three of the most studied targets for neuroprotection in PD through reduction of neuroinflammation.

4.1. Nitric Oxide Production. Under pathological conditions, such as PD, nitric oxide (NO) produced by inducible nitric oxide synthases (iNOS) combines with superoxide to form the highly toxic peroxynitrite, which directly contribute to

oxidative damage and neuroinflammation. In PD patients, there is increased immunoreactivity for iNOS and 3-nitrotyrosine in the substantia nigra, likely the result of microglial activation [28]. Increased iNOS and 3-nitro-tyrosine are also found in ventral midbrain and striatum of MPTP-treated animals [29]. From a therapeutic standpoint, pretreatment of animals with iNOS inhibitors, such as 7-nitroindazole, or genetic deletion of iNOS was partially protective against MPTP and paraquat neurotoxicity [29]. However, iNOS inhibitors have not advanced into clinical trials for PD, mainly because of the potential for cardiotoxicity.

4.2. TNFα Production. Activated microglia release a number of cytokines and chemokines, most notably the pro-inflammatory cytokine TNFα. Studies in post-mortem and living PD patients consistently found that TNFα levels are elevated in the brain, serum, and cerebrospinal fluid [30]. In preclinical models, genetic deletion of TNFα or its receptors was partially protective against MPTP toxicity [31]. However, the use of anti-TNF therapeutics is hindered by poor penetration of the blood-brain barrier. Furthermore, recent reports of microglial heterogeneity and a potential role of TNF in cell survival have brought into question whether targeting TNF may actually be detrimental [32].

4.3. NADPH Oxidase Activation. NADPH oxidase, also known as NOX2, is a prime generator of ROS in microglia [33, 34]. NOX2 consists of multiple subunits, including $gp91^{phox}$, which serves as the primary catalytic subunit [35–38]. NOX2 is expressed in a variety of cell types in the brain but has particularly high expression in microglia [39]. Microglial NOX2 is increased in post-mortem PD brains, as evidenced by increased immunostaining for $gp91^{phox}$ [40]. The NADPH oxidase pathway influences dopaminergic neurodegeneration by both LPS and MPTP, as mice lacking NOX2 or the catalytic subunit $gp91^{phox}$ exhibit reduced microglial activation and neurodegeneration [33, 41]. Subsequent studies reported that several nonspecific and relatively specific inhibitors of NOX2 were protective in preclinical models of PD, including dextromethorphan, the aforementioned minocycline, apocynin, and diphenyleneiodonium [42]. However, limitations in blood-brain-barrier permeability, potential off-target effects, lack of specificity, and potential disruption of the beneficial effects of NOX2 in the immune response have hampered the clinical development of NOX2 inhibitors.

5. Microglial Ion Channels as Potential New Targets to Reduce Neuroinflammation

Microglia express several ion channels, including K^+, Ca^{2+}, and Na^+ channels, among others, that are increasingly being recognized for their potential to modulate microglial functions [43–48]. Early studies on membrane properties of microglial cells in culture demonstrated a preponderance of inward rectifying K^+ currents and a resting membrane potential of approximately −50 mV [49]. Additional studies demonstrated that microglia isolated from neurosurgical samples in adults expressed Na^+ currents. Here, we briefly discuss microglial K^+, Ca^{2+}, and Na^+ channels and explore their potential as novel targets for neuroprotection.

5.1. Potassium Channels. K^+ channels (K_v), and in particular the inward rectifier K_v (K_{IR}), were one of the first ion channels characterized in microglia [49]. Indeed, K_{IR} appear to be an early marker of activated microglia, as they are reported not to be expressed in resting microglia. There is also a delayed rectifying outward K^+ current that is associated with activated microglia and appears to be mediated by K_v 1.3 and 1.5. K_v 1.3 was reported to be increased in LPS-activated microglia, as well as in microglia activated by HIV TAT and β-amyloid [46, 50]. LPS or phorbol ester-induced respiratory burst was blocked by a variety of K_v blockers, but these had no effect on NO production [51]. Given that these blockers are toxin based and K_v are also present on neurons, further research is needed to determine the potential of K_v as potential targets for neuroprotection *in vivo*.

The vast majority of recent focus on K_v in microglia has focused on the calcium-activated K^+ channels, particularly KCNN4/KCa2 and 3.1, and ATP-sensitive K^+ channels (K_{ATP}) [52]. KCa3.1 was reported to contribute to microglia activation and NO-dependent neurodegeneration in retinal ganglion cells subjected to optic nerve transaction [53]. Importantly, neurodegeneration was reduced by intraocular injection of triarylmethane-34. With regards to K_{ATP} channels, there is more of a controversy over their effects. Most studies found that administration of diazoxide, a classic K_{ATP} channel activator, reduces microglial activation and is neuroprotective in a variety of models involving neuroinflammation [54]. However, a recent report found that blockade of the K_{ATP} channel with glibenclamide following hypoxia-ischemia is neuroprotective [55]. Given the non-specific nature of the agonists and antagonists used and the presence of these K_{ATP} channels on neurons, further research is warranted on targeting these channels for neuroprotection.

5.2. Calcium Channels. At this time, there is limited electrophysiological evidence for voltage-gated Ca^{2+} channels in microglia [56, 57]. However, treatment with the BAY K 8644, a positive modulator of voltage-gated Ca^{2+} channels, enhanced superoxide production in microglial cells that was blocked by nifedipine [56]. Calcium channel blockers, particularly of the L-type, have recently received significant attention as potential targets for neuroprotection in PD [58]. Indeed, administration of L-type Ca^{2+} channel antagonists, including isradipine [59] and nimodipine [60], exerts neuroprotective effects in MPTP mouse models. However, it is not clear whether this effect results from inhibition of Ca^{2+} channels on neurons or microglia.

There is also a intracellular Ca^{2+} release-activated Ca^{2+} current in microglia that appears to be regulated by Orai and TRP channels, particularly TRPM7 [61]. A growing body of evidence suggests that TRP channels regulate microglial function and may contribute to neurodegeneration [62]. As such, TRP channels may represent a new target for reducing neuroinflammation and exerting neuroprotective effects.

5.3. Sodium Channels. Sodium channels (Na_v) are ubiquitously expressed in neurons throughout the central and peripheral nervous systems where their primary function is to generate action potentials for cellular communication. However, Na_v are also expressed in other neuronal cells, such as astrocytes and microglia, where their role is still being established [63]. Recently, microglial ion channels, including Na_v, were reported to participate in the regulation of a wide range of cellular functions in microglia, including morphological transformation, proliferation, migration, and phagocytosis in response to inflammatory stimuli [43, 45]. Additional studies demonstrated that a variety of Na_v blockers, including tetrodotoxin, and a variety of antiepileptic drugs reduce the phagocytic and migratory activity of cultured microglia [43]. Most recently, we demonstrated that increased tetrodotoxin-sensitive Na^+ flux is an early response to LPS application in microglia and that tetrodotoxin can block TNFα secretion [64].

Using isoform-specific antibodies, Black and coworkers [43] reported that cultured rat microglia express Na_v 1.1, 1.5, and 1.6, with 1.6 being the most highly expressed isoform. Na_v 1.6 was also confirmed to be the isoform responsible for alteration of microglial function, as primary cultures from mice lacking Na_v 1.6 exhibited a reduction in LPS-stimulated phagocytosis [65]. *In vivo*, elevated expression of the Na_v 1.6 isoform was found in activated microglia in an animal model of experimental autoimmune encephalopathy (EAE) and in human multiple sclerosis lesions [65]. Indeed, this study found minimal to no staining of quiescent microglia with Na_v. More importantly, this elevated expression was progressive, and Na_v blockers used clinically as antiepileptic drugs, including phenytoin, reduced microglial activation and axonal degeneration in this model. However, further studies found that if phenytoin treatment was removed, there was a rapid exacerbation of EAE symptoms that was accompanied by increased activated microglia [66]. The mechanism of this exacerbation remains to be fully established.

6. Other Potential Targets on Microglia That Regulate Ionic Homeostasis

6.1. NHE and Na^+/Ca^{2+} Exchangers. NHE are important regulators of intracellular pH through controlling transport of H^+ against an influx of Na^+ ions [67, 68]. In the brain, NHE-1 is the most abundant NHE isoform and regulates cytosolic pH in neurons, astrocytes, and microglia. Early studies demonstrated that increased NHE-1 activity during ischemia reperfusion in the heart and brain contributes to reversal of the Na^+/Ca^{2+} exchanger and influx of Ca^{2+} leading to cell death [69, 70]. More recently, activation of microglia by LPS increases NADPH oxidase activity in microglia that is partially inhibited by NHE-1 inhibition [64, 68]. Luo and co-workers also reported that pharmacological inhibition of NHE-1 was partially neuroprotective against ischemic brain injury, in part through dampening the microglial response [41, 71, 72]. A similar effect was observed in mice heterozygous for NHE-1 [71]. Taken together, these data suggest that NHE-1 may be a viable target for neurodegeneration in PD. However, the clinical development of NHE-1 antagonists has been hampered by poor efficacy and significant side effects [73]. Likewise, a recent report demonstrated a neuroprotective effect of SEA0400, a Na^+/Ca^{2+} exchanger inhibitor, in an MPTP model of PD [74]. While encouraging, this neuroprotective effect was not associated with decreased microglial activation, suggesting that it targets the neuronal Na^+/Ca^{2+} exchanger.

6.2. Hv1 Proton Channels. An exciting new ion channel recently described in microglia is the Hv1 proton channel. Hv1 was first shown to be expressed in immune tissues and support the respiratory burst in phagocytic leukocytes [75]. Subsequent studies revealed the requirement of Hv1 for NADPH-oxidase generation of superoxide during the respiratory burst and a role for regulation of intracellular pH [76]. Most recently, Hv1 was reported to be selectively expressed in isolated human and mouse brain microglia [77]. Further, mice lacking Hv1 displayed less neurodegeneration following *in vitro* oxygen-glucose deprivation and *in vivo* following partial cerebral artery occlusion. These neuroprotective effects were associated with decreased NADPH-oxidase-dependent ROS production. Because Hv1 appears to be present only in brain microglia and is required for NADPH oxidase activation, it may be an ideal target for reducing microglial activation and subsequent neurodegeneration without the potential of off-target effects. However, this remains to be established since there may be infiltrating macrophages from the periphery that express Hv1.

7. Conclusions

A large and growing body of evidence supports an integral role for microglial activation and neuroinflammation in the pathogenesis of PD. Unfortunately, this information has not led to successful translation to clinical trials for neuroprotection in PD. There are numerous reasons for this lack of success in translation to the clinic, including pharmacokinetic issues. However, many of the bottlenecks arise from the fact that many of the targets are widely expressed, leading to adverse effects that preclude their use in PD. Emerging data on the presence of unique localization of ion channels on microglia and the potential for their expression to be increased in neurodegeneration may provide a new avenue for specifically targeting microglia and dampening the ongoing inflammatory process in PD. However, further work is required to determine whether ion channel expression or function in microglia is altered in PD and in which type of microglia (Th1 or Th2) they are expressed. In turn, this may provide additional means of targeting activated proinflammatory Th1 microglia and preserving the potential beneficial function of Th2 type.

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Review Article

Microglia Control Neuronal Network Excitability via BDNF Signalling

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Microglia-neuron interactions play a crucial role in several neurological disorders characterized by altered neural network excitability, such as epilepsy and neuropathic pain. While a series of potential messengers have been postulated as substrates of the communication between microglia and neurons, including cytokines, purines, prostaglandins, and nitric oxide, the specific links between messengers, microglia, neuronal networks, and diseases have remained elusive. Brain-derived neurotrophic factor (BDNF) released by microglia emerges as an exception in this riddle. Here, we review the current knowledge on the role played by microglial BDNF in controlling neuronal excitability by causing disinhibition. The efforts made by different laboratories during the last decade have collectively provided a robust mechanistic paradigm which elucidates the mechanisms involved in the synthesis and release of BDNF from microglia, the downstream TrkB-mediated signals in neurons, and the biophysical mechanism by which disinhibition occurs, via the downregulation of the K^+-Cl^- cotransporter KCC2, dysrupting Cl^- homeostasis, and hence the strength of $GABA_A$- and glycine receptor-mediated inhibition. The resulting altered network activity appears to explain several features of the associated pathologies. Targeting the molecular players involved in this canonical signaling pathway may lead to novel therapeutic approach for ameliorating a wide array of neural dysfunctions.

1. Introduction

Once simply considered as the "guardians" of the central nervous system (CNS), microglia have more recently emerged as key players in regulating neuronal network excitability. Indeed, physical and chemical alterations in the extracellular environment promote the synthesis and release of several microglia-derived molecules which, in turn, shape neuronal circuit function. The effects of such microglia-neuron interactions were found to be critical in the course of different central disorders, and in particular seminal studies provided significant evidence for a role of microglia in the pathogenesis of seizures (for review see [1, 2]) which was associated with increased glutamatergic transmission through the potentiation of NMDA receptor-mediated activity [3]. However, from a theoretical point of view, raising network excitability can be equally achieved through increasing excitatory inputs or removing inhibitory ones. In fact, unmasking silent interconnections can be better achieved through disinhibition than enhanced excitation. Furthermore, disinhibition has been shown as an upstream substrate of activity-dependent enhancement of excitation in several plasticity paradigms [4–7]. Thus, in addition to the glutamatergic hypothesis, it can be postulated that microglia alter neuronal excitability by affecting synaptic inhibition. This hypothesis has been explored during the last decade and the results of several investigations have uncovered molecular mechanisms underlying microglia-mediated disinhibition.

Synaptic inhibition in central neurons is mediated by γ-amino-butyric acid (GABA) and glycine (Gly) which activate ionic channels ($GABA_AR$ and GlyR) permeable to anions, namely, chloride (Cl^-) and bicarbonate (HCO_3^-). Under physiological conditions, Cl^- flows inwardly and HCO_3^- outwardly, along with their electrochemical gradient. Cl^- contribution is by far the more conspicuous, and, consequently, the reversal potential of GABA/glycine (EGABA/Egly) in adult

neurons is set below the resting potential (Vr) near the Cl^- equilibrium (ECl). It follows that when $GABA_AR$/GlyR are activated, Cl^- produces a net hyperpolarization. Although in principle correct, this brief summary of the ionic mechanisms of synaptic inhibition provides a quite static representation of GABA/Gly-mediated transmission and importantly does not take into account that, EGABA/EGly and Vr being only few millivolts apart, even a small change in anion concentrations may have a profound functional impact [8, 9]. In this respect, the critical variable is represented by the intracellular Cl^- concentration and the critical property is the capacity of the cell to maintain this concentration low. In the event that intracellular Cl^- rises, it follows that (i) EGABA/EGly shifts toward or beyond Vr; (ii) the Cl^- gradient across the membrane collapses; and (iii) the previously negligible depolarizing HCO_3^- current becomes more relevant. On the whole, an increase in the intracellular Cl^- concentration weakens the strength of GABA/Gly-mediated inhibition or, in the extreme case, turns it into paradoxical excitation [10].

How do neurons control intracellular Cl^- concentration? Chloride homeostasis in cells is maintained by a group of membrane carriers known as cation-chloride cotransporters (CCCs [11, 12]). The K^+-Cl^- cotransporter 2, KCC2, is the main CCC isoform expressed in central neurons [13, 14]. KCC2 extrudes Cl^- following the K^+ gradient, and its activity typically maintains a low intracellular Cl^- concentration, which is the prerequisite for an effective GABA/Gly-mediated inhibition. Now, KCC2 activity is not static, but it can be profoundly modulated by different physiological or pathological challenges. The most spectacular example of such plasticity has been extensively described during development [14–16]. KCC2 is little expressed in prenatal and early postnatal brains, but during maturation it undergoes a developmental increase, which parallels the switch in GABA/Gly-mediated transmission from excitatory to inhibitory [14]. These mechanisms are thought not only to play a pivotal role in the activity-dependent development of central synapses during CNS maturation [17] but also to favor a proper wiring by triggering spontaneous rhythmic activity in motor networks [18] and to promote synaptic integration of new born neurons in those area of the brain in which adult neurogenesis occurs [19, 20]. On the other hand, reduction of KCC2 activity has been associated with several neurological diseases and conditions, originally epilepsy and neuropathic pain [10], and more recently motor spasticity [21], stress [22], and schizophrenia [23, 24]. Several lines of evidence accumulated during the last decade have indeed demonstrated that the increase in excitability in these pathological conditions can be largely explained by a loss of inhibition, and KCC2 has been recognized as a key molecular target underlying this loss [25, 26].

The findings in recent years that KCC2 can be dynamically modulated by several intercellular signaling pathways have been particularly interesting [4], the most prevalent being brain-derived neurotrophic factor (BDNF) signaling onto neuronal TrkB receptors [27–31]. Even more intriguing is the finding that, in certain conditions, BDNF in the CNS is not only released by neurons but also by microglia [32]. In this review we summarize and discuss the more relevant findings supporting the role of microglia in conditioning KCC2 function, as well as consequently inhibitory neurotransmission, through the release of BDNF. Several convergent findings uncover a canonical signaling mechanism by which the immune system can control neuronal network excitability by regulating the strength of inhibition.

2. Role of BDNF in the Control of KCC2 Function

BDNF is a neurotrophin with important functions in neuronal survival and differentiation. However, beyond its classical neurotrophic role, BDNF is directly involved in the control of neuronal activity and synaptic plasticity as a neuromodulator [33–35]. These functions are described in several areas of the CNS, such as hippocampus [36], cortex [37], amygdala [38], cerebellum [39], and spinal cord [34], and are involved in different forms of plasticity [40, 41]. Although initial studies mainly focused on glutamatergic synapses, the effects of BDNF on GABAergic transmission have lately received increasing attention [40]. Interestingly, early work on these effects performed in the rat hippocampus yielded a number of conflicting results, unveiling a more complex picture than expected. Indeed, in juvenile rodents BDNF was found to favor a substantial depression of GABAergic transmission via either pre- or postsynaptic mechanisms [42, 43]; conversely, studies in immature neurons showed an overall potentiating effect [44, 45]. To explain such a discrepancy, it was hypothesized that the effect of BDNF onto GABAergic transmission in hippocampal neurons might be developmentally regulated in parallel with the switch in GABAergic transmission from excitatory to inhibitory [46]. Thus, BDNF depresses GABAergic transmission in mature neurons when GABA is inhibitory and potentiates it in immature neurons when GABA is depolarizing, favoring activity-dependent synapse formation which has been relayed to GABA-mediated Ca^{2+} entry in developing neurons [37, 44, 46]. The fact that the changes in BDNF effects on GABA-mediated transmission are coincident with the developmental switch in GABAergic current polarity raised the question of whether BDNF has an effect on KCC2 function and/or expression. This was indeed demonstrated by Rivera and colleagues [29, 30]. The authors provided evidence that, in hippocampal slices, BDNF rapidly downregulates KCC2 expression through the BDNF preferred receptor TrkB (tyrosine kinase B receptor), thus reducing neuronal Cl^- extrusion capacity [29]. The effect required the activation of two downstream cascades involving src homology 2 domain containing transforming protein/FGF receptor substrate 2 (Shc/FRS-2) and phospholipase C γ- (PLCγ-) cAMP response element-binding protein signaling, respectively [30]. Interestingly, the activation of the Shc pathway alone was surprisingly found to promote the upregulation of KCC2, which might elegantly explain the opposite effects of BDNF across brain development based on the specific intracellular pathways involved [30]. One important point of this study is that the membrane level of KCC2 undergoes a fast turnover rate, and this turnover is accelerated by exogenous BDNF or by an

increased neuronal activity during which BDNF is released [30]. A logical consequence is that such a fast regulation of KCC2 activity, which happens in few hours or less, is not compatible with the physiological time course required for altering gene expression, and a number of alternative mechanistic models have been proposed including protein phosphorylation, trafficking, and quaternary structure [47]. In particular, KCC2 activity and membrane localization seem to depend on the tyrosine phosphorylation level, and BDNF has been shown to promote KCC2 dephosphorylation, which in turn reduces surface protein expression [48]. Thus, KCC2 phosphorylation influences protein trafficking by either increasing endocytosis or reducing insertion [48]. Alternatively, KCC2 transport activity has been directly correlated with the capacity of the protein to form oligomers at the membrane level [49]. Thus, Cl^- extrusion capacity is improved if KCC2 is organized in oligomers, and an increased oligomers/monomers ratio parallels the KCC2 upregulation during development [49]. Interestingly, KCC2 clustering is strongly reduced in the presence of a point mutation on the KCC2 tyrosine phosphorylation site, suggesting that phosphorylation and oligomerization might simply be different parts of the same process controlling the transporter activity [50]. Finally, KCC2 activity can also be rapidly affected by the activation of the Ca^{2+}-dependent protease calpain [11], and these pathways may be under the control of BDNF/TrkB signaling [51].

Altogether, these findings provided clear evidence that Cl^- homeostasis can be rapidly regulated by an extracellular signal, such as BDNF, thus inducing short- or long-term changes in neuronal activity that cannot be simply explained in terms of classical synaptic plasticity but rather as a novel form of "ionic plasticity" [16].

After the initial studies on the effects of BDNF on Cl^- homeostasis in CA1 pyramidal neuron of the hippocampus [29, 30], similar mechanisms were subsequently observed in different regions across the CNS, including the spinal dorsal horn [27] and ventral horn [21], the ventral tegmental area [52], the cortex [53, 54], and the cerebellum [39]. These findings attracted attention to the fact that BDNF may play a pivotal role as a regulator of neuronal Cl^- homeostasis in the brain and, by ricochet, of inhibition and hence neuronal network excitability.

3. Microglia Are a Central Source of BDNF

The expression of BDNF in synaptic vesicles and its synaptic release from different neuronal populations [34, 55] support the role of the neurotrophin in activity-dependent downregulation of KCC2 [30]. However, BDNF is not only expressed by neurons but is also found in astrocytes [56] and microglia [32]. Microglial BDNF was first shown in microglia cultures [57, 58] and soon confirmed in different regions of the CNS during the course of various neurological disorders, such as viral encephalitis [59], traumatic injury [60, 61], ischemia [62], multiple sclerosis [63], Parkinson's disease [64], neuropathic pain [27], and spasticity [21]. That microglia are a potential source of BDNF is a crucial point to predict the role of the neurotrophin in neurological disorders. Indeed, microglia primary function is to sense and react to alterations of the extracellular milieu with a protective and defensive role. In the presence of factors signaling potentially harmful, microglia undergo morphological and functional alterations collectively identified under the term of "microglia activation," and, depending on the signaling pathways involved, this process may lead to secretion of specific messengers, including BDNF [65]. Once released, the neurotrophin in turn sculpts neuronal circuit excitability via the signaling cascade described above.

The synthesis and release of BDNF in microglia appear to be tightly associated with the purinergic receptor P2X4R [65–67]. Purinergic receptors are endogenously activated by ATP (Adenosine-5′-triphosphate), which is typically stored in the cytoplasm of neuronal and nonneuronal cells and released in the extracellular space following tissue damage [68]. Alternatively, ATP may be released by neurons [69] or astrocytes [70]. Microglia sense extracellular ATP trough different types of purinergic receptors [68], such as P2Y12Rs, which can detect tiny gradient of extracellular ATP and promote microglia migration [71, 72], or P2X7Rs, which trigger morphological changes in microglia from a resting to an activated state [73]. Microglial P2X4Rs, instead, do not appear to be involved in the morphological alterations leading to the activated phenotype, but rather their involvement is a functional consequence of microglia activation [65]. Indeed, P2X4Rs are normally expressed at negligible levels in resting microglia, and they need to be upregulated to promote BDNF synthesis and release [65]. Which external factors are involved in the upregulation of P2X4R in activated microglia is still a matter of debate. Chemokines released from injured neurons, such as CCL2 and CCL21, have been regarded as potential inductors of P2X4R expression [74, 75]. In particular, CCL21 application *in vivo* and *in vitro* strongly promoted P2X4R upregulation in spinal microglia [74]. Interestingly, in both CCL21 [74] and P2X4R [65] deficient mice microglia activation is not compromised, which implies a mechanistic separation between the morphological changes and the subsequent downstream effects. Also CCL2, which instead plays an important role in microglia activation after injury [76, 77], has been suggested to participate in the P2X4R upregulation process; however, CCL2 does not seem involved in *de novo* expression of the protein, but rather it has been suggested to promote P2X4R trafficking from intracellular stores to the cell membrane [75]. Finally, a few nonneuronal endogenous molecules have been also identified as potential inductors of P2X4R in microglia, namely, the proinflammatory cytokines INF-γ [78], the mast cell-derived tryptase activated PAR2 [79], and fibronectin, a component of the extracellular matrix [80, 81]. At the nuclear level, the interferon regulatory factor 8 (IRF8) has been recently proposed as a key transcription factor involved in the upregulation of P2X4Rs in activated microglia [82].

Once upregulated, P2X4Rs can efficiently respond to extracellular fluctuation in ATP concentration and initiates the intracellular cascade leading to BDNF synthesis and

release. Being particularly highly Ca^{2+} permeable, P2X4 channels cause a significant Ca^{2+} influx and the downstream activation of Ca^{2+}-dependent intracellular pathways, among which the phosphorylation of p38 MAP kinase, which is directly involved in the synthesis and release of BDNF [67]. In addition Ca^{2+} influx through P2X4Rs is also necessary to directly facilitate the release of BDNF by acting on the vesicle-releasing machinery, which is typically an NSF-attachment protein-(SNARE-) mediated exocytosis [67]. Alternative pathways (i.e., ERK1/2) have been also suggested to promote BDNF synthesis in cultured microglia [83, 84]; however, these hypotheses need to be properly confirmed *in vivo*.

4. The Special Case of Neuropathic Pain

Based on the findings outlined above, the following conclusions can be drawn: (1) various extracellular signals may activate microglia and upregulate P2X4Rs; (2) P2X4R activation triggers the release of BDNF from microglia; (3) BDNF-TrKB signaling alters KCC2 function leading to a reduced Cl^- extrusion capacity which dampens $GABA_AR$/GlyR mediated inhibition. Assuming that all these events happen in sequence, one should expect that microglia, under certain functional states, influence synaptic inhibition. This is indeed the case of neuropathic pain [66]. Nociceptive transmission is normally conveyed to higher centers through spinal nociceptive pathways. In the most simple configuration, this involves peripheral neurons located in the dorsal root ganglia, which contact second-order neurons in the spinal dorsal horn, and a spinal projection neurons which transmits the information to the thalamus. In the spinal dorsal horn, pain transmission is controlled by a network of local inhibitory interneurons which assure the separation of nociceptive sensory pathways from nonnociceptive sensory pathways by releasing GABA and Gly [85]. Indeed, a spinal administration of $GABA_AR$ or GlyR antagonists induces tactile allodynia [86, 87], a clinical condition in which innocuous stimuli are perceived as painful. Tactile allodynia is a classical symptom of neuropathic pain and indicates an erroneous encoding of low threshold stimuli through the nociceptive channel. Several causal events have been postulated to promote spinal disinhibition, including presynaptic mechanisms affecting the amount of transmitter released and intracellular pathways regulating postsynaptic GABA and glycine receptor function/expression [7]. In our laboratory, we found that altered Cl^- homeostasis in the superficial spinal dorsal horn appears as a key mechanism underlying neuropathic pain symptoms [88] and that this alteration results from the release of BDNF from microglia [27]. Microglia had already been implicated in the pathogenesis of neuropathic pain [89–91], and in particular the upregulation of P2X4Rs in microglia was early identified as a crucial step in the central sensitization process [92]. P2X4Rs are in fact necessary for the development of mechanical allodynia after nerve injury and are required for the release of BDNF from microglia [65, 67]. BDNF in turn binds TrkB receptors in neurons of the superficial dorsal horn, thus compromising KCC2 function and altering Cl^- homeostasis [27]. Blocking the microglia-to-neuron cascade at any level reverses established allodynia in neuropathic animals by restoring spinal inhibitory GABAergic/glycinergic transmission [27, 28]. Subsequent studies have provided additional evidence that this form of spinal disinhibition happens in a different model of pathological pain, such as spinal cord injury [93], diabetes-induced neuropathy [94], and orofacial pain [95]. Moreover, we have very recently shown that the pain hypersensitivity induced by morphine (better known as morphine-induced hyperalgesia) is mediated by the same P2X4Rs-BDNF-TrkB-KCC2 cascade, thus recapitulating the sequence of events described in neuropathic pain [28]. In the latter study, we used a transgenic mouse in which BDNF expression was genetically ablated in microglia only, and we showed that, without microglial BDNF, morphine hyperalgesia does not take place. The involvement of spinal microglia in this specific form of hypersensitivity is due to the expression of opioid receptors in microglia [84] whose activation promotes P2X4Rs [28]. In turn, morphine appears to act on microglia via a nonopioid receptor-dependent pathway to enable BDNF release upon P2X4Rs activation [28].

In conclusion, ten years of investigations on the spinal mechanisms of nociceptive transmission have provided compelling evidence that neuropathic pain critically depends on microglia-to-neuron signals which alter GABA/glycine-mediated inhibition.

5. Microglia-BDNF-KCC2 Signaling in the Pathogenesis of Multiple Neurological Conditions

The microglia-to-neuron communication discovered in the dorsal horn of the spinal cord can be virtually replicated in all those regions of the CNS where functional TrkB receptors are expressed and may play a role in the development of multiple central disorders [10]. Accumulating evidence in recent years supports this hypothesis.

In the spinal motor system, a TrkB-KCC2 interaction has been described in motoneurons following spinal cord injury [21]. Here, the reduced Cl^- extrusion capacity due to the downregulation of KCC2 was associated with hyperreflexia and spasticity, a clinical condition burdening a large number of patients with spinal trauma [96]. The authors did not investigated the origin of BDNF in their model; however microglia are clearly involved in the pathophysiology resulting from spinal cord injury [97], and a role for microglial P2X4Rs has also been envisaged [98], suggesting a microglial BDNF link.

In the brain, alterations in Cl^- homeostasis have been shown to underlay epilepsy in animals and humans [99]. Based on experiments *in vitro* on hippocampal slices [29, 30], TrkB-KCC2 signaling was proposed as the molecular mechanism underlying hyperexcitability in epilepsy [30]. In this model, however, KCC2 downregulation was shown to be activity dependent, thus implying a neuronal source of BDNF whose release is directly related to the level of network excitability. On the other hand, epilepsy has multiple etiologies and might develop in different brain areas.

A role for microglia can be therefore predicted in those pathological conditions which imply a neuronal damage and the subsequent reorganization of synaptic function, as in the case of a traumatic event [100]. Indeed, a TrkB-dependent downregulation of KCC2 has also been described in traumatic brain injury [101], a condition in which neuronal death and inflammation clearly promote the activation of microglia [102]. Interestingly, in animal models of traumatic brain injury, microglia were found to express P2X4Rs and phosphorylated p38 [103, 104], which is known to be the main upstream signal for BDNF synthesis and release in microglia [67].

Finally, a BDNF-mediated impairment of Cl^- homeostasis has been shown to underlie the central mechanisms of opiate dependence in the ventral tegmental area (VTA) [52]. Although the main source of BDNF remains here elusive, chronic exposure to opioids is known to activate microglia and to induce the synthesis of BDNF [28, 84], which, in turn, impairs Cl^- homeostasis in central neurons [28]. In addition, it has recently been described that functional modifications in microglia are involved in mechanisms of opiate dependence in the nucleus accumbens where the early exposure to morphine in young rats was shown to influence drug-seeking behavior in adulthood increasing the risk of drug-induced reinstatement [105].

Taken together, these evidence indicate that BDNF-TrkB signaling drives disinhibition by targeting KCC2 function. Such an effect does not directly depend on the source of BDNF (neuron, astrocytes, or microglia) but rather on the intracellular pathways linking TrkB to KCC2 [29]. This is exemplified in immature neurons where BDNF-TrkB signaling, rather than causing KCC2 downregulation, stimulates the synthesis of KCC2 and favors the developmental switch of GABAergic transmission from excitatory to inhibitory [106]. In contrast, microglial BDNF has gained special attention as underlying neurological diseases in adult tissue, and this is mainly due to the specific role played by microglia in the CNS. Indeed, microglia-driven disinhibition via BDNF-TrkB signaling can be regarded as a peculiar consequence of microglial reaction to injury or to certain pharmacological treatments, potentially occurring in different areas of the CNS. The "pathological" consequences of such process are usually dramatic, leading for instance to an altered nociceptive behavior or to seizure. It remains enigmatic what the normal "physiological" meaning of the release of BDNF from microglia and the subsequent downregulation of KCC2 is. The primary role of microglia is in fact to react in response to a variety of external challenges supposedly with the aim of protecting neurons. In this context, the release of BDNF can be considered as a part of a neuroprotective strategy, being neurotrophins classically involved in neuronal survival process [107]. A neuroprotective and reparative role for microglial BDNF has indeed been postulated during the course of encephalitis [59], brain ischemia [62], and traumatic injury [60]. Interestingly, the posttraumatic loss of KCC2 in mature neurons induced by BDNF and the subsequent GABA-mediated depolarization was found necessary for neuronal survival of injured neurons, a mechanism which is strongly reminiscent of the trophic effect of excitatory GABA during CNS development [101]. In contrast, the central inflammatory reaction in the spinal dorsal horn following peripheral nerve injury appears to be substantially maladaptive and detrimental. Indeed, the activation of spinal microglia following nerve injury produces a release of BDNF onto spinal neurons which are not directly injured. The main effect of microglial activation in this case is thus the suppression of spinal inhibition and the activation of nociceptive pathways, leading to the clinical development of neuropathic pain [27]. Microglia therefore appear as an ambiguous actor, in some cases protective and in other cases having deleterious actions [108]. An accurate prediction of the balance between neuroprotection and neurotoxicity appears thus important to understand how microglia intervene in diseases to develop appropriate therapeutic strategies.

Yet, regardless of the positive or negative outcome of microglia action on neuronal survival and repair, the activation of the P2X4R-BDNF-TrkB-KCC2 cascade allows microglia to critically control network excitability and to unmask hidden neuronal circuits that are normally kept silent by the physiological Cl^--mediated inhibition (Figure 1) [109].

6. Future Directions

The signaling cascade described in this review represents a molecular substrate underlying the mechanism by which microglia target GABAergic/glycinergic neurotransmission. However, it is likely that BDNF released from microglia also challenge network excitability by mechanisms other than KCC2. In particular, BDNF-TrkB signaling also targets NMDA receptors [65, 110], and microglial BDNF has been suggested to underlie certain forms of pathological pain via the activation of spinal NMDA [111]. The outcome of both KCC2 downregulation and NMDA potentiation is an overall increase in network excitability. This raises the question of whether modulations of KCC2 and NMDA functions are independent processes or are reciprocally connected. Several lines of evidence support the latter hypothesis [112, 113], and future investigations are encouraged to further explore such interactions in different neurological disorders. In addition to BDNF, microglia are known to directly or indirectly modulate synaptic transmission through the release of tens of other different molecules [114]. Most of past studies have differently focused on the effect of these molecules in the modulation of glutamatergic transmission. In this respect, a role has been described for cytokines [115], glycine [116], NMDA receptor agonists [117], adenosine [118], and ATP [119]. On the other hand, a growing body of studies reported that cytokines might also directly modulate GABAergic transmission [115]: interleukin 1β was found to depress GABA release in a model of autoimmune encephalitis [120] and to potentiate GABAergic transmission in CA1 [121] or in hypothalamic neurons [122]; both interleukin 6 and interleukin 1β were seen to reduce GABA- and Gly-mediated currents in the spinal dorsal horn [123]; tumor necrosis factor α was shown to promote $GABA_AR$ endocytosis in hippocampal neurons thus weakening the inhibitory synaptic strength [124]. In addition, microglia also produce lipophilic gaseous molecules, such as

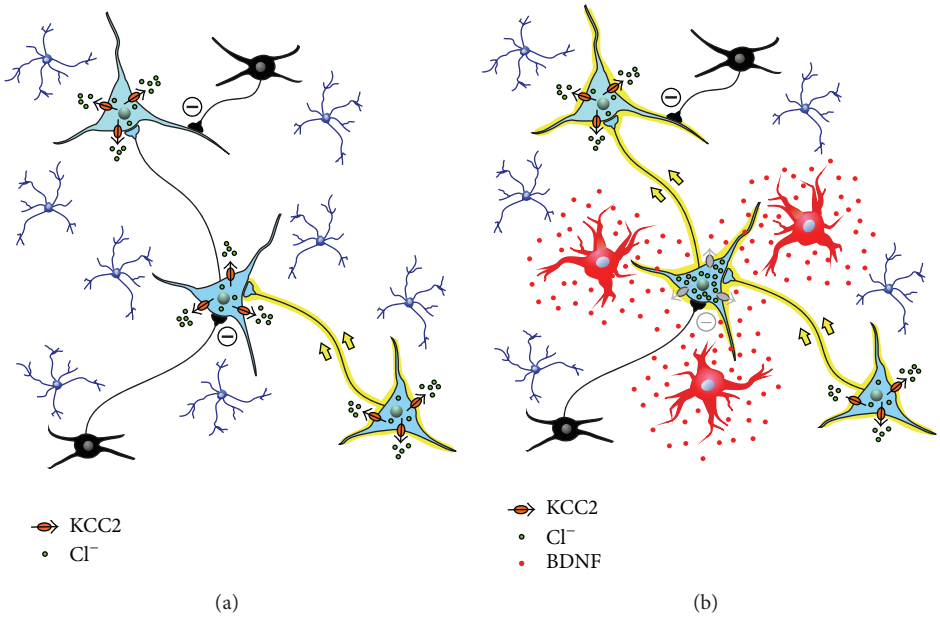

Figure 1: Microglia control neuronal network excitability via secretion of BDNF. The left panel illustrates a schematic neuronal network in the mature CNS under normal conditions: microglia (blue ramified cells) are in their resting state; small inhibitory interneurons release GABA or Gly to repress the flow of signals across the network; normal KCC2 activity extrudes Cl^- (black arrows) to maintain the Cl^- gradient, and, consequently, Cl^- flows in through $GABA_AR$/GlyR channels to inhibit activity. The right panel illustrates the same network after an external event has induced microglial activation (red cells) and the release of microglial BDNF: BDNF-TrkB signaling causes downregulation of KCC2; Cl^- accumulates in neurons and the Cl^- gradient collapses; $GABA_AR$/GlyR-mediated inhibition is less effective in controlling neuronal firing, and previously silent neuronal pathways are unmasked (yellow arrows).

nitric oxide [125–127], and lipidic inflammatory mediators, such as prostaglandins [127, 128]. Interestingly, prostaglandin E2 directly suppresses glycine-mediated transmission in the spinal dorsal horn, a mechanism centrally involved in the development of inflammatory pain [129, 130]. Deeper insights into the role played by each of these messengers in normal and pathological conditions are required to improve our understanding of the role of microglia-to-neuron communication.

Yet, the effects reported in different studies for most of these microglia-derived molecules are often quite dissimilar and critically influenced by the experimental paradigms, drug concentrations, and neuronal populations considered [114]. In addition, the mechanisms leading to the release of specific molecules, as well as the molecular pathways activated in neurons, are still poorly understood, making it difficult to draw a coherent picture for their role in synaptic transmission. Conversely, the P2X4R-BDNF-TrkB-KCC2 cascade described here appears to connect altered extracellular conditions with microglia activation, neuronal excitability, and eventually the development of a pathological behavior. Collectively, these findings open new important therapeutic avenues for the control of neuropathic pain [25] and epilepsy [99]. Yet, many questions are left unanswered and need to be addressed to better delineate the range of applications for an effective microglia-targeted therapeutic strategy; in particular: which neurological disorders are associated with a microglia-driven loss of inhibition? In which brain areas? Do all microglia have the same potential to synthesize and release BDNF when exposed to a given extracellular challenge? Or, instead, are microglia a heterogeneous population with multiple phenotypes playing different roles in different CNS areas and in different pathological states?

Tackling the multiform universe of microglia-neuron interactions and understanding the underlying molecular pathways offer the opportunity to identify specific biomarkers for neurological disorders and potential targets for novel therapeutic approaches.

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Review Article

Bidirectional Microglia-Neuron Communication in the Healthy Brain

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Unlike other resident neural cells that are of neuroectodermal origin, microglia are resident neural cells of mesodermal origin. Traditionally recognized for their immune functions during disease, new roles are being attributed to these cells in the development and maintenance of the central nervous system (CNS) including specific communication with neurons. In this review, we highlight some of the recent findings on the bidirectional interaction between neurons and microglia. We discuss these interactions along two lines. First, we review data that suggest that microglial activity is modulated by neuronal signals, focusing on evidence that (i) neurons are capable of regulating microglial activation state and influence basal microglial activities; (ii) classic neurotransmitters affect microglial behavior; (iii) chemotactic signals attract microglia during acute neuronal injury. Next, we discuss some of the recent data on how microglia signal to neurons. Signaling mechanisms include (i) direct physical contact of microglial processes with neuronal elements; (ii) microglial regulation of neuronal synapse and circuit by fractalkine, complement, and DAP12 signaling. In addition, we discuss the use of microglial depletion strategies in studying the role of microglia in neuronal development and synaptic physiology. Deciphering the mechanisms of bidirectional microglial-neuronal communication provides novel insights in understanding microglial function in both the healthy and diseased brain.

1. Introduction

Microglia comprise a unique subset of glial cells as the resident macrophages of the central nervous system (CNS). Although their developmental origin has been debated for several decades, the general contemporary consensus is that microglia originate from two sources to populate the CNS: an early source in the embryonic yolk sac and a later source from myeloid progenitors that invade the CNS during embryonic and postnatal development. Subsequent to this early colonization, the resident microglial population remains stable and is maintained through adulthood [1–3].

Traditionally, microglia were studied for their role as pathologically responsive cells with virtually no interest in their functions in the healthy brain. However, given their presence in various species from the invertebrate leech to advanced mammals, as well as their emergence during early development, their functions cannot simply be restricted to pathological settings [4]. The last decade has witnessed a dramatic increase in microglia studies in the healthy brain. These studies suggest an interesting possibility that microglia and neurons engage in dynamic communication essential for nervous system development and maintenance.

In the following pages, we survey the recent microglia literature that highlight the interaction between microglia and neurons in the healthy brain (Figure 1). Here, we emphasize the two-way communication that goes on between these cells, beginning with how neurons can modulate microglial state and activity, for example, by specific chemokine, classic neurotransmitters, and purinergic signaling. We then review the evidence that microglia, both by making direct physical contact with neuronal elements and releasing certain paracrine signals, can in turn alter neuronal behavior including the establishment of neuronal circuits. Moreover, we discuss

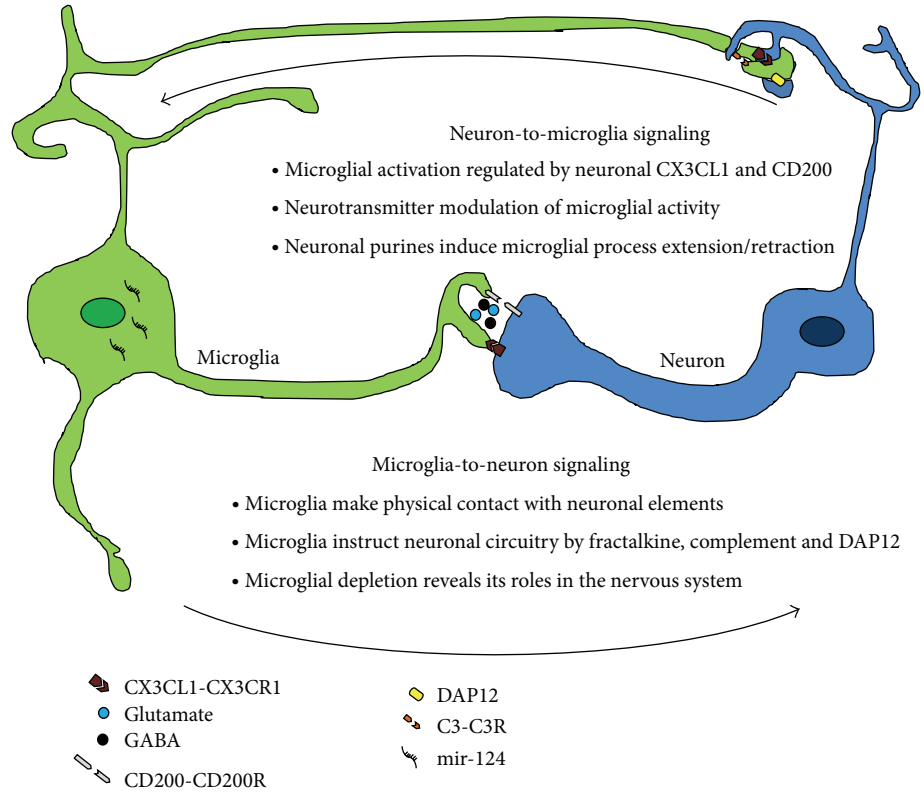

Figure 1: Bidirectional microglia-neuron communication in the healthy brain. Microglia-neuron interactions occur in both directions. Neurons can regulate microglial activation state through the unique ligand-receptor pairs (CX3CL1-CX3CR1 and CD200-CD200R), microRNA-124 (mir-124), neurotransmitters (glutamate and GABA), and purinergic signaling. Conversely, microglia also regulate neuronal activities. It is shown that the microglia is physically making contact with neuronal components. Moreover, fractalkine (CX3CL1-CX3CR1), complement (C3-CR3), and DAP12 signaling which occur distinctively between neurons and microglia are critical for the proper development and maintenance of neuronal circuits. Finally, the roles of microglia in the healthy brain are being elucidated by the several microglial depletion techniques.

microglial depletion studies as an approach to understand microglial importance in neuronal development, function, and maintenance (Figure 1).

2. The Neuron-to-Microglia Communication Axis

While the full repertoire of microglial functions in the CNS is yet to be elucidated, several reports have provided persuasive evidence that microglial functions are modulated by neuronal activities. These studies indicate that neurons can (a) regulate and/or maintain microglial activation states by secreting factors that influence basal microglial properties, (b) release neurotransmitters that influence microglial behavior, and (c) release purines that direct microglial chemotaxis during acute injury. Here, we perform a detailed review of neuronal-to-microglial signaling, though we recommend other reviews that have addressed the subject in detail in microglial-neuronal chemokine signaling [5, 6].

2.1. Neuronal Signals Regulate Microglial Activation State. One of the interesting microglial phenomena is that despite their exquisite sensitivity to perturbations in CNS homeostasis, by which they undergo rapid phenotypic and functional transformation into activated cells, they remain remarkably quiescent or "unactivated" while performing elaborate surveillance roles in the healthy brain. It is now recognized that this "unactivated" state is under the control, at least in part, of neuronal factors, including CD200 and fractalkine (CX3CL1). CD200 is a glycoprotein expressed on the neuronal cell surface in the CNS and functions by activating its receptor, CD200R, which is mainly expressed by microglia in the CNS parenchyma [7]. Genetic ablation of CD200 increased microglial activation, showing several molecular (increased expression of microglial activation markers such as CD11b and CD45) and morphological (decreased ramification) features of activation [8]. As with the above results, retinal microglia have also been shown to possess features of activation in CD200 knockout mice [9, 10]. These results suggest that neuronal CD200 acting through microglial CD200R keep microglia in a quiescent, unactivated state. Like the CD200-CD200R signaling axis, the CX3CL1-CX3CR1 signaling axis has also been implicated in the control of microglial activation state. CX3CL1 is expressed on neurons in the CNS, and CX3CR1 is expressed exclusively on

microglia in the brain parenchyma. CX3CR1-deficient mice showed increased microglial cell-autonomous neurotoxicity in three different models of inflammation [11]. Therefore, neuronal release of CX3CL1 in the healthy brain may maintain microglia in a nonneurotoxic quiescent state. In addition, the CX3CR1 receptor seems to regulate microglial basal motility. For example, exogenous application of CX3CL1 resulted in increased motility in CX3CR1 heterozygotes but had no effect in knockouts suggesting that neuronal release of CX3CL1 not only maintains microglia in a quiescent state but also contributes to basal microglial surveillance. Consistently, microglial process dynamism is reduced in the CX3CR1 null but not CX3CR1 heterozygote mice in a retinal explant system [12]. The downstream effectors of microglial quiescence by neurons remain to be clarified. One intriguing possibility involves the regulation of microglia quiescence by microRNAs such as mir-124. Expression of this microRNA was found to distinguish CNS resident microglia from peripheral macrophages that expressed mir-223 but not mir-124 [13]. mir-124 was found to instruct microglial quiescence but was downregulated in activated microglia. Indeed, when neurons were cocultured with macrophages, mir-124 was upregulated and macrophages took on a more quiescent phenotype. Therefore, it is proposed that circulating blood cells with a macrophage phenotype migrate into the CNS during development and then gradually adopt a quiescent phenotype upon exposure to neuronal factors. Together, these studies suggest that as "transplants" into the CNS, microglia cells are "tamed" by signals from the principal elements of the nervous system.

Interestingly, in addition to keeping microglia in a quiescent state, neurons may also activate microglia during development likely with a different purpose. Neural precursor cells (NPCs) are present in certain neurogenic niches such as the hippocampal dentate gyrus. In such regions, microglia were recently shown to display more activated (as detected by CD68 expression) phenotypes [14]. The authors extended this correlation between NPC presence and microglial activation by *in vivo* NPC transplantation experiments. Here, NPCs were shown to increase microglial activation markers (e.g., CD68) in the striatum of injected mice. Moreover, the activated phenotype was reconstituted in mice injected with NPC conditioned media [14]. Specifically, NPC-derived vascular endothelial growth factor (VEGF) was found to be necessary and sufficient for the aforementioned phenotypic changes though the functional significance of the NPC-induced microglia activation is still largely unknown.

2.2. Classic Neurotransmitters and Microglial Motility. A subject of increasing interest is the possibility that neuronal communication can also regulate microglial activity. However, whether microglia are capable of responding to neurotransmitters has only begun to be investigated in recent years, and the current data point to differences in global and local regulation of microglia activity by neurotransmitters. Before considering the primary evidence for modulation of microglial behaviors by specific neurotransmitters, we discuss three principal studies that showed that microglial activity is influenced by neuronal transmission. (i) Using two-photon imaging of neurons and microglia in the mouse visual cortex, Wake et al. [15] observed microglia making physical contact with neuronal elements. To test the requirement of neuronal activity for such contacts, the authors inhibited neuronal activity by either injecting TTX (which blocks sodium channels and thus action potentials) into the eye or lowering the body temperature of mice during imaging. Microglia displayed significantly reduced contacts with neurons under such conditions. (ii) Tremblay et al. [16] observed interaction between microglia and neuronal spines in the mouse visual cortex that was modified during visual experience. When mice were deprived of sensory input by dark adaptation, microglial motility was reduced and microglial processes were modified to display phagocytic structures suggesting the engulfment of material which may include neuronal elements. Interestingly, light reexposure restored microglial motility though phagocytic structures persisted. (iii) Most recently, neuronal activity-dependent microglial behaviors were reported in the zebrafish optic tectum [17]. As in the mouse [15, 16], the dynamic processes of zebrafish microglia made contacts with neurons through their bulbous endings. Additionally, when imaging was done in TTX, microglial bulbous endings were significantly reduced while repeated stimulation of a single eye by light resulted in increased microglial bulbous endings in the contralateral tectum [17]. Together these studies demonstrate that both physiological (e.g., eye deprivation or repetitive stimulation) and pharmacological (e.g., TTX application) alterations in neuronal activity can modulate microglial behavior. Regarding the modulation of microglial activity by neuronal activity, it should be pointed out that other studies reported that microglial sampling volume remained unaltered during TTX application *in vivo* [18] and microglial motility remained unchanged by neuronal activities induced by high frequency stimulation induced [19, 20].

Recent reports consistently suggest that global neurotransmission alters microglial motility. Using *in vivo* imaging in the mouse cortex, the first direct evidence showed that global inhibition of GABA-ergic neurotransmission resulted in an increase in the volume of tissue sampled by individual microglia [18]. Whether this effect is mediated by the direct action of inhibitory neurotransmitter on microglia or by other indirect factors like ATP released as a cotransmitter or secondary signal has yet to be clarified. Further evidence supported the role of global neurotransmission in regulating microglial activity in mouse retinal explants. As in the mouse cortex, retinal microglial morphological activity was increased by GABA inhibition. In addition, global inhibition of endogenous glutamatergic transmission decreased while exogenous glutamate receptor agonists increased microglial motility [21]. These authors suggested that neurotransmissional effects on microglial activity occurred through ATP. Together, these observations suggested that both excitatory and inhibitory neurotransmissions may act in concert to determine overall microglial activity.

Although the evidence for regulation of microglial motility by global levels of neuronal activity is mounting, the data for regulation of such activity by local neurotransmission is not as clear. Wu and Zhuo [20] first began to address this

question by combining electrophysiology and imaging of resident microglia in brain slices. The authors found that local application of glutamate or GABA in the vicinity of ramified microglia did not induce membrane currents or chemotaxis of microglial processes. Moreover, activity-dependent synaptic plasticity induced by high-frequency stimulation failed to elicit changes in microglial motility. A subsequent study extended the observation in hippocampal slices to spinal cord slices as microglia failed to respond in a morphological significant way to a wide range of neurotransmitters by local application [19]. Despite these initial observations, recent *in vivo* imaging in the zebrafish optic tectum reported increased microglial activity to localized glutamate uncaging [17]. The differences may result from different methods of glutamate application, that is, through a pipette versus via uncaging, species differences, that is, mouse versus zebrafish, differences in tissue preparations that is, *in vivo* versus *ex vivo* slices, or differences in the age of tissue studied, that is, young animals (5–8 days post fertilization) versus adult (6–10 weeks old). This latter point is attractive since it has now been shown that even (astro) glial metabotropic glutamate signaling is developmentally regulated [22]. Moreover, like in the young zebrafish tectum, glutamate was found to elicit chemotactic responses from microglia in two-week-old spinal cord slices though the source of glutamate under such conditions remains to be determined [23].

In summary, although not conclusive, the current data suggest that microglial motility is decreased by global inhibitory neurotransmission and increased by excitatory neurotransmission, implying that such global communication between neurons also regulates the elaborate basal motile activity of microglia. Yet, whether neurotransmitter influence on microglial motility is developmentally regulated has not been clearly evidenced. In addition, although there has been interest in ionotropic receptors on tissue microglia (e.g., the reports by Fontainhas et al. [21] and Wu and Zhuo [20] failed to observe expression of functional glutamate receptors in microglia), further investigation is needed to determine the role of metabotropic glutamate/GABA receptors on tissue microglia and how they may integrate neuronal signals to modulate microglial functions.

2.3. Traumatic Neuronal Signals "Activate" Microglia during Injury. While clarity on neuron-to-microglia signaling via classic neurotransmitters during normal physiology is lacking, evidence for the communication during acute injury via purinergic signaling has been well established (Figure 2). We consider it in this review of microglia-neuron communication in the *healthy* brain because neuronal demise and the concomitant release of purines may occur physiologically, especially during development. However, the discussion here is also applicable during nervous system injury, disease, and pathology.

Beginning with the work of Geoffrey Burnstock in the 1970s, a role for adenosine triphosphate ATP (which is a ubiquitous energy source) and its metabolites (e.g., adenosine diphosphate [ADP] and adenosine) in the extracellular space acting on cell surface receptors has now been established. These molecules are capable of eliciting different cell responses through two receptor types: ion channel P2X receptors that mediate ionic flux and G protein coupled P2Y receptors that activate G proteins and their downstream effectors (reviewed in [24]). Under normal physiological conditions, the concentrations of these metabolites are maintained at high levels within cells and at relatively lower levels in the extracellular space by a complex system that includes degrading enzymes, metabolite uptake, and generation of the respective purine [24]. However, during acute neuronal injury, these metabolites are released into the extracellular space at concentrations that could activate the respective receptor(s), many of which are expressed by microglia [25]. It is now clear that neuronal demise is detected and responded to by microglia in the event of the release of these purines.

By the late 1990s, experiments with cultured microglia suggested that microglia expresses purinergic (P2) receptors as high concentrations of extracellular ATP induced intracellular Ca^{2+} elevation [26] and cell death [27] in a receptor-dependent manner. Further studies provided evidence for ATP- and ADP-induced microglial chemokinesis (motility) and chemotaxis (directed migration) in cultured microglia exposed to varying concentrations of purines [28]. Moreover, metabotropic purinergic (P2Y) receptors are implicated in the chemokinetic mechanism. Using *in vivo* imaging of microglial behavior, Davalos et al. [29] confirmed the relevance of ATP-induced microglial chemotaxis in the mouse cortex. The authors showed that laser-induced injury to brain tissue resulted in robust microglial branch extension towards the site of injury; the process chemotaxis was able to be abolished by apyrase, an ATP/ADP degrading enzyme. ATP-induced microglial chemotaxis was then confirmed in acute mouse brain slices and was further shown to involve ATP-induced outward potassium currents [30]. Using a similar acute brain slice preparation in rats, Kurpius et al. [31] also demonstrated that following tissue slicing, which inevitably induces neuronal injury, microglia cells are able to "home" rapidly to neuron-rich regions presumably by sensing endogenously released neuronal purinergic signals. Subsequently, ATP-induced microglial chemokinesis has been confirmed in the mouse spinal cord [19, 32] and retina [21] as well as in other animal models including the zebrafish [33] and leech [34] indicating the widespread existence of this signaling mechanism. The specific receptors involved in purine-induced chemotaxis have also been identified. Using a genetic approach, Haynes et al. [35] provided very powerful evidence *in vitro*, *ex vivo* (acute slice preparation), and *in vivo* for the regulation of ATP-induced microglial chemotaxis by the P2Y12 metabotropic receptor as branch extension or migration towards purinergic sources was abolished or significantly delayed in P2Y12 knockout microglia. Subsequently, using pharmacological approaches, Wu et al. [30] confirmed P2Y12 involvement in ATP-induced microglial chemotaxis in brain slice preparation.

As the forgoing has shown, ATP/ADP is capable of inducing microglial chemotaxis. However, whether ATP/ADP is sufficient for this cause has also been a topic of interest. It is well known that ATP and ADP are rapidly degraded by endogenous enzymes present in the extracellular space.

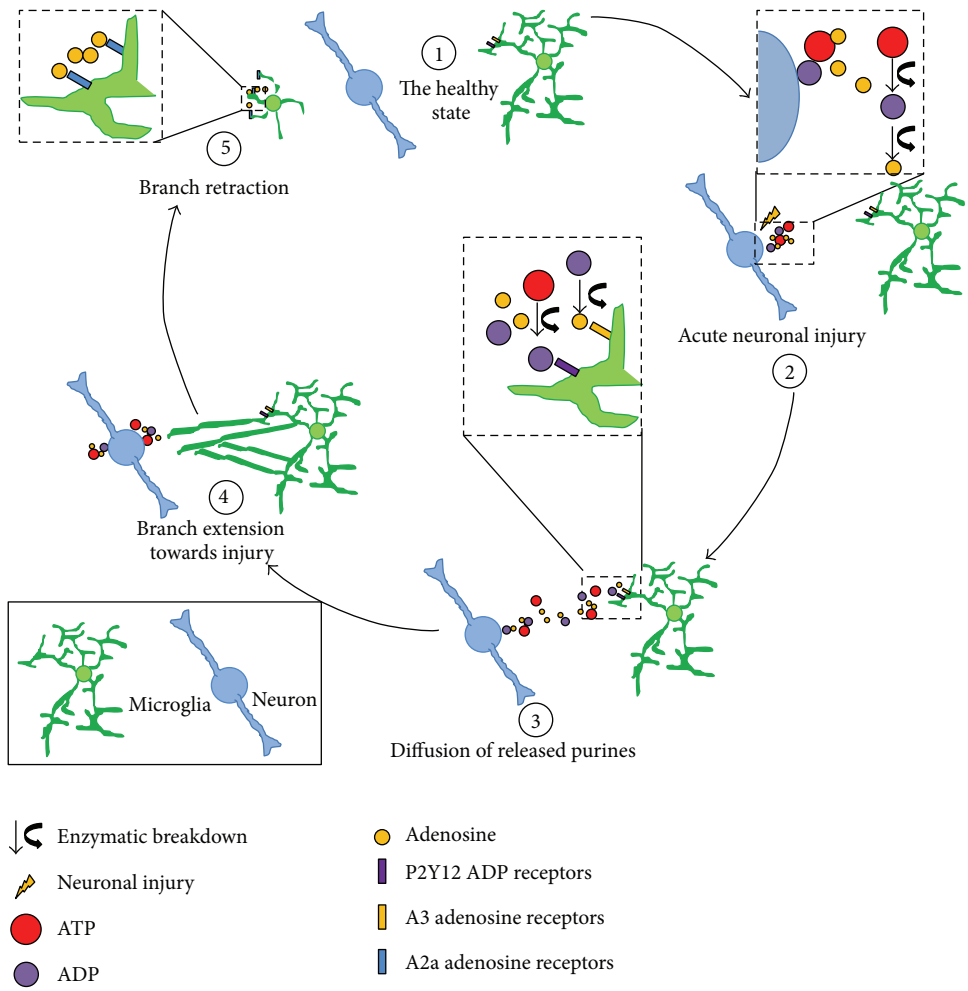

Figure 2: Neuron-to-microglia purinergic signaling regulates microglial extension and retraction. (1) In the healthy brain, microglia exist in close proximity to neurons. (2) In the event of neuronal injury, neurons release purines including ATP which can be degraded by endogenous enzymes into ADP and adenosine (magnification at top right). (3) Released purines diffuse in the extracellular space and can activate P1 (A3) and P2 (P2Y12) receptors on microglia that act in concert (magnification in center). (4) Purinergic activation leads to microglial branch extension towards the injury site. (5) Following microglial activation, adenosine can also activate A2a receptors that mediate microglial branch retraction.

The hypothesis that adenosine signaling may act in concert with P2Y12 signaling was first studied by Färber et al. [36]. The authors found that mice genetically deficient for a purine degrading enzyme that generates adenosine also showed deficient chemotaxis to ATP and ADP which was reconstituted by exogenous adenosine application. These results were extended *in vivo* in a focal ischemia model where microglia cells accumulate in neuron dense regions and microglial accumulation was significantly reduced in mice deficient in the ability to breakdown ATP/ADP to adenosine. More recently, the A3 adenosine receptor has been identified by Ohsawa et al. [37] using pharmacological approaches to be the specific adenosine receptor regulating microglial chemotaxis. A second adenosine receptor, the A2a receptor, has also been implicated in microglial process dynamics, and A2a receptor activation results in branch retraction [38].

Although purinergic signaling has been shown to be important for microglial motility during injury, an intriguing possibility exists that even in the healthy brain, physiological release of ATP is important. First, degradation of purines (ATP and ADP) by apyrase reduces basal microglial motility [31]. Moreover, zebrafish tectal neurons (but not microglia) were shown to express pannexin channels which release ATP upon glutamate uncaging suggesting that neurons may also communicate with microglia via purinergic signaling under physiological conditions [17]. Together, these results suggest that purinergic signaling is the most firmly established route of neuron-to-microglia signaling and may serve as a paramount mechanism by which the nervous system is maintained in a proper homeostatic state by microglia (Figure 2).

3. The Microglia-to-Neuron Communication Axis

By far, the literature is more extensive on the neuron-to-microglia communication axis in brain tissue or *in vivo*. Nevertheless interesting details are emerging on the specific ways

in which microglia may instruct neuronal function. We consider this communication axis along three lines. First, we discuss some of the recent imaging evidence that microglia make direct physical contact with neurons which suggest neuro-modulatory roles for microglia during normal physiology. Next, we summarize some of the emerging data indicating that microglia regulate neuronal circuitry through identified signaling pathways, including fractalkine, complement receptor, and DAP12. Finally, we highlight some data that indirectly indicate the physiological role of microglia in neuronal function and synaptic activities by microglia depletion strategies.

3.1. Microglia Cells Make Transient Physiological Contact with Neurons. Microglia have been long recognized to make physical contact with phagocyte postmortem or dying neurons [39]. However, whether they make physical contact with healthy neurons would require the development of advanced imaging techniques. Live two-photon imaging in the uninjured mouse cortex already revealed that microglial processes were extremely dynamic and constantly undergoing remodeling by repeated branch extension and retraction [18, 29]. This remodeling was thought to be essential for microglial sensing of the microenvironment.

Although microglial-to-neuronal soma contact in the living brain was observed by Nimmerjahn et al. [18], evidence for direct microglia-to-synaptic element contact in the living brain was first provided by Wake et al. [15] using two-photon imaging in the mouse cortex. The authors observed that, although seemingly undergoing random branch extension-retraction dynamics, microglial processes made direct and repeated contacts with dendritic spines. Interestingly, subsequent observations revealed that, in the developing brain, microglia-to-neuronal spine contacts were prolonged and microglia could modify the morphology of such spines during the third and fourth postnatal weeks [16, 40]. These results suggest direct microglia-to-synaptic element contact and a developmentally regulated mechanism in these interactions [15, 16]. Combining electron and two-photon microscopy, Tremblay et al. [16] were able to show that microglia indeed make contact with neurons, including synaptic spines, in the visual cortex in a manner that is dependent on visual experience. Moreover, similar interactions were reported in the both the visual and auditory cortices during adulthood and normal aging [41]. What then is the relevance of microglia-to-neuron contact? Wake et al. [15] found that following an hour of transient ischemia, microglia-to-neuron contact was prolonged from a duration of about 5 minutes in the healthy brain to about 80 minutes following transient ischemia and suggested that microglia function to monitor the functional state of neuronal synapses. In addition, work in the visual cortex showed that the physical contact of microglia with dendritic spines is able to alter the spine size in the healthy brain [16]. Microglial processes were found to preferentially localize to smaller dendritic spines that undergo the most dramatic changes in size during microglial contact. Moreover, chronic imaging indicated that a quarter of the microglia-contacted spines were eliminated over two days suggesting that microglia actively participate in the regulation of spine number and size in the healthy brain [16].

A more recent study in the zebrafish optic tectum also reported that microglial processes made bulbous contacts with neuronal soma under physiological conditions that increased with increasing neuronal activity [17]. Additionally, the authors reported that microglial processes made preferential contact with more active neurons as measured by calcium flux increases. Interestingly, microglia-contacted neurons displayed less activity following contact than non-contacted neurons, leading the authors to propose that a function of microglial-neuronal contact involves homeostatic mechanisms to downregulate neuronal excitability. However, future studies will have to identify the molecular mechanisms by which microglia "calm" excited neurons.

3.2. Microglia Cells Contribute to Neuronal Circuitry Establishment. In addition to the possibility that microglia are involved in regulating acute neuronal activity by the physical contact of neurons, microglia cells are also important in the more long term wiring of neuronal circuits. Knockouts of specific microglial receptors have now been studied to determine the contribution of microglia to neuronal circuitry development. Here, the emerging picture indicates that microglia cells are cellular components that participate in establishing functional neuronal circuits through several molecular pathways, including fractalkine receptor, complement receptor, and DAP12.

A recent study showed that fractalkine receptor knockouts displayed a transient reduction in hippocampal microglial numbers from the beginning of the second through to the end of the fourth postnatal week [42]. In the same mice, the authors observed increased dendritic spine density on hippocampal neurons during the second postnatal week [42]. High resolution microscopy data further showed microglia phagocyte synaptic components and that the increased spine density in fractalkine knockout mice is likely due to a defect in microglial phagocytosis of synapses during development. Evidence for microglial engagement with synaptic components using high-resolution microscopy has also been documented by other studies [16, 41, 43]. These initial reports on defective neuronal development in the hippocampus of fractalkine receptor-deficient mice were extended to the cortex in another study where proper maturation of thalamocortical circuits in the barrel cortex of developing mice was shown to require functional fractalkine signaling [44]. The behavioral consequence of microglial fractalkine signaling has also been investigated. Rogers et al. [45] showed that even the loss of a single functional fractalkine receptor allele resulted in significant deficiencies in motor learning, contextual fear, and memory. These behavioral observations were correlated with an impairment in cellular LTP, modulated molecularly by an increase in proinflammatory IL-1β release, and activity in fractalkine receptor heterozygotes and homozygotes relative to wildtypes. Moreover, this signaling axis has been shown to be important in mediating the enhancement of synaptic plasticity and spatial memory induced by rearing in an enriched environment [46].

As with fractalkine signaling, complement signaling has been reported in microglial pruning of developing neuronal synapses. During development, extranumerary synapses are pruned in the functional development of the nervous system. Microglia have now been identified in the process of synapse elimination, a process that involves neuronal communication to microglia via complement signaling. First, it was identified that C1q, an upstream member of the complement signaling cascade, colocalized with synapses in the developing CNS. Interestingly, genetic ablation of C1q resulted in an excess number of synapses during adolescence, a result that was recapitulated in mice deficient with C3, a downstream member of the complement cascade [47]. To determine the mechanism of elimination, a follow-up study further showed that microglia engulfed synaptic material in a C3-receptor-dependent manner during early postnatal development [43]. Thus, synapses to be eliminated were proposed to be tagged for elimination by complement proteins which serve as a signal to microglia for engulfment and subsequent elimination.

Apart from the fractalkine and complement signaling axes, a role for microglial DAP12 has also been reported in the development of functional neuronal synapses. DAP12, expressed mainly on hematopoietic cells, was shown to be exclusively expressed on microglia in the hippocampus around birth [48]. Intriguingly, in genetically deficient DAP12 mice, developmental apoptosis of neurons was decreased [49] but synaptic plasticity was enhanced [48]. Interestingly, DAP12 function has also been linked to TREM2, a known regulator of microglial phagocytosis [50, 51]. Another mechanism of DAP12's regulation of synaptic plasticity was suggested to involve brain derived neurotrophic factor (BDNF) signaling via its receptor (TrkB) on neurons as DAP12 deficient mice also had reduced TrkB expression at synaptic sites. DAP12 contains a tyrosine-based motif, a docking site for Syk tyrosine kinases, promoting activation of PI3 K and ERK pathways [52]. How these signaling pathways are coupled to BDNF pathway needs to be further investigated.

Microglial roles in normal neuronal circuitry are becoming increasingly appreciated from several recent studies correlating microglial function with behavior. For example, aberrant microglia cells were shown to be responsible for pathological grooming behavior in mice and could be rescued by bone marrow transplantation [53]. Similarly, microglial dysfunction, especially in phagocytosis, was implicated in a mouse model for Rett syndrome, an autism spectrum disorder [54]. Within the context of normal development, microglia cells were instructive in the determination of masculine features and behavior in developing rats. Here, compared to females, microglial numbers were significantly increased in the male preoptic area which is responsible for sex-specific development. Interestingly, the male preoptic area also had an increase in dendritic spines suggesting that microglia may actually stabilize existing and/or induce the formation of dendritic spines [55].

3.3. Microglial Depletion and Its Neuronal Effects. A useful approach to gain insights into microglial modulation of neuronal activity is to perform microglia depletion experiments. Currently, there are five methods being used for the ablation of microglia *in vivo* or in cultured brain slices. (1) One method involves the use of CD11b-HSVTK mice in which the herpes simplex virus thymidine kinase (HSVTK) is placed under the control of the CD11b promoter expressed exclusively by microglia in the brain [56]. Thymidine kinase converts ganciclovir into cytotoxic kinases leading to cell suicide. Thus, ganciclovir exposure can serve as an inducible cell suicide technique in HSVTK-expressing microglial cells in transgenic CD11b-HSVTK mice. (2) A second method involves the use of DTR mice in which the human dipthera toxin receptor (DTR) is expressed under the control of CD11b [57]. Human DTR expressing microglial cells can be selectively ablated by localized injection of the dipthera toxin in the mice. (3) A third method involves the use of PU.1 knockout mice that lack the PU.1 hematopoietic-lineage specific transcription factor resulting in a lack of mature hematopoietic cells including microglia [58, 59]. However, the mice die by late gestation or shortly after birth. (4) A fourth method involves the use of CSF1R knockout mice. CSF1R knockout mice in which a null mutation in a macrophage-specific receptor, the colony stimulating factor-1 receptor (CSF1R), results in effective elimination of brain microglia embryonically and postnatally [60]. Interestingly, these mice are viable for up to the third postnatal week. (5) The last method involves ablation using clodronate. Clondronate can function as an intracellular mediator of apoptosis. Once encapsulated in liposomes, they can be engulfed by phagocytes that degrade the liposomes to release its contents. This approach was first used in selectively depleting macrophages [61] but has now been applied to microglia ablation [62–64]. Clondronate has so far been used extensively in slice cultures and has only recently been employed for microglial depletion in the embryonic [62] and neonatal [63] brain. However, whether this method can be used in the adult brain to elucidate microglial roles in the healthy brain remains to be determined.

Although microglial ablation studies have been directed towards effects on disease progression, including multiple sclerosis [56], ischemic stroke [65], Alzheimer's disease [66, 67], ALS [68], epilepsy [69], bacterial meningitis [70], and brain tumor [71], the discussion of microglia in brain disease is beyond the scope of the current review. Therefore, we will mainly focus on recent studies using the microglia ablation strategy to gain important insights on the role of microglia in normal physiology, including brain development and synaptic transmission.

Microglia depletion studies showed that microglia is critical for brain development. By using the clondronate liposome ablation method, microglia can be effectively eliminated from neonatal cerebellar slices within three days [72]. Microglial depletion in this fashion prevented the death of Purkinje neurons suggesting that microglia at this early postnatal period are active in developmental neuronal death. The mechanism involves the activation of NADPH oxidase and the release of reactive oxygen species in killing neurons. The notion of microglia in brain development was further supported by a recent study using CSF1R knockout mice [60]. These mice were devoid of microglia by 65–99%

depending on brain region. In the postnatal knockout mice, there are defective brain structures including enlarged ventricles and compressed parenchyma in the olfactory bulb and cortex. Therefore, the results indicate the critical role of microglia in the maintenance of brain architecture during development; though since CSFR is also expressed in the periphery, a role for cells outside the CNS has not been ruled out.

Using microglia ablation strategies, recent studies also shed new light on the role of microglia in synaptic transmission. Pascual et al. [73] investigated the effects of microglial depletion on synaptic transmission using PU.1 knockout mice. Given the late embryonic or early postnatal lethality of mice with a disrupted PU.1 gene, the authors cultured hippocampal slices from PU.1 knockout mice at birth. As expected, microglia were lacking in these mice even after 10–14 days in culture. Interestingly, spontaneous neuronal activity in the form of excitatory postsynaptic potentials (EPSPs) remained unchanged between microglial "wildtype" and "knockout" slices. However, in the presence of inflammatory stimuli, such as LPS, microglia were able to alter neuronal EPSPs within minutes which was absent in slices lacking microglia. The mechanism involves ATP release from microglia and the participation of astrocyte in the modulation of synaptic transmission. These results indicate that microglia are able to directly sense inflammatory signals and rapidly translate that information indirectly to neuronal physiology. Another recent study has incorporated the clondronate ablation approach to provide insights to microglial regulation of neuronal activity. Using an organotypic hippocampal slice culture preparation with clondronate ablation of microglia, Ji et al. [64] reported that, in the absence of microglia, hippocampal neurons exhibited enhanced frequency of synaptic currents, while replenishment of microglia reverses the effect of microglial depletion on synaptic functions, suggesting that microglia reduce or properly "tune" synaptic activity. Consistently, culturing neurons in the presence of microglia resulted in a reduction in the number of synapses [64]. The study corroborates results from earlier studies that demonstrated microglial engulfment of synaptic material during early postnatal development of the murine hippocampus and lateral geniculate nucleus [42, 43]. It is worth noting that the studies by Pascual et al. [73] and Ji et al. [64] reported conflicting consequences to the absence of microglia on basal synaptic activity. Although it is not entirely clear why this is the case, it is possible that the discrepancy may be due to the different methods of microglial depletion and the timing of depletion. In summary, several approaches (pharmacological and genetic) are now available to begin to address neurotransmission in the absence of microglia. Thus far, the modulatory role of microglia in neurotransmission remains a poorly investigated endeavor but is sure to uncover novel insights into microglial functions in the nervous system. However, it should be kept in mind that depletion strategies that result in microglial death may alter the neural tissue milieus inadvertently altering neuronal activity. Therefore, data achieved using microglia depletion studies should be received with caution and performed perhaps with complementary approaches.

4. Conclusion

The role of microglia during injury and disease has been studied for a few decades. However, the dynamics of microglia-neuron communication in the healthy brain have only gained attention in recent years with already interesting results. The current evidence indicates that the communication between microglia and neurons is bidirectional involving several immunomodulatory factors and signaling axes including purinergic, neurotransmitter, chemokine, and complement signaling. Yet, many unanswered questions remain including the repertoire of microglial functions in the healthy brain during neural development and maintenance, the role of neurotransmitter signaling on microglial activity, and the immediate consequence of microglial engagement with synaptic elements making this field of research a veritable treasure throve in the next decade.

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Review Article

Microglia and Spinal Cord Synaptic Plasticity in Persistent Pain

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Microglia are regarded as macrophages in the central nervous system (CNS) and play an important role in neuroinflammation in the CNS. Microglial activation has been strongly implicated in neurodegeneration in the brain. Increasing evidence also suggests an important role of spinal cord microglia in the genesis of persistent pain, by releasing the proinflammatory cytokines tumor necrosis factor-alpha (TNFα), Interleukine-1beta (IL-1β), and brain derived neurotrophic factor (BDNF). In this review, we discuss the recent findings illustrating the importance of microglial mediators in regulating synaptic plasticity of the excitatory and inhibitory pain circuits in the spinal cord, leading to enhanced pain states. Insights into microglial-neuronal interactions in the spinal cord dorsal horn will not only further our understanding of neural plasticity but may also lead to novel therapeutics for chronic pain management.

1. Microglia-Synapse Interactions in Healthy CNS

Microglia are derived from myeloid precursor cells in the periphery and penetrate the central nervous system (CNS) during embryogenesis [1]. They account for approximately 10–20% of all cells in the CNS, however their distribution varies from one region to another [2, 3]. Microglial density is particularly high in the hippocampus, basal ganglia, substantia nigra, and spinal cord [2, 4]. Microglia are regarded as the resident macrophages in the CNS and, similar to peripheral macrophages, they display different morphology depending upon their physiological states. In the resting physiological state, microglial cells are ramified with slender, radially projecting processes with similar thickness, length, and ramification, whereas in pathological states, microglia can be activated presenting an amoeboid morphology characterized by large soma, short/thick, and radially projecting processes with few ramifications [4–6]. Although most studies have focused on the role of activated microglia and synaptic transmission, both resting and activated microglia dynamically interact with synapses shaping their connectivity and function [7].

Microglial processes constantly and dynamically survey their environment and interact with nearby synapses [8, 9]. In mature CNS, it has been observed that microglial processes interact with axon terminals and dendritic spines in a transient manner, for an average of five minutes and at a rate of approximately one microglial contact per hour [10]. Notably, microglia processes are driven by neuronal activity and can simultaneously interact with both presynaptic and postsynaptic elements. Reducing neural activity by inhibiting sensory inputs or lowering body temperature results in retraction of microglial processes and decreases the frequency of contacts between microglial processes and synapses [10]. It is well known that astrocyte processes envelop synapses and actively modulate physiological synaptic transmission; however, whether and how microglia directly influence physiological synaptic transmission is still unclear.

Several studies demonstrated that microglial processes can engulf synapses and participate to their phagocytic elimination in an experience-dependent manner in the mature healthy brain [11–13]. Interestingly, a progressive accumulation of microglial phagocytic-like structures was observed in both mouse visual and auditory cortices by age-related loss of vision and hearing, respectively [14]. This suggests that

microglia shape neuronal circuits not only during postnatal development but aslo along the lifespan. Together, these observations suggest that periodic interactions between microglia and synapses exist in the absence of pathological insult. These interactions may be compromised following nervous system injury or disease. The physiological role of microglia in spinal cord circuitry development and pain transmission remains to be investigated.

2. Nociceptive Pain and Persistent Pain

Our bodies play host to a wide variety of sensory information that is detected every moment by the peripheral nervous system. Primary sensory neurons that are responsible for the detection and transduction of painful stimuli (e.g., cold, heat, mechanical, and chemical), which are somatosensory stimuli that cause potential danger to the organism, are called nociceptors [15]. Nociception is of vital importance for survival; thus, it has become a highly regulated pathway within the nervous system of humans. Nociceptive input elicits pain as well as emotional, neuroendocrine, and autonomic responses. Persistent nociceptive input in this system after intense noxious stimulation or tissue injury results in activity-dependent plasticity or a progressive increase in the response of the system to repeated stimuli [16]. As a result of the neural plasticity, a normally innocuous low-threshold stimulation such as light touch could trigger a painful response (mechanical allodynia). Pathological pain or chronic pain results from neural plasticity both in the peripheral nervous system (i.e., peripheral sensitization) and CNS (central sensitization) [17]. The circuitry in the spinal cord dorsal horn connects incoming primary afferents to outgoing projection neurons that ascend to the brain. The projection neurons in the superficial dorsal horn (lamina I) also receive input from interneurons in the lamina II [18]. Importantly, spinal cord dorsal horn neurons undergo marked plastic changes in the pathological conditions, leading to hyperactivity of the projections neurons. Thus, dorsal horn is not only a critical relay center in nociceptive transmission [19] but also an important player in the development and maintenance of central sensitization [16, 17]. Several animal models are used for the study of neuropathic pain. In chronic constriction injury (CCI), the sciatic nerve is constricted by several loose ligatures. In spared nerve injury (SNI), the tibial and common peroneal divisions of the sciatic nerve are ligated and cut while sparing the sural division. In spinal nerve ligation (SNL), a spinal nerve, usually L5, is ligated where it exits the spinal column. Each of these models produces robust mechanical allodynia for weeks following injury; however, the underlying mechanisms may vary. Here we will focus on the contribution of spinal cord microglia to central sensitization in nerve injury-induced neuropathic pain and tissue injury-induced inflammatory pain states.

3. Spinal Cord Microglial Activation in the Context of Persistent Pain

Under pathological conditions, especially nerve injury conditions, microglia undergo "microgliosis", a complex set of changes that allow the cell to respond rapidly and perform a broad range of functions such as shielding injury sites, phagocytosing cellular debris, and releasing inflammatory signals to initiate and/or propagate the immune response. Traditionally, microgliosis has been determined by a change in morphology from ramified to amoeboid [20]. Several pain models of persistent neuropathic pain including CCI, SNI, SNL, or spinal cord injury as well as chronic morphine-induced hyperalgesia and tolerance are associated with the development of microgliosis in the dorsal horn of the spinal cord [21–23]. Figure 1 shows microglial reaction in the dorsal horn of the lumbar spinal cord seven days following CCI of the sciatic nerve in CX3CR1-GFP mice. CX3CR1 promoter activity is restricted to microglia [24]. GFP expression revealed evenly distributed resident microglia in the contralateral side of the spinal cord that exhibited a quiescent or resting type morphology (left, Figure 1). However on the side ipsilateral to injury (right, Figure 1), microglia of the dorsal horn and ventral horn showed enlarged and amoeboid morphology, indicating their activation. Nerve injury also induces marked increases in the density and number of microglia, due to proliferation and possible migration (Figure 1) [25]. Of interest, nerve injury-induced microgliosis is very mild in young animals (2-3 weeks old) [26]. It should be noted, however, that there are also a number of persistent pain conditions that are not associated with spinal cord microgliosis, such as adjuvant-induced inflammatory pain and chemotherapy-induced neuropathic pain [27, 28].

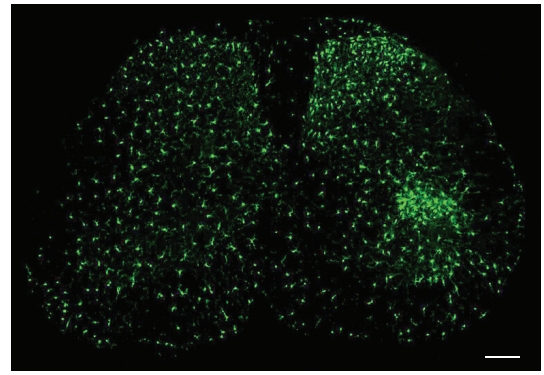

Figure 1: Nerve injury induces marked microglial reaction in the ipsilateral lumbar spinal cord 7 days after chronic constriction injury (CCI). Microglia as demonstrated by CX3CR1 expression in CX3CR1-GFP mice. The contralateral side (left) shows the typical resting microglial morphology, and the ipsilateral side (right) illustrates the enlarged and amoeboid morphological features of activated microglia cell bodies. Also note that on the ipsilateral side the number and density of microglia in the medial superficial dorsal horn and lateral ventral horn, where injured primary afferent axons terminate, are significantly increased. Scale, 100 μm.

Mitogen-activated kinase (MAPK) pathways are important for intracellular signal transduction and play critical roles in neuronal plasticity and inflammatory responses. The MAPK family consists of three separate signaling pathways: extracellular signal-regulated kinases (ERK), p38, and c-Jun N-terminal kinase (JNK). MAPK activation is correlated with

most if not all persistent pain conditions [29, 30]. Several neuropathic pain models, including spinal nerve injury and spared nerve injury, exhibit a robust increase in p38 activation (phosphorylation) in microglia of the dorsal horn beginning at twelve hours, peaking at three days, and slowly declining over several weeks [29, 31–33]. Intrathecal administration of p38 inhibitors has been shown to attenuate neuropathic pain [29, 33]. Minocycline, a broad-spectrum antimicrobial tetracycline compound that inhibits microglial activation, also decreases pain behavior following nerve injury [34, 35], possibly by inhibiting p38 activation [33]. However, minocycline is not able to reverse existing pain states [36]. Interestingly, intrathecal administration of minocycline in models of inflammatory pain, where morphological activation of microglia is not evident, also prevents the development of mechanical sensitization, by inhibiting spinal cord microglial p38 activation [37, 38]. Thus the release of inflammatory mediators from microglia may also occur without morphological alteration. The morphological changes associated with microgliosis may be mediated by the activation of ERK/MAPK [30], and nerve injury was shown to induce ERK activation in spinal microglia in the early phase [39]. It seems there may be multiple activation states whereby microglia do change the manner with which they participate in neural plastic changes, but do not reach a morphologically activated phenotype.

The p38 MAPK pathway can be activated by a host of molecules known to increase pain sensitivity, including the proinflammatory cytokines TNFα and IL-1β, CCL2 (also known as monocyte chemoattractant protein 1 (MCP-1)), fractalkine (CX3CL1), inducible nitric oxide synthase (iNOS), and matrix metalloprotease-9 (MMP-9) as well as the ATP receptors P2X4 and P2X7 [40–47]. As shown in Figure 2, some of these microglial activators, such as ATP, CCL2, fractalkine, and MMP-9, could be released from primary afferent neurons [48, 49]. Once activated, the p38 pathway induces the expression of proinflammatory transcription factors, enzymes, and signaling molecules, including NFkappaB, COX2, iNOS, BDNF, TNFα, IL-1β, and IL-6 [38, 41, 50]. p38 activation in microglia also results in increased release of BDNF and TNFα in microglia [22]. Microglial production of proinflammatory cytokines can further recruit microglia, activate surrounding astrocytes, and promote the sensitization of central nervous system nociceptive circuits.

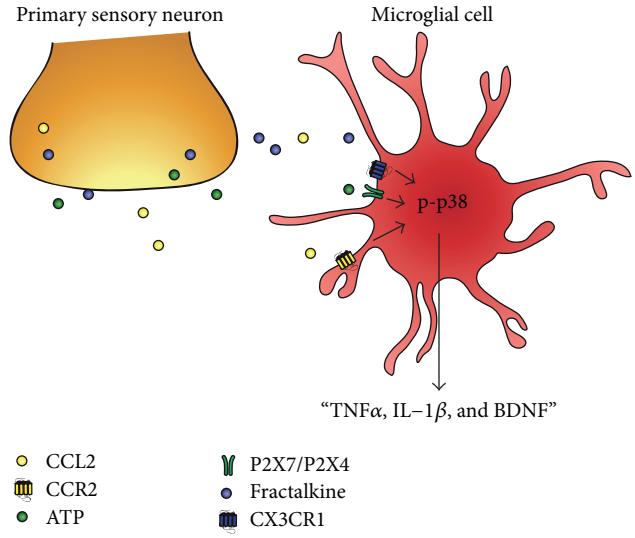

Figure 2: Schematic of primary afferent releasing factors that are responsible for microglial activation. Microglial cells are activated by factors released from hyperexcitable primary afferents such as CCL2 (MCP-1), ATP, and CX3CL1 (fractalkine). Respective activation of CCR2, P2X4/P2X7, and CX3CR1 receptors on microglia causes phosphorylation of p38 in microglia, leading to increased synthesis and release of TNFα, IL-1β, and BDNF.

4. Dorsal Horn Microglial-Synapse Interactions in the Context of Persistent Pain

The development of central sensitization in persistent pain is characterized by increased excitatory synaptic transmission and decreased inhibitory synaptic transmission in the dorsal horn of the spinal cord. In order to modulate pain sensitivity and participate in central sensitization, glia must interact with neural pain circuits via modulation of neurotransmission. Glial mediators can modulate synaptic transmission at very low concentrations. While neurotransmitters such as glutamate, GABA, and glycine produce synaptic effects at μM concentrations, glial cytokines, chemokines, and growth factors can affect synaptic activity at nM concentrations [51–53]. Accumulating evidence indicates a critical role of TNFα, IL-1β, and BDNF, all of which are released from activated microglia, in inducing the hyperactivity of dorsal horn neurons and thus in the development of pain hypersensitivity primarily in the setting of neuropathic pain, through modulation of both excitatory and inhibitory neurotransmission in the dorsal horn [40, 54, 55].

4.1. TNFα. TNFα is present both in healthy brain tissue and in disease states. TNFα is known to play a role in synaptic plasticity, which has been studied mainly in hippocampal slices. Glial TNFα has been shown to enhance synaptic efficacy by increasing the surface expression of GluR1-possessing AMPA receptors via TNFR1-mediated PI3 K activation [56, 57]. Glial TNFα also causes endocytosis of $GABA_A$ receptors resulting in a decrease in inhibitory synaptic currents [57]. Homeostatic synaptic scaling of excitatory synapses increases their strength in response to network activity reduction or decreases their strength in response to increased network activity. In response to decreases in network activity, glial TNFα was shown to increase AMPA-mediated currents by increasing the number of calcium permeable AMPA receptors at the cell surface [58]. However, its role may be more permissive rather than instructive in this change [59].

The effects of TNFα have also been studied in the dorsal horn of the spinal cord (see Figure 3). Intrathecal injection of TNFα causes the development of thermal and mechanical hyperalgesia [51]. To investigate the synaptic mechanisms of TNFα, many studies have used *ex vivo* spinal cord slice

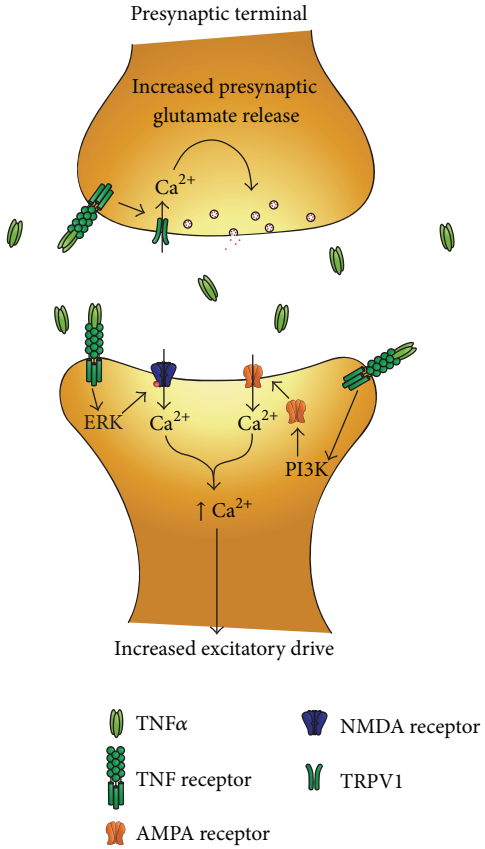

Figure 3: Schematic of TNFα induced potentiation of spinal cord synaptic transmission. Microglial release of TNFα increases excitatory neurotransmission in the dorsal horn via both presynaptic and postsynaptic mechanisms. At presynaptic sites, TNFα increases glutamate release via TRPV1 activation and there will be a subsequent increase in intracellular Ca^{2+}. At postsynaptic sites, TNFα increases the activity of AMPA and NMDA receptors via activation of PI3 K and ERK on glutamatergic neurons to increase excitatory drive.

preparation. Incubation with TNFα increases the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) in lamina II excitatory interneurons [60]. This could be indicative of a change in presynaptic glutamate release. TNFα increases sEPSC frequency via activation of transient receptor potential cation channel subfamily V member 1 (TRPV1) in presynaptic terminals, possibly through activation of adenylyl cyclase, protein kinase (PKA), or extracellular signal-related kinase (ERK) [60]. Activation of TRPV1 results in increased presynaptic calcium influx and, therefore, increased vesicular glutamate release [60].

TNFα also acts on the postsynaptic neurons in the spinal cord. In a carrageenan model of inflammation, TNFα recruited Ca^{2+} permeable AMPA receptors to dorsal horn neurons resulting in increased sEPSC amplitude [61]. NMDA currents in lamina II neurons are also enhanced by application of TNFα [51], and TNFα increases NMDA receptor (NMDAR) activity through phosphorylation of ERK in dorsal horn, neurons [62]. Thus via pre- and post synaptic mechanisms TNFα increases excitatory neurotransmission in the dorsal horn.

In spinal cord slices, TNFα not only enhances sEPSCs but also suppresses the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) [63]. This was found to be mediated by a decrease in spontaneous action potentials in GABAergic neurons via activation of TNF receptor 1 (TNFR1) and activation of p38 MAPK [63]. Neurons in the dorsal horn possess both TNFR1 and 2 (TNFR2), however, TNFR1 seems to make a greater contribution to enhancing nociceptive signaling in the dorsal horn [64].

In spinal cord slices from TNFR1 KO mice, TNFα was unable to elicit increases in sEPSCs or increases in NMDA currents [64]. However in TNFR2 KO mice, TNFα was still able to produce a small increase in sEPCS, and it elicited a normal increase in NMDA currents [64]. Both TNFR1 and TNFR2 knockout (KO) animals show decreased pain behavior in response to complete Freund's adjuvant and formalin induced inflammatory pain as well as intrathecal injection of TNFα [64]. Thus, microglial release of TNFα in the dorsal horn both enhances excitatory neuronal/synaptic activity and suppresses inhibitory neuronal/synaptic activity to enhance central sensitization primarily through the activation of TNFR1 on nociceptive dorsal horn neurons.

Long-term potentiation (LTP) in the spinal cord is implicated in pathological pain [65]. LTP in the spinal cord can be triggered by stimulation of the primary afferent fibers with the typical high-frequency titanic stimulation [66] and also by low-frequency stimulation (a more physiological firing pattern of nociceptors) [67] as well as by formalin or capsaicin administration to the paw or by nerve injury [16, 17, 67]. TNFα is also important for the induction of spinal LTP [68], and both TNFR1 and TNFR2 are required for titanic stimulation-induced LTP [60, 69]. While the prevailing view is that TNFα release from glia activates TNF receptors on neurons to promote LTP, new evidence has recently been found that TNF receptor expression on glial cells is also necessary for the generation of spinal LTP [69]. In the presence of fluorocitrate, a pharmacological blocker of glial activation, TNFα failed to potentiate AMPAR-mediated synaptic currents [69]. This suggests that there is an intermediate step which may facilitate TNFα-induced sensitization of dorsal horn neurons. TNFα could act on glial cells to elicit the release of additional glial mediators to facilitate its pronociceptive effects. Furthermore, pretreatment of slices with the microglial selective inhibitor minocycline inhibits LTP produced by high-frequency stimulation, however, application of TNFα reversed the effect [70], again suggesting that microglial TNFα release is important for long-term synaptic plasticity.

It is important to note that the effects of TNFα vary among regions of the CNS particularly at high pathological concentrations. While constitutive TNFα release may be permissive for plastic changes in neurotransmission, the activation of microglia can result in 10-fold higher TNFα concentrations and, at these high concentrations (greater than 0.3 nM), TNFα may change its mode of action [71]. When cultured in the presence of microglia, astrocytic glutamate released is dramatically amplified [72]. In hippocampal slices,

high concentrations of TNFα may cause prostaglandin E2 mediated glutamate release from astrocytes [73] to the degree that it causes excitotoxic damage in neurons [72]. It has been repeatedly demonstrated that at high concentrations (10–100 ng/mL), TNFα inhibited hippocampal LTP [74–76]. However, in spinal dorsal, horn both low and high concentrations of TNFα increased C-fiber induced LTP in nerve-injured rats [68].

4.2. IL-1β. IL-1β is induced in astrocytes, neurons, and microglia in inflammatory and neuropathic pain [77–80]. Intrathecal administration of IL-1β induces both thermal and mechanical hyperalgesia [81, 82]. Inhibition of IL-1β signaling through administration of IL-1 receptor antagonists decreased allodynia in neuropathic pain models [77, 83–85]. IL-1β enhances nociceptive activity in the dorsal horn through some of the same mechanisms as TNFα. Application of IL-1β to spinal cord slices increases sEPSC frequency, which indicates enhanced excitatory neurotransmission through increased release of neurotransmitter [51]. Importantly, IL-1β, but not TNFα, increased the amplitude of sEPSCs, suggesting additional postsynaptic regulation [51]. Also similar to TNFα, IL-1β enhances NMDA receptor currents in dorsal horn neurons [86], albeit by a different mechanism. IL-1β induces phosphorylation of the NR1 subunit of the NMDA receptor [87, 88]. NR1 is an essential subunit of the NMDA receptor, and phosphorylation of NR1 increases NMDA-mediated inward currents. In both inflammatory and neuropathic pain models, phosphorylation of NR1 increases NMDA receptor activity, facilitating excitatory neurotransmission in the dorsal horn [89–91].

IL-1β also suppresses inhibitory neurotransmission in the dorsal horn. Application of IL-1β to spinal cord slices inhibits both the frequency and the amplitude of spontaneous postsynaptic currents (sIPSCs) [51]. Therefore it functions via both pre- and postsynaptic mechanisms. Furthermore, application of IL-1β is capable of reducing both GABA and glycine mediated currents in dorsal horn neurons [51]. Overall, IL-1β enhances pronociceptive neurotransmission both through enhancing excitatory neurotransmission and suppressing inhibitory neurotransmission.

4.3. BDNF. Brain derived neurotrophic factor (BDNF) is a secreted protein and part of the family of neurotrophins which act on neurons to promote survival, growth, and differentiation of new neurons and synapses [92]. In the brain, BDNF is important for synaptic plasticity and long-term memory [93, 94]. However, in the spinal cord following nerve injury, it plays a deleterious role.

Following nerve injury, microglia upregulate their expression of the ionotropic ATP receptor P2X4 concurrently with the development of allodynia [95]. P2X4 receptor stimulation results in the activation of the p38 MAPK pathway and increases the synthesis and release of BDNF from microglial cells [96]. Application of BDNF to the spinal cord is capable of producing mechanical allodynia in otherwise naïve animals [97]. Furthermore, intrathecal administration of microglia activated by ATP into naïve animals produces mechanical allodynia, which can be alleviated by blockade of the BDNF receptor TrkB [95]. Thus microglial produce BDNF following nerve injury and BDNF is sufficient to produce pain behavior.

Microglial derived BDNF contributes to pain hypersensitivity through the disinhibition of nociceptive processing in the dorsal horn. BDNF acts on lamina I pain transmission neurons; neurons that carry the output message of the dorsal horn to higher brain centers where pain is perceived. BDNF, through the activation of its receptor TrkB, decreases expression of the potassium-chloride cotransporter 2 (KCC2) resulting in a rise in intracellular chloride [53, 98]. This shift in the neuronal anion gradient is sufficient to suppress inhibition in the majority of lamina I projection neurons, and in some neurons the shift is large enough that their response to application with GABA becomes excitatory rather than inhibitory [53, 99, 100]. The outcome is suppression of the intrinsic inhibitory circuit in the dorsal horn. Protein levels of KCC2 are down-regulated in the spinal cord following both spinal cord injury and nerve-injury models in parallel with the development of thermal and mechanical hypersensitivity [101, 102].

In naïve animals, lamina I projection neurons respond to painful but not innocuous stimuli. The suppression of inhibitory drive and thus exaggerated responses of lamina I neurons to normally noxious stimuli explains the development of hyperalgesia, an exaggerated response to normally noxious stimuli, but it does not explain the development of allodynia, the painful response to normally innocuous stimuli. Persistent pain sufferers report three cardinal features of their pain: hyperalgesia, allodynia, and spontaneous pain. *In vivo* recordings from lamina I projection neurons show that following nerve-injury, these neurons begin to respond to nonnoxious stimuli, increase their response to noxious stimuli, and discharge spontaneously [103]. Though the mechanism is not fully elucidated, these results can be recapitulated by disrupting the chloride homeostasis of lamina I projection neurons as well as by the addition of ATP stimulated microglia, which presumably secrete BDNF [103]. Thus microglial derived BDNF may participate further in the development of persistent pain conditions, yet the entire mechanism remains to be elucidated.

5. Concluding Remarks

Hyperactivity of peripheral nociceptive fibers due to inflammation or injury causes the release of factors such as CCL2, ATP, and fractalkine into the dorsal horn of the spinal cord [48, 49]. The binding of these factors to their respective receptors CCR2, P2X4, P2X7, and CX3CR1 on microglia causes activation of proinflammatory cascades and often gliosis [40–47]. p38 MAPK acts as a signal integrator of microglia stimulation. Activation of p38 results in the production and release of cytokines, chemokines, and proinflammatory mediators [22] that amplify pronociceptive signals in the dorsal horn.

We examined the mechanisms of three key signaling molecules released by microglial cells in the context of pain: TNFα, IL-1β, and BDNF. TNFα and IL-1β increase presynaptic release of excitatory neurotransmitter as well as increasing excitatory postsynaptic currents through the recruitment of

AMPA receptors and enhancing NMDA currents [51, 60–62, 86]. Both also suppress inhibitory neurotransmission through distinct mechanisms. TNFα decreases spontaneous activity in GABAergic neurons while IL-1β suppresses GABA and glycine mediated inhibitory currents [51, 63]. BDNF in the dorsal horn functions a wholly different mechanism to suppress inhibitory neurotransmission. Microglial release of BDNF decreases expression of KCC2 [53, 98]. This shifts the chloride gradient of the cell sufficiently to make the normally inhibitory currents resulting from GABA application negligible or even excitatory. Therefore, these proinflammatory mediators promote central sensitization through multiple mechanisms to increase excitatory drive and decrease inhibitory drive in dorsal horn nociceptive circuitry.

TNFα, IL-1β, and BDNF also affect long-term neuronal plasticity in the dorsal horn. Activation of their respective receptors, TNFR1, IL-1, and trkB, on neurons leads to the phosphorylation of ERK which can enter the nucleus and produce phosphorylation of cAMP response element binding protein (CREB) [51, 104–106]. Activation of the transcription factor CREB occurs following nerve injury [107]. Its activation leads to the transcription of genes such as COX-2, NK-1, and trkB which increase neuronal excitability and further promote sensitization [106, 108, 109]. These proinflammatory mediators encourage both short-term and long-term sensory plasticity in the dorsal horn resulting in an increase in ascending nociceptive neurotransmission to the brain. This central sensitization is a key component in the development of hyperalgesia and allodynia associated with persistent pain disorders, and microglia are emerging as a key contributor to central sensitization.

6. Future Directions

Recently, studies have found that microglia, like peripheral macrophages, are capable of distinct functional states referred to as classical or alternative activation. Classical activation is initiated by IFNγ, IL-1β, and LPS which increase production and release of proinflammatory molecules such as TNFα and IL-1β [110, 111]. Alternative activation is induced by IL-4, IL-13, IL-10, and TGFβ which activate anti-inflammatory cascades or tissue repair mechanisms in microglia [110, 112]. The anti-inflammatory actions of IL-4 and IL-13 are mainly a result of their ability to antagonize changes induced by proinflammatory cytokines and suppress the production of IL-1β and TNFα from microglia [113–117]. Additionally, IL-4 treatment increases the mRNA expression of repair genes, which are also markers of alternative activation in macrophages, such as arginase 1and mannose receptor 1, found in inflammatory zone 1 and Ym1 [118, 119]. Administration of IL-10 and TGFβ or uptake of apoptotic cells induces an alternative activation state sometimes called acquired deactivation, that as well as being anti-inflammatory is also an immunosuppressive state [110]. However, the majority of studies on alternative activation have been performed using peripheral macrophages, and anti-inflammatory signaling cascades in microglia have not been widely investigated and remain poorly understood. Furthermore, it also remains to be seen if these anti-inflammatory cytokines are capable of regulating synaptic plasticity in the dorsal horn.

A second class of anti-inflammatory, proresolution mediators are the lipid derivatives resolvins and neuroprotectins. Administration of resolvin E1 (RvE1) or neuroprotectin/protectin D1 (NPD1/PD1) can block TNFα induced synaptic plasticity as well as long-term potentiation in the dorsal horn [120–122]. Additionally, RvE1 is capable of blocking TNFα induced increases of postsynaptic NMDA currents via regulation of ERK [121, 122]. RvE1 and NPD1 can further block LPS-induced TNFα release in microglia [123, 124]. While blocking proinflammatory cytokine production remains a major avenue of clinical pain management, anti-inflammatory and proresolving lipid mediators may provide new avenues for controlling clinical pain.

Conflict of Interests

All the authors have no competing financial interests in this study.

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