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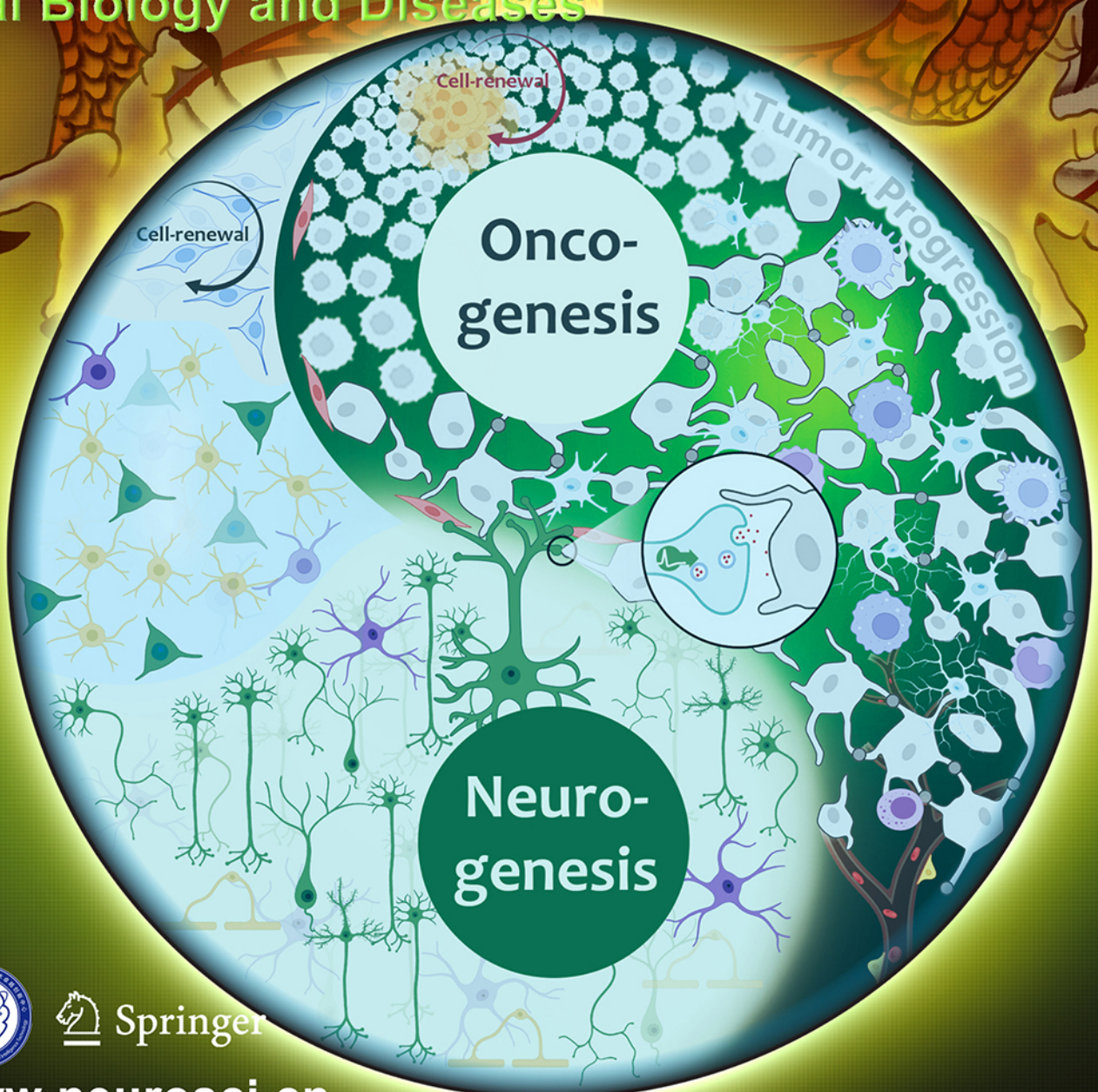
## Special Issue on Novel Insight into Glial Biology and Diseases

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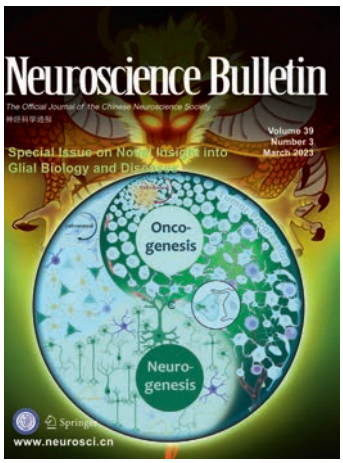
## About the Cover

Studying the function of glial cells is key to understanding how the brain works and what goes wrong in brain disease. This special issue aims to provide novel insight into glial biology and diseases. The cover of this special issue illustrates the relationship between neurogenesis and the oncogenesis of glioma, reviewed in this issue by Zhuang *et al*. The round ball on the cover was inspired by the traditional 'taiji 太极', highlighting the transforming mechanism behind the normal and abnormal processes. Inside the ball, gliomas exist in a complicated microenvironment, including immune cells, vascular cells, and neurons, and there are special communications between neurons and glioma networks. The dragon above trying to grab the ball indicates the importance of the neurobiology of gliomas. See pages 393–408. (Cover provided by Prof. Ying Mao and Hui Yang)

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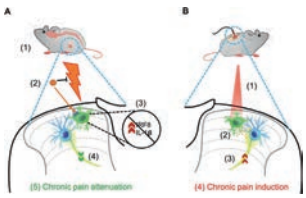
## Special Issue on Novel Insight into Glial Biology and Diseases

## Guest Editor: Prof. Cheng He



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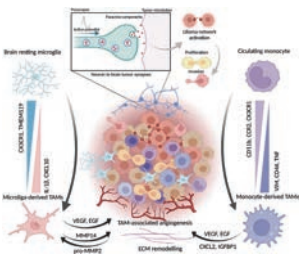

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## Reviews

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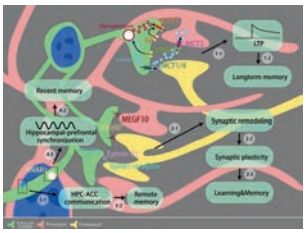
## Insight





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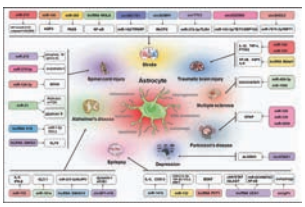

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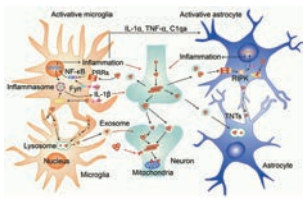

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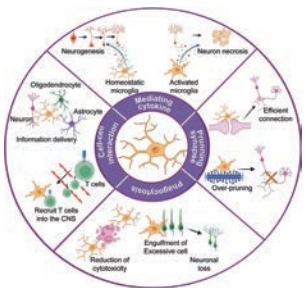

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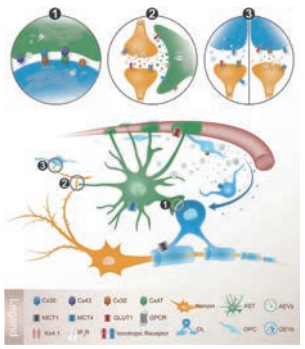

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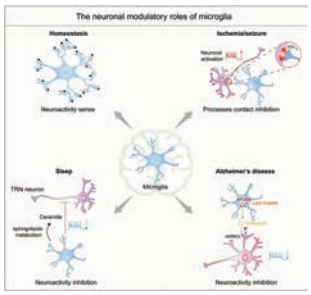
## Correction



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EDITORIAL

# Novel Insight into Glial Biology and Diseases

**Cheng He[1] · Shumin Duan[2]**

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In the central nervous system (CNS), there are mainly two types of nerve cells. One is neurons, the other is glial cells. Neurons are electrically excitable, whereas glial cells are not, and this is the fundamental difference between the two types of cells. Glial cells, consisting of astrocytes, oligodendrocyte lineage cells, and microglia, contribute more than half of the total cells in the mammalian CNS. Since Rudolf Virchow coined the term 'Neuroglia' in the last century [1], studies on the structure and function of glial cells have suggested that these cells are much more than supporting cells in the CNS. Astrocytes contribute to the maintenance of brain homeostasis; oligodendrocytes form myelin for axons; microglia are primary residence immune cells in the CNS. Glial cells are vital for the regulation of a variety of physiological and pathological processes in the CNS [2, 3]. This special issue consists of 14 Reviews and 1 Insight that provides the latest advances in glial biology and disease.

Glial cells exhibit high heterogeneity, as evidenced by varied origins, locations, developmental stages, and functional states [4–6]. Therefore, the selection of specific molecular markers is crucial for studying glial cells. In this issue, Huang *et al.* summarized the immunological markers of central glial cells and discusses the pros and cons of their applications [7]. Particularly, the distinct markers for oligodendroglia help to characterize the specialization, differentiation, and maturation of oligodendrocyte progenitors, and aid in understanding the delicate process of myelin regeneration in diseases. Non-coding RNAs (ncRNAs), with epigenetic and translational regulatory activity, play a critical role in glial physiology and pathology [8]. In view of the lack of protein markers of the varied functional states in glial cells [7], studies on the expression and function of ncRNAs may also provide specific markers for the characterization of glial cells in distinct functional states.

During early CNS development, neural stem cells first give rise to neurons and are followed by glial progenitor cells, which further produce oligodendrocyte progenitor cells or astrocyte progenitor cells, and then differentiate into oligodendrocytes and astrocytes, respectively [9]. The alteration of tumor-related genes in neural stem cells and glial progenitor cells leads to the development of gliomas [10]. Meanwhile, gliomas also adopt molecular signaling pathways in glial cells to achieve proliferation and migration [11]. Therefore, elucidation of the molecular biology of glial cells may benefit the understanding of glioma origin, microenvironment, and progression, as well as developing new therapeutic strategies.

Glial cells actively regulate neural circuit functioning. As a component of the tripartite synapse, astrocytes modulate synaptic transmission and plasticity effectively with their actions on neurotransmitter clearance and gliotransmitter release, beyond maintaining ion homeostasis in the extracellular space [3]. Astrocytes contribute to information processing as well as memory formation and storage through their interaction with neurons at the local and network levels [12]. Moreover, microglia perform surveillance of neuronal activity and respond rapidly to neuron-derived signals such as glutamate, purine, and norepinephrine, by undergoing

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biochemical and morphological changes to modulate neuronal activities in turn [13]. Glia-neuron interactions also participate in many pathological processes. For example, astrocytes are involved in the regulation of nociceptive transmission and network function to amplify pain signals under chronic pain conditions [14]. In this issue, Parusel *et al.* also discuss the role of microglia in the pathogenesis of neuropathic pain and stresses the importance of the specific regional and temporal manipulations of microglial function in the study of microglia-related neurological diseases [15].

Myelination enables the saltatory conduction of action potentials along axons, and dynamic changes of the myelin sheath, including myelin segment number, distribution, length, and thickness, affect conduction velocity and information processing in neural circuits [16]. Myelin also serves as a metabolic support for axons that underlie developmental and neurodegenerative diseases [17]. In this issue, Yang *et al.* compare the differential regulation of oligodendrocyte and Schwann cell biology in CNS and PNS myelination, particularly the interaction between growth factor and amino-acid signaling pathways, and demonstrate their roles in the metabolic support of axons [18]. With a focus on multiple sclerosis, a typical demyelinating disease, Sun *et al.* comprehensively summarize the glial roles in the connections between inflammation and neurodegeneration and propose that therapeutic improvement in multiple sclerosis requires a strategy combined with immune intervention, early neuroprotection, and the promotion of remyelination [19].

Glial cells are responsible for defense functions in the CNS, contributing to the maintenance of homeostasis. When glial cells detect xenobiotics, damaged cells, or toxic proteins, they are activated to remove them by phagocytosis; meanwhile, activated glial cells also release a variety of inflammatory factors [3, 20]. However, the mechanism underlying the interaction between phagocytosis and inflammation remains elusive. In this issue, Wang *et al.* discuss the association of glial activation and α-synuclein pathology in Parkinson's disease [21]. They suggest that glial cells activated by α-synuclein at the early stage promote the phagocytosis and clearance of the protein aggregates, while sustained glial activation by α-synuclein results in chronic inflammation, leading to the inhibition of phagocytosis and α-synuclein accumulation. Microglia are versatile effector cells in the degenerating brain that have been identified as a "double-edged sword" in the progression of Alzheimer's disease, a culprit in Parkinson's disease, and a lesion component in multiple sclerosis [22]. Therefore, microglial depletion and subsequent repopulation are proposed as promising therapeutic interventions for neurodegenerative diseases [22].

The concept of a glial-vascular unit, based on the framework of a neurovascular unit, emphasizes the central role of glial cells. The interactions between astrocytes, microglia, and perivascular cells are actively involved in the regulation of cerebral blood flow, formation of the blood-brain barrier, and clearance of toxic wastes [23]. Dysfunction of the glial-vascular unit has been demonstrated in ischemic stroke, spinal cord injury, Alzheimer's disease, and major depression disorders [23]. As the fourth type of glial cells in the mammalian CNS, in addition to astrocytes, oligodendrocytes, and microglia, NG2 glial roles in the pathological process of cerebral small vessel diseases have been appreciated [24]. Aberrant NG2 glial cells lead to the failure of remyelination and immunomodulation, suggesting strategies targeting these cells to alleviate white matter lesions in cerebral small vessel diseases [24].

Generally speaking, neural networks refer to neurons forming electrically excitable circuits through synapses. However, emerging evidence shows that glial cells themselves can also form networks. For example, through connexin-mediated gap junctions, connections are formed between astrocytes and oligodendrocytes [25]. These glial networks directly exchange metabolic substances and transduction signals, which are closely associated with many neurological disorders. In this issue, Hu *et al.* discuss the effects of glial network functioning on myelin development, blood-brain barrier integrity, and neuronal energy supply [26]. They highlight the possible role of the glial connections in the occurrence and development of myelin-related neurological diseases.

In summary, a growing body of evidence demonstrates the importance of glial cells in nervous system function and disease. Glial cells are essential for the regulation of synaptic transmission and neural circuit functioning. Future studies to characterize glial heterogeneity, metabolism, development, and aging, as well as the coupling between glial and neuronal networks, by using new techniques, would allow for a more complete and in-depth understanding of brain function and diseases.

**Acknowledgements** This editorial was supported by STI2030-Major Projects (2022ZD0204700).

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REVIEW

# Chemogenetic and Optogenetic Manipulations of Microglia in Chronic Pain

**Sebastian Parusel[1,2] · Min-Hee Yi[1] · Christine L. Hunt[3] · Long-Jun Wu[1,4,5]**

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**Abstract** Chronic pain relief remains an unmet medical need. Current research points to a substantial contribution of glia-neuron interaction in its pathogenesis. Particularly, microglia play a crucial role in the development of chronic pain. To better understand the microglial contribution to chronic pain, specific regional and temporal manipulations of microglia are necessary. Recently, two new approaches have emerged that meet these demands. Chemogenetic tools allow the expression of designer receptors exclusively activated by designer drugs (DREADDs) specifically in microglia. Similarly, optogenetic tools allow for microglial manipulation *via* the activation of artificially expressed, light-sensitive proteins. Chemo- and optogenetic manipulations of microglia *in vivo* are powerful in interrogating microglial function in chronic pain. This review summarizes these emerging tools in studying the role of microglia in chronic pain and highlights their potential applications in microglia-related neurological disorders.

**Keywords** Chronic pain · Microglia · Optogenetics · Chemogenetics · DREADDs · ReaChR

## Introduction

Neuropathic pain is a chronic condition that results in pain hypersensitivity and allodynia (pain responses to normally innocuous stimuli) after nerve damage that can occur after a host of insults, such as physical injury, diabetes, or autoimmune diseases [1]. When tissue damage has healed, however, neuropathic pain does not resolve [2]. A growing body of evidence indicates that microglia, as central nervous system (CNS) resident immune cells, play an important role in the pathogenesis of neuropathic pain [3–5]. Indeed, specific ablation or inhibition of microglia prevents the development of neuropathic pain [6–8]. In addition, recent progress highlights intimate microglia-neuron interactions in chronic pain [5, 9].

Microglia undergo functional changes during chronic pain states. In homeostatic conditions, microglia dynamically respond to changes in the microenvironment with their remarkably motile processes [10–13]. However, in response to peripheral nerve injury, microglia become activated and promote chronic pain. A major known mechanism for microglia to contribute to this process is through the release of cytokines and other mediators, such as interleukin-1 beta (IL-1β), IL-6, tumor necrosis factor alpha (TNFα), prostaglandin $E_2$, brain-derived neurotrophic factor, and reactive oxygen species. These signals can lead to chronic pain [5, 14]. In addition to diffusible molecules, microglia also contribute to chronic pain hypersensitivity by adopting new functional roles, such as altered transcriptional activation and phagocytosis [9]. However, it is important to note that microglial activation during chronic pain states is not always

Sebastian Parusel and Min-Hee Yi have contributed equally to this work.

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detrimental because they are indeed heterogeneous [15, 16]. Recent findings identified a subpopulation of activated microglia playing a beneficial role in resolving chronic pain after peripheral nerve injury [17].

The cellular mechanisms of microglia in chronic pain have been investigated through pharmacological approaches. For example, systemic inhibition of microglia and macrophages by the broad inhibitor minocycline attenuates pain hypersensitivity [7, 8]. However, minocycline also has inhibitory effects on other cells, such as neurons, astrocytes, and T-cells [18–20]. Inhibitors of microglia through the colony-stimulating factor 1 receptor (CSF-1R), such as PLX5622 [21, 22] and neutralizing colony-stimulating factor 1 (CSF-1) antibody, also reduce microglial activation and proliferation in the spinal dorsal horn after nerve injury and alter pain responses [21, 23]. Specifically, mechanical allodynia and thermal hyperalgesia are attenuated in CSF-1 inhibitor-treated mice with chronic pain [21, 23–26]. However, CSF-1R inhibition induces off-target effects in other peripheral immune cells expressing the receptor [22, 27].

The importance of spinal microglia in the development of chronic pain has also been demonstrated by using genetic approaches to remove key microglial genes such as P2X4 [28], P2X7 [29], CX3CR1 [30], TRPM2 [31], P2Y12 [32], and Hv1 [33]. In addition, multiple Cre lines including CX3CR1 [34], Sal1 [35], TMEM119 [36], HexB [37], and P2Y12 [38] have been developed to target microglia. However, the development of advanced tools that provide temporal accuracy and spatial specificity is still needed. In the past few years, precise and selective methods for manipulating microglia have been used to study their involvement in chronic pain. Here, we introduce recent advances in how microglia control the pathophysiology of pain by using chemogenetic and optogenetic approaches.

## Chemogenetic Approaches in Microglia

Chemogenetic approaches refer to the expression and activation of DREADDs [39, 40]. DREADDs allow the selective interrogation of multiple G-protein-coupled receptor (GPCR) signaling cascades, including Gq, Gi, and Gs in various cell types [40]. DREADDs can be specifically activated in a cell type of choice by locally or systemically applying a specific ligand, such as clozapine N-oxide (CNO), with minimal off-target effects. Chemogenetic approaches have historically been used in neurons to interrogate the neuronal circuitry underlying behaviors [41–43]. Similarly, numerous studies have also applied DREADD approaches in astrocytes to investigate their physiological alterations in GPCR-mediated $Ca^{2+}$ signaling [44], memory [45–47], neuroinflammation [48], and pain [49, 50]. Microglia express a number of GPCRs that are important for various microglial functions [51]. In particular, the microglial signature P2Y12 receptor is a Gi-coupled GPCR involved in the chemotaxis of processes towards ATP/ADP, which can occur after injury [52] and during the development of neuropathic pain [32, 53, 54]. Of the available chemogenetic GPCRs, Gi- (e.g. hM4Di) and Gq-signaling (e.g. hM3Dq) DREADDs have been used to investigate the functions of microglia in the CNS (Fig. 1).

### Chemogenetic Manipulation of CNS Microglia

Several studies have used chemogenetic approaches in microglia (Table 1). The Watkins's lab was the first to use Gi and Gq DREADDs in rat microglia by viral expression [55, 56]. Spinal microglia were transfected with adeno-associated virus (AAV) 9 containing DREADDs driven under the CD68 promoter. AAVs have been successfully used to target various cell types in the CNS. However, microglial transduction *in vivo* is complicated, and it may not achieve robust transfection levels [57]. However, in the Watkins studies, microglial DREADD transfection by AAV led to functional DREADD expression in the spinal cord. Microglial Gi DREADD activation can attenuate pro-inflammatory signaling including through the nuclear factor of the kappa light polypeptide gene enhancer in B-cell inhibitor alpha, NLR family pyrin domain-containing 3 (NLRP3), and IL-1β [55]. On the other hand, Gq DREADDs mediate microglial activation and cytokine release [58, 59], potentially through the mobilization of intracellular $Ca^{2+}$. Thus, Gq DREADD activation induces pro-inflammatory mediator production, while Gi DREADD activation inhibits lipopolysaccharide- (LPS) and chemokine (C-C motif) ligand 2-induced inflammatory signaling *in vitro* [56]. One potential confounder in these studies is the possibility that microglia might react with an immune response to AAV transfection [57]. However, it has been reported that AAV vectors (in contrast to adenovirus-based vectors) cause minimal immune reactions [60]. Nonetheless, careful use of adequate controls (DREADD expression without DREADD ligand administration) is essential to distinguish the effects of chemogenetic manipulation from the side effects of immune reaction to viral infection.

Other studies have more commonly applied gene knock-in approaches to selectively express Gi/Gq DREADD in microglia [61–63]. The C-X3-C motif chemokine receptor 1 (CX3CR1) is highly expressed by microglia in the CNS and cells of mononuclear origin in the periphery [64]. The use of constitutive $Cx3cr1^{cre/+}$:$R26^{LSL\text{-}hM4Di/+}$ mice results in Gi DREADD expression in all CX3CR1-expressing cells including microglia and monocytes [61]. To exclusively express Gi DREADD in microglia but not peripheral monocytes, researchers have used inducible $Cx3cr1^{creER/+}$:$R26^{LSL\text{-}hM4Di/+}$ mice [63]. Due to the fact that blood $CX3CR1^{+}$ cells have rapid turnover while microglia are longer-lived, it is possible to achieve greater microglial specificity by

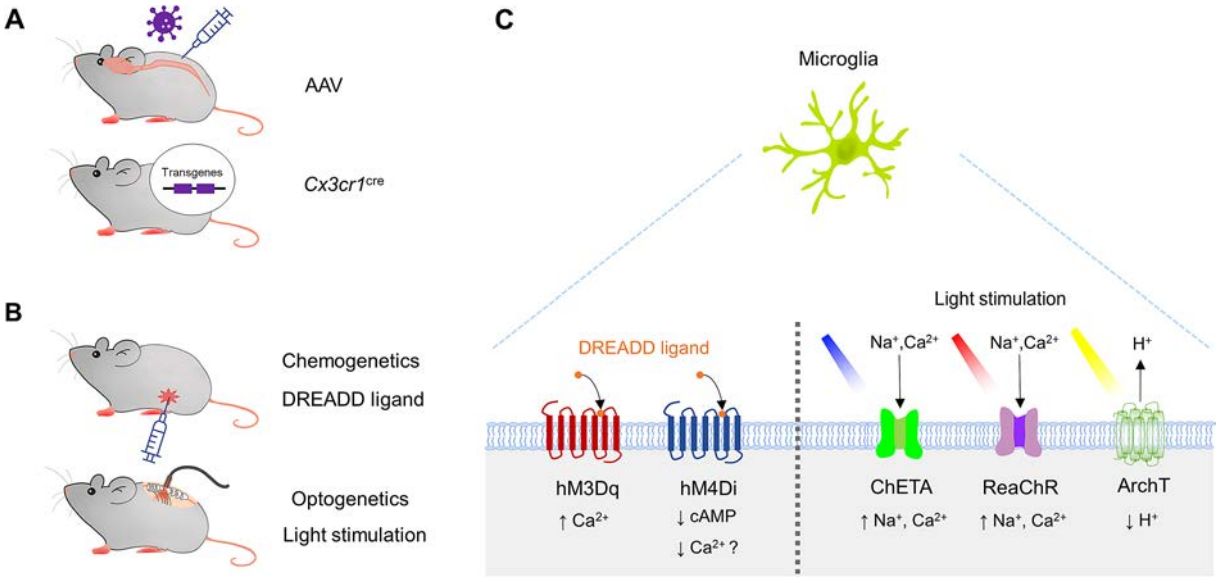


**Fig. 1** Chemogenetic and optogenetic approaches in microglia. **A** Viral-vector-mediated (e.g. AVV) or gene knock-in approaches (e.g. *Cx3cr1*$^{cre}$) introduce DREADDs or opsins specifically in microglia. **B** Activation of DREADDs (chemogenetics) or opsins (optogenetics) in microglia by a DREADD ligand (e.g. CNO) or specific light stimulation. **C** In microglia, a DREADD ligand binds to hM3Dq or hM4Di, to activate Gq- or Gi-coupled signaling; light stimulation of opsins opens non-selective cation channels (such as ReaChR or ChETA) or proton pumps (such as ArchT)

waiting for cell turnover after tamoxifen administration [34]. Indeed, DREADDs are expressed and co-localized only with Iba1$^+$ microglia in the spinal cord and brain 4 weeks after tamoxifen injection [63]. Using these genetic knock-in mice expressing Gi/Gq DREADDs in microglia, further studies have interrogated their function and underlying mechanisms in chronic pain (Fig. 2) [61–63].

In addition to pain research, chemogenetic methods were also recently applied to microglia to investigate their functions in inflammation, vascular interaction, and aversive behaviors. For example, inducible *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}hM3Dq/+}$ mice were used for the functional expression of microglial Gq DREADDs [65]. Gq DREADD activation induced intracellular $Ca^{2+}$ elevation and the phagocytosis of FluoSpheres in primary microglia. Unexpectedly, chronic Gq DREADD activation attenuated the LPS-induced increase of pro-inflammatory cytokines, including TNFα, IL-1β, and IL-6 in the mouse brain. In line with these results, chronic Gq DREADD activation in microglia robustly increased social exploration 2 h after LPS-induced inflammation [65]. Recently, a Cre-inducible lentiviral vector has been used to express DREADDs in dorsal striatal microglia of *Cx3cr1*$^{creER/+}$ mice [66]. Using this approach, both microglial Gq DREADD activation in naïve mice or LPS administration led to conditioned place aversion. Interestingly, microglial Gi DREADD activation before LPS administration prevents the development of conditioned place aversion without affecting markers of systemic inflammation [66]. Chemogenetic methods have been also applied to microglia to investigate neurovascular coupling changes [67]. *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}hM3Dq\text{-}CGaMP5g\text{–}tdTomato/+}$ mice were used for the expression of microglial Gq DREADDs. Activation of Gq DREADDs in microglia that interact with arterioles and microvessels in the cortex led to the withdrawal of perivascular microglial processes around arterioles and reduced the cerebral blood flow in response to whisker stimulation. Taken together, these bi-directional chemogenetic approaches have proven to be powerful tools in interrogating microglial function in the brain.

## Chemogenetic Manipulation of Microglia in Chronic Pain

The first study applying chemogenetic approaches through the viral expression of DREADDs in microglia studied the contribution of microglia to morphine-induced persistent sensitization in rats [55]. While opiates have been regularly used for pain treatment, they paradoxically induce nociceptive sensitization known as opioid-induced hyperalgesia [68]. Exposure to opioids in healthy individuals has been demonstrated to lead to hyperalgesia in many clinical studies, although large-scale trials cannot be performed in human subjects for ethical reasons [69, 70] Morphine-induced persistent sensitization is associated with microglial inflammasome activation in the spinal cord [55]. Microglial Gi DREADD activation reduces pro-inflammatory signaling and prevents morphine-induced persistent sensitization. Similar to microglial Gi DREADD

**Table 1** Chemogenetic applications in microglia

| References | DREADD expression | DREADD type | DREADD ligand administration (CNO) | Effects |
|---|---|---|---|---|
| Grace *et al.* [55] | hM4Di DREADDs *via* CD68-driven AAV9 (Spinal cord) | Gi | 20 μg/h i.t. for 5.5 d | Prevention of morphine-induced central sensitization |
| | | | 20 μg/h i.t. for 7 d | Attenuation of pro-inflammatory signaling |
| Grace *et al.* [56] | hM4Di DREADDs *via* CD68-driven AAV9 (Spinal cord) | Gi | 1 mg/kg i.p. or 60 μg i.t. | Inhibition of pro-inflammatory signaling and reversal of neuropathic pain |
| | hM3Dq DREADDs *via* CD68-driven AAV9 (Spinal cord) | Gq | 1 mg/kg i.p. or 60 μg i.t. | Induction of pro-inflammatory signaling and induction of chronic pain |
| Binning *et al.* [65] | *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}hM3Dq/+}$ | Gq | 1 mg/kg i.p. | Increase in phagocytic activity |
| | | | 1 mg/kg i.p. daily for 4 d | Attenuation of LPS-induced pro-inflammatory signaling |
| Saika *et al.* [61] | *Cx3cr1*$^{cre/+}$:*R26*$^{LSL\text{-}hM4Di/+}$ | Gi | 10 mg/kg i.p. or 20 nmol i.t. | Attenuation of neuropathic pain after PSL |
| Saika *et al.* [62] | *Cx3cr1*$^{cre/+}$:*R26*$^{LSL\text{-}hM3Dq/+}$ | Gq | 1 mg/kg i.p. or 2 nmol i.t. | Induction of chronic pain |
| Yi *et al.* [63] | *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}hM4Di/+}$ | Gi | 5 mg/kg i.p. daily for 3 d | Delayed development of neuropathic pain (DREADD activation 3 d prior to SNT)<br>Attenuation of neuropathic pain (DREADD activation 3 d after SNT) |
| Klawonn *et al.* [66] | *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}hM4Di/+}$ | Gi | 2 mg/kg i.p. | Prevention of LPS-induced place aversion |
| | hM3Dq DREADDs *via* Cre-inducible lentivirus (Dorsal striatum) | Gq | 2 mg/kg i,p, | Induction of place aversion |
| Császár *et al.* [67] | *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}hM3Dq\text{-}CGaMP5g\text{–}tdTomato/+}$ | Gq | 0.5 mg/kg i.p. or 1 μg/kg i.p. (DCZ) | Increase in microglial intracellular $Ca^{2+}$<br>Withdrawal of microglial processes around arterioles |

CNO, clozapine N-oxide; AAV, adeno-associated virus; DREADD, designer-receptor-exclusively-activated-by-designer-drug; i.t., intrathecal; i.p., intraperitoneal; LPS, lipopolysaccharide; PSL, partial sciatic nerve ligation; SNT, spinal nerve transection; DCZ, deschloroclozapine

activation, pharmacological blockade of toll-like receptor 4, P2X7, or the inflammasome can all independently block morphine-induced sensitization [55]. Thus, the mechanism underlying the action of Gi DREADD in microglia may be related to Gi inhibition of $Ca^{2+}$ elevation for pro-inflammatory cytokine production or release [58, 71].

Using viral expression of both Gq and Gi DREADD specifically in microglia, the group further studied the role of microglia in chronic pain in rats [56]. Gi DREADD activation in microglia rapidly reversed allodynia in neuropathic pain conditions. Mechanistically, microglial Gi DREADD activation attenuated the level of inflammatory mediators including nitric oxide (NO) and IL-1β. Microglial Gq DREADD activation, on the other hand, was able to induce allodynia in naïve male rats and increased the expression of pro-inflammatory mediators, such as NO, TNFα, Il-1β, and IL-6 [56].

After Cre-inducible DREADD mice were generated in 2016 [72], both Kiguchi's group and our group investigated microglial function in chronic pain in mice selectively expressing DREADDs in microglia. Using constitutive *Cx3cr1*$^{cre/+}$:*R26*$^{LSL\text{-}hM4Di/+}$ mice, Kiguchi's group showed that microglia expressing Gi DREADDs in the spinal cord, upon activation, alleviate pain sensitization after partial sciatic nerve ligation (PSL) [61]. Using inducible *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}hM4Di/+}$ mice, we showed that Gi DREADDs are specifically expressed in microglia in adult mice [63]. Microglial Gi DREADD activation 3 days before L4 spinal nerve transection (SNT) delayed the development of allodynia while activation 3 days after SNT attenuated mechanical allodynia [63].

Several potential mechanisms underlying microglial Gi DREADD in pain attenuation have been proposed [63]. First, activation of microglial Gi DREADD signaling

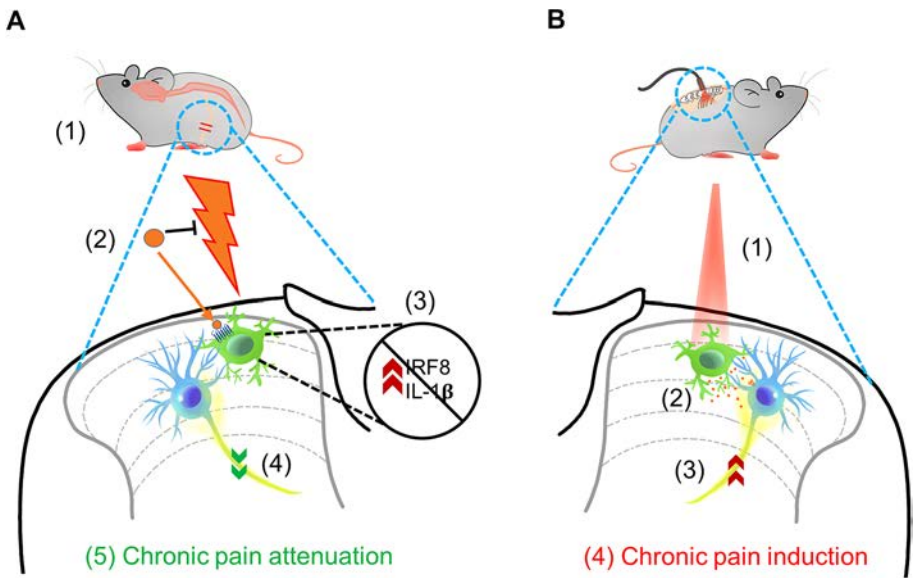


**Fig. 2** Mechanisms underlying chronic pain regulation by chemogenetic and optogenetic manipulation of microglia. **A** Chemogenetic activation of Gi DREADD attenuates neuropathic pain after peripheral nerve injury. After L4 spinal nerve transection (SNT) (1), CNO activation of Gi DREADD-expressing microglia (2) leads to microglial inhibition. Subsequently, SNT-induced microglial upregulation of IRF8 and IL-1β is inhibited (3). Thus, chemogenetic inhibition of microglia normalizes neuronal hyperactivity (4) and attenuates chronic pain behaviors after SNT (5). **B** Optogenetic activation of spinal microglia triggers chronic "microgliogenic" pain. Optogenetic stimulation of spinal microglia expressing ReaChR with red light (625 nm) (1) activates microglia and increases the $Ca^{2+}$-dependent release of IL-1β (2), which sensitizes neuronal activity (3), leading to chronic pain behaviors (4)

prevents microglial proliferation, known as the main source of microgliosis in neuropathic pain [4, 23]. Second, SNT-upregulated expression of interferon regulatory factor (IRF) 8, a transcription factor implicated in microglial transition to a reactive state [73], is prevented by Gi DREADD activation. Similarly, Il-1β as a critical mediator of neuropathic pain [73, 74], is also decreased by microglial Gi DREADD activation. Third, C-fiber-evoked field potentials *in vivo* are reduced upon activation of microglial Gi DREADD. These results complement and expand previous findings that selective microglial activation in the spinal cord promotes synaptic strengthening and synaptic plasticity between primary afferent C-fibers and spinal neurons [25, 75]. Together, these results indicate that Gi DREADD manipulation in microglia attenuates chronic pain by inhibiting microglial proliferation, neuroinflammation, and synaptic potentiation (Fig. 2A).

Microglial function in chronic pain was further investigated by using Gq DREADD in constitutive *Cx3cr1*$^{cre/+}$:*R26*$^{LSL\text{-}hM3Dq/+}$ mice [62]. After Gq DREADD activation, naïve male mice displayed allodynia and hyperalgesia. Further analysis showed that Gq DREADD activation led to a significant upregulation of inflammatory mediators (IL-1β, TNFα, CCL3, and CCL4) and microglial markers (Iba1, CD11b, IRF5, and IRF7). Importantly, these results point to a sex-specific mechanism, as both the behavioral correlates of pain and their potential underlying inflammation occurred in male but not female mice. After microglial ablation by PLX3397, an inhibitor of CSF1-R, Gq DREADD activation by CNO administration does not induce chronic pain hypersensitivity or the upregulation of inflammatory markers in male mice, further providing evidence that the chemogenetic activation of microglia is necessary for the development of chronic pain.

These findings are part of a larger literature finding an interesting sex-dependent microglial function in chronic pain. Although microglial activation (microgliosis) develops in both sexes in neuropathic pain models, emerging reports suggest that microglial inhibition resolves pain only in male mice. For example, microglia-targeted inhibitors (minocycline as well as inhibitors of TLR4, P2X4, or p38 mitogen-activated protein kinases) are effective in attenuating neuropathic pain in male but not female rodents [76–78]. One potential explanation is the involvement of T-cells in the development of chronic pain in females only, while males depend on microglia-related mechanisms [76]. In line with this idea, activation of Gi DREADD in CX3CR1$^{+}$ cells attenuates mechanical allodynia after PSL only in male mice [61]. While Gq DREADD microglia can initiate sex-dependent differences in pain responses, we have recently found that microglial Gi DREADD activation attenuates neuropathic pain in both male and female mice after SNT [63]. This discrepancy might be explained by different genetic manipulations (constitutive *Cx3cr1*$^{cre/+}$ *versus* inducible *Cx3cr1*$^{creER/+}$ mouse lines impacting different sets of cell

classes) or different CNO dose paradigms (one time-point only at 10 mg/kg *vs* three times at 5 mg/kg/day). Further, no sex differences have been reported in chronic pain attenuation after CX3CR1$^+$ cells are genetically ablated [6]. Future studies are needed to understand these potential discrepancies and determine the circumstances under which microglia may engage in sex-dependent chronic pain responses.

## Optogenetic Approaches in Microglia

Optogenetics fuse genetic and optical procedures to allow the manipulation of specific cell populations, conferring the unique capability to sense and respond to light through light-sensitive proteins in behaving animals [79]. All known organisms express photon-sensitive receptor proteins, called rhodopsins. The main types of opsins found in microorganisms are bacteriorhodopsins and halorhodopsins, which are light-driven ion pumps/channels such as channelrhodopsins (ChRs), and sensors such as sensory rhodopsin [80]. Optogenetic approaches have been widely used to drive the depolarization or hyperpolarization of selected neurons in response to specific wavelengths of light, allowing scientists to interrogate complex circuits underlying behavior [81, 82] including pain sensation [83]. Recent advances have also enabled optogenetic approaches to be applied to glial cells such as astrocytes and microglia [84–87]. For example, optogenetic approaches have been used to dissect astrocyte functions in breathing [84], memory [45], and epilepsy [85]. Furthermore, a recent study used ChR2, a non-selective, depolarizing cation channel, to selectively activate astrocytes. Depolarized spinal astrocytes elicited chronic pain behaviors by inducing ATP release [86]. In the periphery, optogenetic activation of ChR2-expressing macrophages in the heart improves the electrical connections underlying conduction [88]. Here, we introduce how optogenetics has been applied to microglia to dissect their function in the CNS, with a particular focus on chronic pain (Fig. 1).

### Optogenetic Manipulation of CNS Microglia

Ionotropic signaling is an overlook mechanism underlying microglial interactions with the brain microenvironment [89]. Unlike neurons, microglia have few voltage-gated $Na^+$ or $Ca^{2+}$ channels *in vivo*. Microglia mediate ionic fluxes using multiple ion channels including $K^+$ channels [90, 91], proton channels [92], transient receptor potential channels [93], pannexin-1 [94, 95], and purinergic ionotropic receptors [96]. The changes in microglial membrane potential in response to ion channel activation under pathological conditions are associated with the reactive microglial transition. For instance, prolonged increased $K^+$ channel conductance often precedes the reactive state transition [51, 97]. $K^+$ channels are also essential for microglial process surveillance and chemotactic responses to extracellular ATP/tissue injury [91, 98, 99]. However, it is not known whether the changes in membrane potential are either necessary or sufficient for microglial activation. Recent advances applying optogenetic approaches to microglia allow us to address these early questions [87, 100].

Only a few studies have used optogenetic approaches in microglia so far (Table 2). In a proof-of-concept study by Yamanaka's group, ChR2 was expressed specifically in microglia by using transgenic *Iba1-tTA:tetOChR2(CS128S)-EYFP* mice [101]. Blue light stimulation depolarized microglia indicating its functional expression, but no further studies were conducted using these mice. For the first time, red-activated ChR (ReaChR) was expressed in microglia using *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}ReaChR/+}$ mice [87]. The advantage of using ReaChR (a newer generation of non-selective cation channels) compared with ChR2 is its activation by red light, which has better penetration deeper into tissue with

**Table 2** Optogenetic applications in microglia

| References | Opsin Expression | Opsin Type | Light Stimulation | Effects |
|---|---|---|---|---|
| Tanaka *et al.* [101] | *Iba1-tTA:tetOChR2(CS128S)-EYFP* | ChR2 | 50 mW blue laser<br>500 ms pulses at 1 s intervals | Microglial depolarization |
| Yi *et al.* [87] | *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}ReaChR/+}$ | ReaChR | 625 nm, red LED<br>45 ms light on,<br>5 ms light off,<br>20 Hz for 30 min | Microglial depolarization<br>Increased microglial IL-1β expression<br>Induction of chronic pain |
| Laprell *et al.* [100] | *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}ChETA\text{-}tdTomato/+}$ | ChETA | 480 nm, blue LED<br>1 Hz light flashes for 20 min | Microglial depolarization<br>Slowed chemotaxis response to laser burn |
| | *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}ArchT\text{-}EGFP/+}$ | ArchT | 575 nm, yellow/green LED | Microglial hyperpolarization<br>No effect on chemotaxis response to laser burn |

ChR2, channelrhodopsin-2; ReaChR, red-activated channelrhodopsin; IL-1β, interleukin-1 beta ChETA a modified form of channelrhodopsin-2; ArchT, archaerhodopsin

less light scatter than the blue/green light for ChR2 activation. In addition, the ReaChR current can be maintained with far less inactivation occurring during light stimulation [102]. Selective microglial ReaChR expression can be achieved using *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}ReaChR/+}$ mice. In the spinal cord, rhodopsin protein is only co-localized with Iba1$^+$ microglia [87]. ReaChR expression is also functional as red-light stimulation induces inward currents in spinal microglia, resulting in their depolarization. In addition, pro-inflammatory cytokines such as IL-1β are secreted by primary microglia after red-light stimulation and this requires extracellular $Ca^{2+}$ influx [87]. Thus, optogenetic depolarization of spinal microglia is sufficient for $Ca^{2+}$-dependent cytokine release.

Similarly, ChETA (a modified form of ChR2) was expressed in microglia using *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}ChETA\text{-}tdTomato/+}$ mice [100]. ChETA activated by blue-light induced microglial depolarization and slowed the chemotaxis of processes in response to laser-induced tissue damage [100]. Microglia rapidly hyperpolarize when sensing ATP or neuronal hyperactivity [103]. Indeed, P2Y12-coupled $K^+$ channel activation is part of the mechanism for rapid chemotactic reactions to laser injury or basal motility [91, 98, 99]. A slower chemotaxis response induced by optogenetic microglial depolarization indicates that ATP-mediated hyperpolarization is not only a concomitant phenomenon of microglial activation but is required for the rapid expansion of microglial processes towards injury. One caveat of using blue light stimulation is the potential off-target effects. For instance, a study showed that microglia alter inflammatory-related gene expression with different levels of blue light stimulation [104]. Nevertheless, the results using ChETA indicate a potential correlation between the membrane potential and the chemotaxis of microglial processes. The light-activated proton pump archaerhodopsin (ArchT) has also been expressed in microglia using *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}ArchT\text{-}EGFP/+}$ mice [100]. Unlike ChR2 and its variants, ArchT activation by yellow/green light results in hyperpolarization. However, the ArchT-mediated hyperpolarization of microglia does not alter the electrophysiological responses of microglial to laser-induced tissue damage, nor does it affect chemotactic responses.

Microglia sense neuronal activity and the brain environment *via* $Ca^{2+}$ signaling [59, 105]. Indeed, increased $Ca^{2+}$ in microglia is strongly correlated with pathophysiological activation such as neuroinflammation [106], seizures [59], stroke [107], and neurodegeneration [108]. Since ChR2 and its derivatives are $Ca^{2+}$-permeable ion channels [109], optogenetic activation of microglia allows for the direct manipulation of $Ca^{2+}$ influx. As a result, ReaChR activation of microglia leads to $Ca^{2+}$-independent cytokine release [87]. On the contrary, in $Ca^{2+}$-free extracellular solutions, microglial chemotaxis to damage sites is significantly slowed, similar to the increased ionic influx during ChETA activation [100]. Therefore, these results suggest that optogenetic depolarization of microglia inhibits $Ca^{2+}$ elevation, thus slowing the chemotaxis of microglial processes. The underlying mechanism might be due to the reduction of the driving force for $Ca^{2+}$ during depolarization in microglia. Interestingly, previous studies found that the removal of extracellular $Ca^{2+}$ alone induces the convergence of microglial processes, similar to that reported in seizures and stroke [110, 111]. Future experiments using *in vivo* $Ca^{2+}$ imaging are needed to directly investigate whether optogenetic activation of microglia increases or decreases microglial $Ca^{2+}$ signaling.

### Optogenetic Manipulation of Microglia in Chronic Pain

By using *Cx3cr1*$^{creER/+}$:*R26*$^{LSL\text{-}ReaChR/+}$ mice to exclusively express ReaChR in microglia, spinal microglia can be depolarized in real time to examine their function in pain behaviors (Fig. 2B). Red-light stimulation can be delivered locally to the lumbar spinal cord through optic fibers. After light stimulation (30 min at 20 Hz,) mechanical allodynia is evident one hour after stimulation and lasts for up to one week in mice [87]. These results are remarkable in that short-term optogenetic stimulation of spinal microglia alone induced long-lasting pain behaviors. The mere stimulation of spinal microglia through optogenetics in the absence of any inflammatory challenge, or nerve damage-elicited chronic pain [87, 112], suggests the intriguing possibility of "microgliogenic" pain that originates from microglial activation in the CNS.

Mechanistically, optogenetic stimulation of microglial ReaChR increases microglial proliferation, neuronal activity, and nociceptive transmission [87]. For example, C-fiber-evoked field potentials and neuronal C-fos expression in the dorsal horn are significantly increased after microglial optogenetic stimulation. Interestingly, IL-1β expression is increased 1–3 days after light stimulation of microglial ReaChR, which could be due to increased expression of NLRP3 inflammasome components and caspase-1. The IL-1 receptor antagonist IL-1ra is sufficient to prevent increased C-fiber-evoked field potentials by light stimulation and alleviate light-induced mechanical allodynia. Thus, optogenetic stimulation of spinal microglia triggers IL-1β release, which increases the neuronal activity underlying chronic pain behaviors (Fig. 2B). In sum, optogenetics allows specific and temporally-controlled manipulation of microglia to study their function in pain. This may provide additional benefit over chemogenetic approaches in that the optical stimulation has better spatial and temporal resolution.

## Conclusions and Outlook

Chemogenetics and optogenetics are two emerging approaches recently applied in the field of microglia

research. DREADDs and opsin expression can be limited to microglia either by viral injection (e.g., AAV) or by promoter-driven conditional expression (e.g., CX3CR1). Unless activated, these proteins have no biological effects. Upon activation, existing DREADDs and opsins allow for a range of modulatory effects on microglia, including depolarization, hyperpolarization, and GPCR signal transduction. Chemo- and optogenetic manipulations of microglia are able to inhibit nerve injury-induced neuropathic pain or directly trigger chronic "microgliogenic" pain. However, it is important to note the limitations of chemo- and optogenetic approaches, as both use artificially-engineered proteins activated by designed stimuli. With chronic DREADD approaches, it has been suggested that repeated administration of CNO may lead to clozapine accumulation, which may have side effects unrelated to DREADDs [113]. The optogenetic stimulation of microglia might be unnaturally strong. In addition, it should be noted that effects may vary depending on the type of opsin and the frequency/intensity of light stimulation.

Here, we highlight the future of investigations of microglia by applying chemo- and optogenetic tools. (1) Microglia play a central role in many pathophysiological processes, such as in epilepsy [114], stroke [115], neurodegeneration [116, 117], depressive-like behaviors [118], memory deficits [119], and autoimmune neurology [120]. These new microglial tools will help illuminate the microglial mechanisms of neurological disorders. (2) Supraspinal microglial activation is also implicated in chronic pain [121–123]. Future studies will apply microglial tools to study their function in pain sensation, aversion, and comorbidities during chronic pain conditions. (3) Given the heterogeneity of microglia in neuropathic pain [15, 16] and their beneficial role in resolving chronic pain [17], it is unknown whether this heterogeneity also occurs when manipulating microglia using chemo- or optogenetic tools. Future studies will apply microglial tools to harness the beneficial function of alternatively-activated microglia during chronic pain conditions. (4) The ability of chemo- and optogenetic tools to directly manipulate $Ca^{2+}$ levels allows the investigation of the role of $Ca^{2+}$ signaling in microglia. Interrogation of the downstream Gi and Gq signaling in microglia is also made possible. Thus, the recent advances in microglial chemo- and optogenetic manipulations highlight the importance and novelty of these emerging tools in studying the function of microglia in neurological diseases, particularly in chronic pain.

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REVIEW

# Immunological Markers for Central Nervous System Glia

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**Abstract** Glial cells in the central nervous system (CNS) are composed of oligodendrocytes, astrocytes and microglia. They contribute more than half of the total cells of the CNS, and are essential for neural development and functioning. Studies on the fate specification, differentiation, and functional diversification of glial cells mainly rely on the proper use of cell- or stage-specific molecular markers. However, as cellular markers often exhibit different specificity and sensitivity, careful consideration must be given prior to their application to avoid possible confusion. Here, we provide an updated overview of a list of well-established immunological markers for the labeling of central glia, and discuss the cell-type specificity and stage dependency of their expression.

**Keywords** Glial cells · Oligodendrocytes · Astrocytes · Microglia · Markers

## Introduction

Glial cells, or glia, were first described by neuroscientists including Rudolf Virchow, Santiago Ramón y Cajal, and Pío del Río-Hortega over a century ago [1]. With time, it was demonstrated that glial cells are diverse in type and function. It is now clear that these cells constitute more than half of the total number in the mammalian central nervous system (CNS) [2, 3]. Central glia can be classified as macroglia, which refers to both astrocytes and oligodendrocytes, and microglia. They have distinct embryonic origins, developmental trajectories, and functions. During early CNS development, neural progenitor cells or radial glial cells (RGCs) first give rise to neurons, followed by glial progenitor cells. Glial progenitors then produce either oligodendrocyte progenitor cells (OPCs) or astrocyte precursor cells (APCs), which subsequently undergo a series of morphological and molecular changes to become functionally mature oligodendrocytes or astrocytes [4]. In contrast, microglia are CNS-resident macrophages which originate from the yolk sac and fetal liver during early embryogenesis [5, 6].

In the vertebrate CNS, oligodendrocytes form myelin sheathes around axons to facilitate the rapid propagation of action potentials and provide trophic support for the myelinated axons [7]. Deficits of myelin are found in many neurological diseases, such as multiple sclerosis and the leukodystrophies [8]. The development of oligodendrocytes is coordinated by a large cohort of intracellular factors and extracellular signals [9, 10]. In mouse spinal cord, OPCs are initially generated from the OLIG2+ ventral neuroepithelial cells of the motor neuron progenitor (pMN) domain around embryonic day 12.5 (E12.5), and later from the ASCL1+ dp3-dp5 neuroepithelial domains in the dorsal spinal cord at ~ E14.5 [11, 12]. In the embryonic telencephalon, the first group of OPCs originate from the NKX2.1+ medial ganglionic eminence (MGE) and anterior entopeduncular area (AEP) in the ventral forebrain at E12.5–E14.5. The second wave of OPCs are generated from the lateral (LGE) and/or caudal ganglionic eminences at E14.5–E16.5. Starting at ~

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E17.5, neural progenitors in the dorsal cortical region give rise to the third wave of OPCs [13]. The generation of OPCs follows a chronological order from ventral to dorsal along the entire anterior-posterior axis. Once generated, OPCs proliferate rapidly and migrate into the surrounding regions. OPCs start to differentiate into newly-formed oligodendrocytes (NFOs) at E18 in the spinal cord and at ~ P2–P3 in the forebrain. NFOs begin to contact and wrap around neuronal axons, express myelin proteins; and further differentiate into mature oligodendrocytes elaborating compact myelin sheaths. Notably, OPCs display significant functional redundancy throughout the CNS both during development and in adulthood, as the OPCs eliminated in one region are quickly replenished by OPCs from surrounding areas [13–15]. Studies over the past several decades have successfully identified a large number of stage-specific oligodendrocyte markers, critical tools for investigating the molecular and genetic control of oligodendrocyte development and axonal myelination in health and disease [16].

Astrocytes are the most functionally diverse glial cells and are involved in nearly all aspects of CNS physiology and functioning. The functional roles of astrocytes include but are not limited to maintaining the blood-brain barrier (BBB), providing physical and trophic support to neurons, axon guidance, synapse formation and remodeling, modulating synaptic transmission, regulating osmotic pressure, and ion homeostasis [17]. As noted earlier, APCs also originate from late RGCs in the ventricular zone of the CNS after neurogenesis. At the early stage of neural development, APCs undergo rapid local proliferation to expand their population. However, there are several unique features in the development of astrocytes as compared to that of oligodendrocytes. First, astrocytes arise from nearly all of the ventricular regions in the CNS and migrate to their final destinations in the direction of their radial glial processes [17]. Second, there is no functional redundancy among astrocytes in different regions [18]. Astrocytes are allocated to spatial regions in accordance with their sites of embryonic origin in the ventricular zone (VZ), and do not migrate tangentially to adjacent areas throughout life, even after acute CNS injury [19]. Third, RGCs, APCs, and astrocytes share many of the same molecular markers, such as GFAP and ALDH1L1 [20, 21]. Therefore, unlike the development of oligodendrocytes, there are few definite stage-specific molecular markers for the astrocyte lineage, and transcription factors that control the differentiation and maturation of astrocytes have yet to be found. Based on these and other findings, we propose that astrocytes are dormant neural progenitor cells that are influenced by local environments and are functionally adapted to support the local neuronal populations [22].

Unlike oligodendrocytes and astrocytes that are derived from neural progenitor cells, microglia are CNS-resident macrophages of blood origin. The CNS macrophages comprise microglia and border-associated macrophages (BAMs, also termed CNS-associated macrophages) in the meninges, choroid plexus, and perivascular spaces. Although microglia have been studied for decades, their developmental origin has been under debate for quite a long time. Now it is known that the bulk of microglia originate from hematopoietic progenitors in the yolk sac and enter the CNS tissue during early embryonic development [23, 24]. With the closure of BBB, renewal of the microglial population in the CNS mainly depends on their own proliferation [25]. With the aid of a number of cell-specific markers, microglia have been found to play critical roles in the development and functional maintenance of the CNS, including synaptic elimination, neural circuit wiring, axon tract fasciculation, vasculature tip cell fusion, synapse pruning, and inflammation [26].

In the past decade, a significant progress has been made in the studies of glial development, homeostasis, reactivation, regeneration, and other functions in the CNS. Clearly, these studies are highly dependent on the proper use of specific molecular markers for particular developmental stages or functional states. Theoretically, the most important features for a "perfect" marker gene are its high specificity and affinity. However, marker genes often display varying degrees of specificity and efficiency, and they even vary at different developmental stages or between species. Therefore, the proper use of cellular markers is critical for drawing correct conclusions.

Here, we provide a comprehensive and updated review of a list of well-characterized glial markers and discuss the pros and cons of these markers in their applications (Table 1).

## Markers for Cells of Oligodendrocyte Lineage

### SOX10

The SRY-box transcription factor SOX10 is one of the earliest identified pan-oligodendroglial markers and is exclusively expressed in cells of the oligodendrocyte lineage in the CNS [27]. Genetic studies revealed that SOX10 plays a central role in controlling oligodendrocyte development, as conventional deletion of the *Sox10* gene leads to a dramatic delay of OPC differentiation with little impact on OPC generation and distribution in spinal tissue [28]. However, *Sox10*-KO enhances the phenotype of *Sox9* mutants in OPC generation [29, 30], while the double knock-out of *Sox8* and *Sox10* genes in differentiated oligodendrocytes results in demyelination in the mouse CNS [31, 32]. These findings suggest that SOX10 regulates nearly all stages of oligodendrocyte development, including their initial generation from neural progenitors, terminal differentiation, and myelin maintenance. As a specific marker for the oligodendroglial

**Table 1** Cellular markers for glial cells

| Markers | Applications | Specificity and efficiency |
|---|---|---|
| SOX10 | Pan-oligodendrocyte lineage cells | Excellent |
| OLIG2 | Pan-oligodendrocyte lineage cells | Also expressed in NPCs in the ventral forebrain and pMN domain of spinal cord at embryonic stage, cortical MIPCs and brain astrocyte before weaning stage |
| PDGFRA | OPCs | Excellent in rodents, weakly expressed in human cortical MIPCs |
| NG2 | OPCs | Also labels vascular pericytes, especially in embryonic and newborn CNS |
| CC1/QKI7 | NFOs and mature OLs | Expressed in NPCs and immature macroglial cells in neonatal CNS |
| MYRF | NFOs and mature OLs | Excellent |
| ENPP6, BMP4 | NFOs | Perfect, but lack of antibodies |
| O4 | Pre-myelinating OLs | Perfect, but is not suitable for counting *in vivo* |
| NKX2.2 | Pre-myelinating OLs | Fine for *in vivo* labeling, also expressed in some neurons |
| CNP, MBP, PLP, MAG, MOG | NFOs and mature OLs | Excellent. Reflects the biomass of myelin sheaths by immunostaining, and ISH is required for cell counting (except MBP) |
| CAII | Type I/II OLs | Specific to mature OLs myelinating small diameter axons |
| GFAP | White matter astrocytes and reactive astrocytes | Also expressed in late RGCs |
| S100B | Gray matter astrocytes | Does not label white matter astrocytes, also expressed in differentiating oligodendrocytes. Not expressed in RGCs and newborn astrocytes |
| ALDH1L1, ACSBG1 | Pan-astrocyte lineage | Also expressed in RGCs |
| SOX9, NFIA | Pan-astrocyte lineage | Also expressed in RGCs and OPCs; suitable for cell counting |
| FABP7 | Pan-astrocyte lineage | Also expressed in RGCs; transiently and weakly expressed in OPCs |
| GLAST | Pan-astrocyte lineage | Transmembrane protein; also expressed in RGCs |
| GS | Pan-astrocyte lineage | Also expressed in RGCs and myelinated OLs |
| AQP4 | Astrocyte end-feet | Not expressed in RGCs and newborn astrocytes. AQP4 is concentrated in astrocytic end-foot membranes surrounding blood vessels |
| IBA1, CX3CR1 | Pan-microglia | Also expressed in macrophages |
| P2RY12, TMEM119 | Resting microglia | Not expressed in macrophages |
| CD68 | Reactive microglia | Dotted distribution of immunostaining signal |
| CD206, LYVE1 | Macrophages including BAMs | Not expressed in microglia |
| HEXB, Siglec-H | Pan-microglia | Not expressed in macrophages including BAMs |

NPCs, neural progenitor cells; OPCs, oligodendrocyte progenitor cells; NFOs, newly-formed oligodendrocytes; pMN, motor neuron progenitor domain; MIPCs, multipotent intermediate progenitor cells; CNS, central nervous system; OLs, oligodendrocytes; RGCs, radial glial cells; BAMs, border-associated macrophages

lineage, SOX10 is activated in OPCs immediately after their fate specification in the ventricular regions (Fig. 1A, B). SOX10 expression is maintained in OPCs and markedly upregulated at the onset of OPC differentiation. It is worth highlighting that Sox10 is considered to be the most reliable pan-oligodendrocyte marker in both brain and spinal cord, due to its specificity and high efficiency in labeling all cells of oligodendrocyte lineage throughout the life span.

## OLIG2

This gene is probably the most frequently-used marker for cells of oligodendrocyte lineage, partly due to its commercial availability. However, OLIG2 is not always a reliable marker for labeling oligodendroglia. In the spinal cord, OLIG2 expression is initially activated in neural progenitors of the pMN domain at ~ E8.5, but is rapidly lost from their motor neuron progeny during neurogenesis [33, 34]. As development proceeds, OLIG2 expression is retained in OPCs but is slightly downregulated in differentiated oligodendrocytes [35, 36]. In the brain, OLIG2 is broadly expressed in the VZ of the ventral telencephalon, including the LGE, MGE, and AEP regions at embryonic stages [37–39]. In the dorsal cortical region, the earliest OLIG2-positive cells appear at ~ E17.5, coincident with the onset of local gliogenesis [40, 41]. These OLIG2+ pluripotent precursor cells, termed multipotent intermediate progenitor cells (MIPCs) (also named pri-OPCs or pre-OPCs in other studies [42, 43]), are the common precursors of cortex-derived astrocytes, oligodendrocytes, and olfactory bulb interneurons (OBiNs) [40, 44] (OLIG2+ cells in the VZ and subventricular zone (SVZ), Fig. 1B). Despite its rapid downregulation in OBiNs, OLIG2 expression is sustained in glial cell progeny, including newborn astrocytes (APCs)

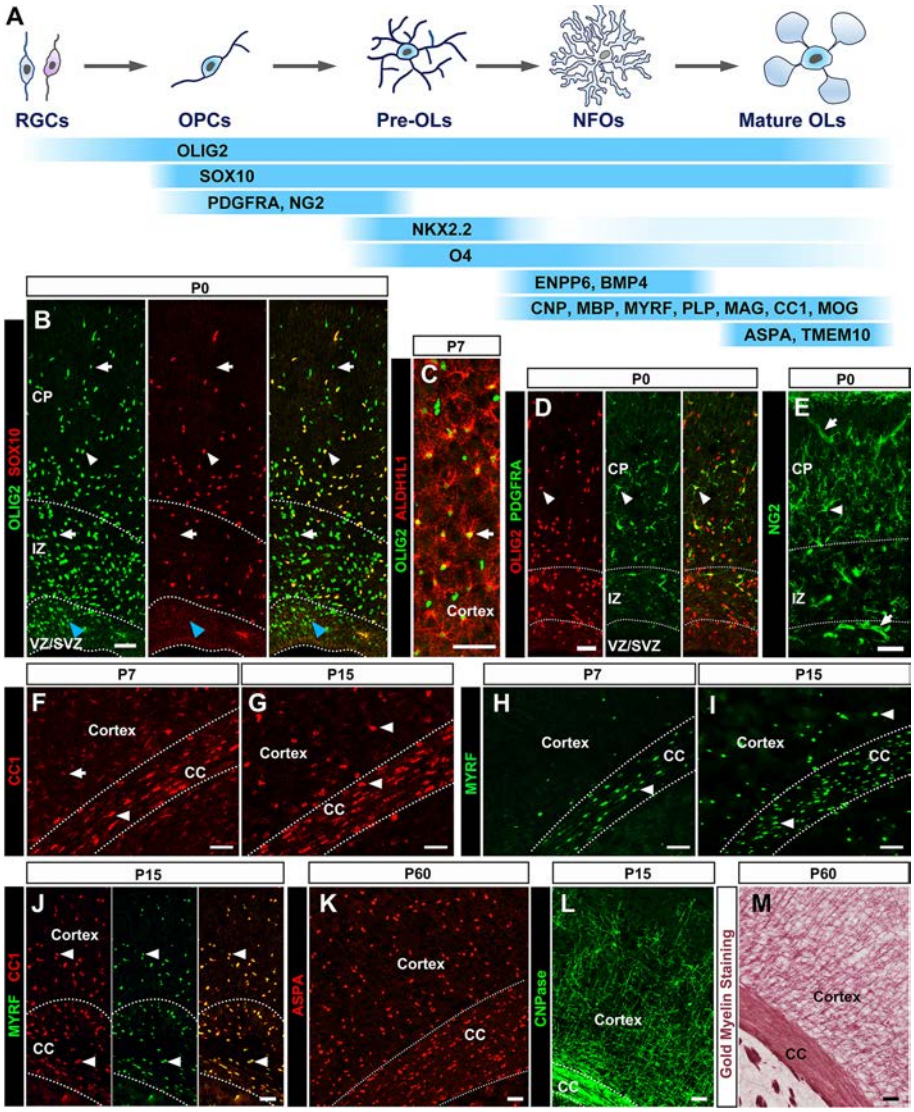


**Fig. 1** Molecular markers for cells of oligodendrocyte lineage. **A** Diagram of oligodendrocyte development and stage-specific markers. **B** OLIG2+ cells include SOX10+ OPCs (white arrowheads), SOX10- astrocytes (arrows), and MIPCs in the SVZ of P0 mouse cortex (blue arrow heads). **C** Nearly all ALDH1L1+ astrocytes express OLIG2 in P7 mouse cortex (arrows). **D**, **E** PDGFRA and NG2 label OPCs in P0 cortex (arrowheads). Note that NG2 is also expressed in vascular pericytes (arrows in **E**), while PDGFRA is not. **F**–**J** CC1 but not MYRF is weakly expressed in astrocytes in P7 cortex (arrows). At P15, all of the CC1+ cells are MYRF+ differentiated oligodendrocytes (arrowheads). **K**, **L** Expression patterns of ASPA and CNPase in mouse cortex. **M** Brain myelin structures visualized by gold-based staining. NPCs, neural progenitor cells; OPCs, oligodendrocyte progenitor cells; Pre-OLs, pre-oligodendrocytes; NFOs, newly-formed oligodendrocytes; OLs, oligodendrocytes; VZ/SVZ, (sub)ventricular zone; IZ, intermediate zone; CP, cortical plate; CC, corpus callosum. Scale bars, 50 μm.

and OPCs [40, 44, 45] (Fig. 1B, C). In the cortical region of neonatal mice, nearly all astrocytes are immunoreactive to OLIG2 until P7 (Fig. 1C). Within the next week or so, the expression level of OLIG2 is rapidly downregulated as they progress further along the astrocyte lineage. By the age of animal weaning, ~ 99% of the OLIG2+ cells in cortical tissue co-express SOX10, and *vice versa*, indicating that OLIG2 predominantly labels oligodendrocyte cells at this stage [46] (Fig. 1B). In summary, OLIG2 is an excellent pan-oligodendroglial marker in the spinal cord, since only a small fraction of pMN-derived astrocytes expresses this gene during embryonic stages [47, 48]. However, OLIG2 cannot be used as a specific oligodendrocyte marker in embryonic or early postnatal brain tissue. Consistent with the regional difference in OLIG2 expression, deletion of the *Olig2* gene affects the development of cortical astrocytes [45, 49], but not that of spinal astrocytes [50, 51].

## PDGFRA and NG2

Platelet-derived growth factor α receptor (PDGFRA) and NG2 proteoglycan (also known as chondroitin sulfate proteoglycan 4) are commonly-used markers for OPCs in the CNS. In rodents, PDGFRA is exclusively expressed in OPCs from their origin from neural progenitor cells in both the embryonic and adult CNS (Fig. 1D) [52, 53]. The function of the PDGFA/PDGFRA signaling pathway is to stimulate OPC proliferation, but meanwhile to inhibit their differentiation. Ablation of either *Pdgfa* or *Pdgfra* at embryonic stages causes premature differentiation of OPCs, resulting in severe hypomyelination due to the reduced proliferation and faster depletion of the progenitor pool [54, 55].

NG2 is another widely-used OPC marker, and NG2+ glial cells produce myelinating oligodendrocytes in adult CNS tissue [46, 56–58]. However, unlike PDGFRA, NG2 is also expressed in vascular pericytes, especially in the embryonic

and newborn brain (Fig. 1E) [59, 60]. In general, PDGFRA is a more reliable OPC marker than NG2 for both *in situ* hybridization and immunostaining, and the pericytes must be culled when NG2 is used in labeling OPCs.

### NKX2.2

Homeodomain transcription factor NKX2.2 is selectively upregulated in differentiating OPCs or pre-oligodendrocytes, but is rapidly down-regulated after oligodendrocyte differentiation [61]. Genetic studies have demonstrated that NKX2.2 controls the timing of OL differentiation, as conditional deletion of *Nkx2.2* causes a developmental delay of OL differentiation [62], and overexpression of NKX2.2 in OPCs results in precocious OL differentiation [55]. NKX2.2 promotes oligodendrocyte differentiation by directly inhibiting *Pdgfra* expression. However, NKX2.2 is also expressed in a subset of neurons in the ventral spinal cord and ventral thalamus [63–65]. Thus, identification of pre-myelinating oligodendrocytes by NKX2.2 expression in the gray matter must be combined with other lineage-specific transcription factors such as OLIG2 or SOX10.

### CC1

The monoclonal antibody anti-adenomatous polyposis coli (APC) clone CC1 is widely used to mark differentiated/mature oligodendrocytes without labeling myelin. However, previous studies have shown that the CC1 antibody does not bind APC [66]. In fact, the expression of APC is completely different from that of CC1 [66–69]. It is now clear that the CC1 antibody recognizes a specific isoform of the RNA-binding protein Quaking (QKI, encoded by the *Qk* gene), namely QKI7 [69]. During early development, *Qk* is broadly expressed in neural progenitor cells and later in their glial progeny, including OPCs and newborn APCs (Fig. 1F) [70]. With time, *Qk* expression is gradually reduced in astrocyte lineages, but is strongly upregulated in differentiated oligodendrocytes (Fig. 1G, J) [71]. As an antibody recognizing the cytoplasmic isoform QKI7 of QK proteins, CC1 is a useful and reliable marker for counting the number of mature oligodendrocytes in postnatal animals (older than P15 in the brain and P7 in the spinal cord). When used in the CNS of neonatal mice, astrocytes and OPCs with weak CC1 expression should be excluded from the mature oligodendrocyte population based on their morphology, gene expression level, and tissue distribution.

### Myelin Proteins

Myelin is made of lipids (>70% of its dry weight) and proteins. Myelin proteins play important roles in cell adhesion, axon-myelin interactions, and the integrity of compact myelin structure. The most abundant proteins in CNS myelin sheaths include PLP (proteolipid protein 1), MBP (myelin basic protein), CNP (2′,3′-cyclic nucleotide 3′-phosphodiesterase, CNPase), MAG (myelin-associated glycoprotein), and MOG (myelin oligodendrocyte glycoprotein) [72]. These proteins constitute >30% of the total myelin-associated proteins [8]. Detection of their expression at the RNA or protein level directly monitors the differentiation state of oligodendrocytes both *in vitro* and *in vivo*. These myelin genes are activated when OPCs start to differentiate, and are sustained in myelinating and myelinated cells. The expression of myelin genes commences at ~E18 in mouse spinal cord or P2–P3 in the cortex by RNA *in situ* hybridization, but their proteins can only be detected ~ 2 days later. Generally speaking, the chronological order of expression of these markers *in vivo* is CNP/MBP, PLP/MAG, MOG/CC1, although the temporal difference is quite small. It should be noted that myelin proteins are predominantly localized in the myelin processes of oligodendrocytes. Thus, immunostaining against these proteins in CNS tissue usually reflects the biomass of myelin sheaths, and is not suitable for cell counting (Fig. 1L). Should the quantitative analysis of cell numbers be required, RNA *in situ* hybridization of these myelin genes (except for *Mbp* mRNA, which is distributed in cellular processes as well) would be a better choice.

### MYRF

The myelin regulatory factor gene (MYRF) is a novel type of membrane-bound transcriptional factor that is evolutionarily conserved from invertebrates to vertebrates. It is initially synthesized as a type-II membrane protein, which subsequently undergoes homo-trimerization and self-cleavage on the endoplasmic reticulum (ER) membrane. Once cleaved on the ER membrane, the N-terminal trimers of MYRF are released and translocate into the nucleus to function as a transcriptional activator [73, 74]. As the downstream target of SOX10, MYRF is a key regulator of myelin gene expression, which is essential for myelin formation and maintenance [75]. In the CNS, MYRF expression is strictly restricted to oligodendrocytes. Its expression is selectively upregulated in oligodendrocytes at the beginning of cell differentiation, and sustained in mature oligodendrocytes, in a pattern similar to that of myelin genes such as *Plp* and *Mbp* (Fig. 1H–J) [75, 76]. Conditional ablation of *Myrf* in OPCs blocks myelinogenesis during development or myelin maintenance/repair in adulthood [76]. Given its higher specificity and nuclear localization, MYRF is considered to be a better marker than CC1 for differentiated oligodendrocytes and cell counting.

### Other Oligodendroglial Markers

In addition to the markers described above, a number of others are also frequently used to identify cells of oligodendrocyte lineage in a stage-specific manner. For instance, the monoclonal antibody O4, like NKX2.2, preferentially labels immature differentiating oligodendrocytes (or pre-oligodendrocytes) before the expression of MBP and PLP [64, 77, 78]. This mouse IgM antibody works well in both culture and tissue sections. Besides, *Enpp6* and *Bmp4* have recently been shown to selectively label newly-formed oligodendrocytes, but their expression is downregulated in more mature myelinating oligodendrocytes [79, 80]. Unfortunately, working antibodies have not been developed for these proteins. As oligodendrocytes undergo terminal maturation, they start to express Opalin (also known as TMEM10) and ASPA (aspartoacylase) [81–84]. The expression of these two new markers in mature oligodendrocytes occurs several days later than that of MBP and PLP *in vivo*, *i.e.* at ~ P5–P7 in spinal tissue or P10–P14 in the corpus callosum. For identification of more mature oligodendrocytes, ASPA seems to be the best choice due to its preponderant localization in the cell body (Fig. 1K). Another marker for mature oligodendrocytes is CAII (carbonic anhydrase 2), and this gene is a specific marker for type I/II oligodendrocytes which are predominantly myelinate small-diameter axons [85, 86]. In addition, a simple myelin staining method, Black-Gold, was developed some years ago, based on the specific affinity of gold phosphate complex with lipidic myelin structures [87]. This chemical staining method is relatively fast and simple compared to immunostaining, and produces much higher resolution than the traditional Luxol Fast Blue staining (Fig. 1M).

## Markers for Cells of Astrocyte Lineage

### GFAP

Glial fibrillary acidic protein (GFAP) was one of the first identified astrocyte markers [88, 89]. For decades, GFAP has been widely used as the standard astrocyte marker in numerous studies because of its robust staining and the lack of better markers. GFAP labels cultured astrocytes *in vitro* and reactive astrocytes in injured or pathological CNS tissues. In normal CNS tissue, GFAP is predominantly expressed by astrocytes in the white matter (fibrous astrocytes) and the parenchyma region near the leptomeninges, but very weakly in gray matter protoplasmic astrocytes. However, GFAP also labels the late radial glial cells in embryonic cortical tissue [40]. It is worth mentioning that GFAP has a high degree of homology to other types of neurofilament proteins (*e.g.* Nestin and Vimentin). Therefore, the specificity of GFAP antibodies must be carefully characterized, due to the potential cross-reactivity issue.

### S100B

Besides GFAP, S100B (S100 protein, beta polypeptide, neural; S100β) has also been widely used for decades as an astrocyte biomarker in both the developing and adult CNS [90, 91]. S100B is a $Ca^{2+}$-binding protein, and has been implicated in several neurological diseases including Alzheimer's disease, Parkinson's disease, and neuropathic pain [92]. However, emerging evidence suggests that S100B is also expressed in cells of oligodendrocyte lineage [93, 94]. Our recent study demonstrated that the earliest S100B+ cells are differentiating oligodendrocytes before P4 in the forebrain, rather than astrocytes as previously thought. Its expression in cortical gray matter astrocytes occurs only after P4, a time-point when astrocytes have already migrated from the germline region [95]. Unexpectedly, we found that almost all of the S100B+ cells in the white matter are SOX10+/MYRF+ oligodendrocytes from the neonatal stage to adulthood. In the postnatal CNS, S100B marks protoplasmic astrocytes in the gray matter and maturing oligodendrocytes in both the gray and white matter (Fig. 2B). Nevertheless, S100B still holds advantages as a marker for astrocytes. First, nearly all protoplasmic astrocytes in the gray matter express S100B in post-weaning animals. Second, S100B protein is predominantly localized in the cell body and thus highly suitable for cell counting. Third, unlike other astrocyte markers, S100B is not expressed by radial glial progenitor cells and newborn astrocyte progenitors (Fig. 2A) [95]. Thus, S100B remains an excellent choice for gray matter astrocytes when combined with SOX10 to exclude its expression in oligodendrocytes.

### ALDH1L1 and ACSBG1

Recent studies on astroglial gene expression profiling have identified two new astroglial marker genes, *Aldh1l1* and *Acsbg1*. *Aldh1l1* (aldehyde dehydrogenase 1 family, member L1) encodes a folate enzyme of tetrahydrofolate synthesis. Several studies have demonstrated the specific expression of ALDH1L1 in astrocytes [96, 97]. We have also confirmed that ALDH1L1+ cells out of the germline region never co-express the pan-oligodendroglial marker SOX10 in both the embryonic and adult brain, indicating their astrocyte identity (Fig. 2C) [95]. ACSBG1, a protein with very long-chain acyl-CoA synthetase activity, has been reported to display an expression pattern similar to that of ALDH1L1 [97]. However, detailed analysis of its spatiotemporal expression pattern is still required to validate its specificity in the astrocyte

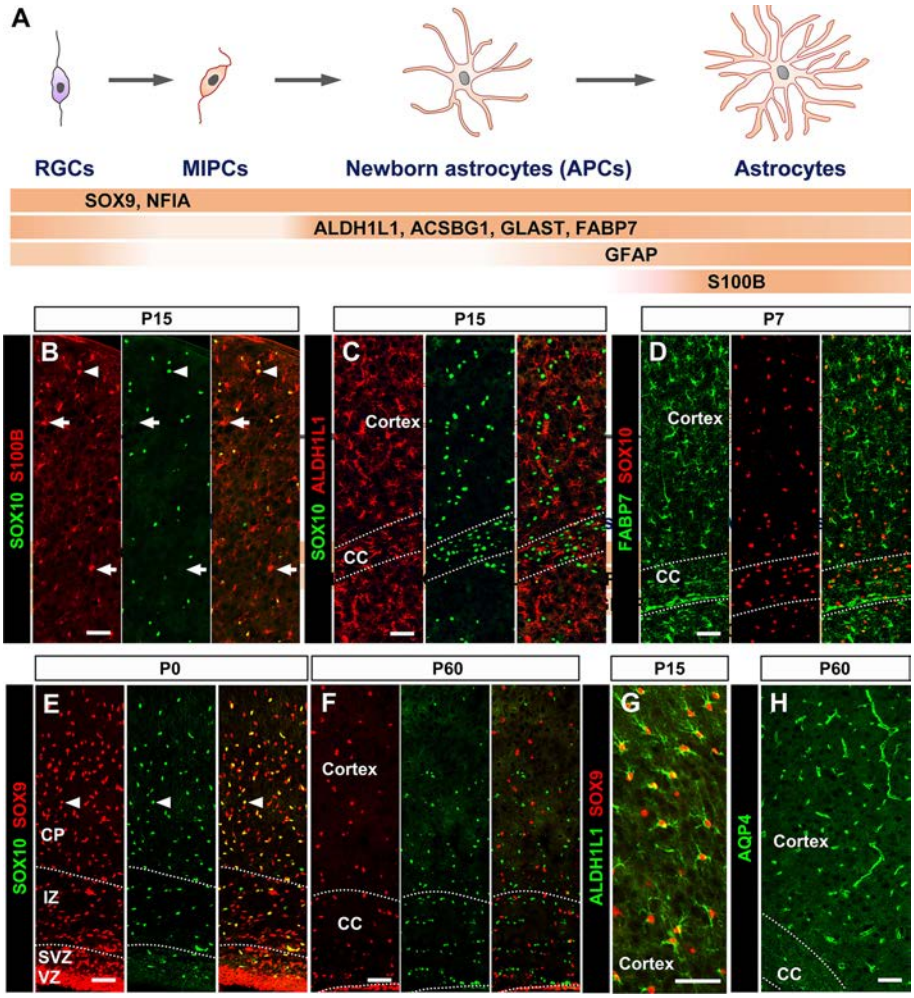


**Fig. 2** Cellular markers for cells of astrocyte lineage. **A** Diagram of astrocyte development and the expression of specific markers. **B** Besides gray matter astrocytes (arrows), many S100B+ cells are co-labeled with the pan-oligodendrocyte marker SOX10 (arrowheads). **C** ALDH1L1 expression is only detected in astrocytes, but not in SOX10+ oligodendrocytes. **D** FABP7 is an operational astrocyte marker. **E**, **F** Nearly all of SOX10+ OPCs co-express SOX9 in P0 forebrain (arrowheads), and SOX9 primarily marks astrocytes in the adult brain. **G** All ALDH1L1+ astrocytes co-express SOX9 in the brain. **H** AQP4 is localized in astrocyte process, and APQ4 immunolabeling reveals the entire network of vessels covered by astrocytic end-feet. RGCs, radial glial cells; APCs, astrocyte progenitor cells; VZ/SVZ, (sub)ventricular zone; IZ, intermediate zone; CP, cortical plate; CC, corpus callosum. Scale bars, 50 μm.

lineage. In addition, it has been noted that the expression of ALDH1L1 decreases in adulthood [96], calling for better astrocyte markers for the adult CNS.

## SOX9 and NFIA

To date, the transcriptional regulation of astrocyte development is still largely unknown. The SRY-box transcriptional factor SOX9 was originally reported to determine glial fate choice in the developing spinal cord. SOX9 is initially expressed in neural progenitor cells, and later in glial cells in the marginal zone, including astrocytes and OPCs (Fig. 2E) [29]. Intriguingly, SOX9 expression is gradually downregulated in the oligodendrocyte lineage as development proceeds and becomes progressively restricted to astrocytes in the adult mouse CNS (Fig. 2F, G) [29, 98]. NFIA (nuclear factor I/A) is a target transcriptional factor of SOX9 and has been implicated in the regulation of astrogliogenesis [99, 100]. Although the expression pattern of NFIA in glial cells is quite similar to that of SOX9, it is also expressed by motor neurons in the spinal cord and deeper layer projection neurons in the cortex [101]. As NFIA down-regulation in oligodendrocytes occurs much slower than SOX9, a higher proportion of SOX10+ OPCs is still more immunoreactive to NFIA than to SOX9 in the adult mouse CNS. Therefore, SOX9 and NFIA could serve as pan-astrocyte markers due to their high immunoreactivity and nuclear localization, but only when OPCs are excluded.

## Other Astrocyte Markers

A number of other astrocytic markers have also been described. GLAST and GLT-1, also known as EAAT1/SLC1A3 and EAAT2/SLC1A2, respectively, are the primary astrocytic glutamate transporters in the adult CNS, accounting for > 90% of synaptic glutamate clearance [102, 103]. While the GLAST expression pattern is consistent with a pan-astrocyte marker, GLT-1 is also expressed in neurons in both the brain and the spinal cord [104, 105]. As a transmembrane protein, GLAST immunostaining displays punctate/reticular-like structures and is not suitable for outlining the shape of astrocytes *in vivo*, especially in adult animals.

Nonetheless, we found that GLAST specifically labels cultured astrocyte *in vitro*. Glutamine synthase (GS, Glul) is another commonly-used astrocyte marker [106]. However, it has recently been reported that GS is activated in mature oligodendrocytes in mouse brain and spinal cord [107, 108]. The onset of GS expression in mature oligodendrocytes is between P21 and P28 in mouse brain, which is later than the appearance of the mature oligodendrocyte marker ASPA. FABP7 (fatty acid binding protein 7, also known as BLBP) has occasionally been used to label astrocytes in some studies [109]. It has also been suggested that FABP7 is expressed in OPCs in both mouse and chicken CNS during development and in adulthood [110]. Nevertheless, FABP7 can still be used as an approximate astrocyte marker, since its expression in OPCs is very weak (Fig. 2D). Other astrocytic markers include Aldolase C (ALDOC), AQP4 (aquaporin 4) (Fig. 2H), and the newly-described transcriptional factor ZBTB20 [111–113]. However, more detailed analyses with molecular and genetic approaches are needed to determine the specificity and expression dynamics of these markers.

## Markers for Microglia

CNS macrophages consist of two distinct types of cell, microglia in the parenchyma and border-associated macrophages (BAMs) in the meninges, choroid plexus, and perivascular spaces. Both cell types are derived from erythromyeloid progenitors during early embryonic development (Fig. 3A) [23, 114]. Therefore, it is not surprising that microglia share many of the same molecular markers with BAMs. It is well documented that two functional states are associated with microglia: resting microglia with ramified morphology, and reactive microglia with an amoeboid shape in response to a pathological environment. In the previous studies, several molecular markers have been identified that can discern microglia from BAMs, or resting from reactive microglia.

### IBA1 and CX3CR1

To date, the most reliable and effective method for detection of microglia relies on the discovery of IBA1 protein by a Japanese group in 1996 and the demonstration of its microglial specificity [115, 116]. IBA1 protein, encoded by the *Aif1* gene (allograft inflammatory factor 1), was initially reported to be involved in the membrane ruffling and phagocytosis of macrophages/microglia [117]. Several high-affinity specific antibodies are commercially available for the IBA1 protein. IBA1 is highly specific and capable of labeling both resting and activated microglia [118]. Since IBA1 protein is distributed in the cell body and the tiny processes of microglia, it allows the observation of morphological changes and the calculation of cell numbers (Fig. 3C). One apparent disadvantage of the IBA1 marker is that it not only labels microglia, but also macrophages, including BAMs and blood-derived monocytes [115]. Another pan-microglia marker is CX3CR1, with the same expression pattern as IBA1 [119]. CX3CR1 is a G protein-coupled receptor involved in cell adhesion and migration. However, antibodies for this protein are scarce. Instead, the reporter mouse line *Cx3cr1-EGFP* has been developed and is widely used for microglia labeling [120].

### P2RY12 and CD68

These markers are frequently used to distinguish between resting and reactivated microglia. P2RY12 is a $G_i$-coupled metabotropic purinergic receptor which mediates microglial

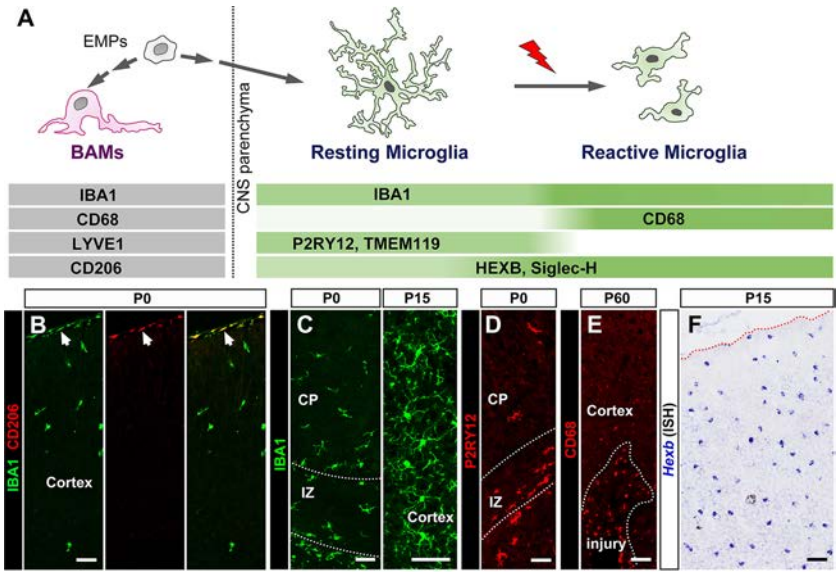


**Fig. 3** Molecular markers for microglia. **A** Diagram of microglial development and lineage-specific markers. **B** CD206 marks BAMs (arrows) but not microglia. **C** IBA1 expression in microglia in P0 and P15 mouse brain. **D** P2RY12 expression in microglia of P0 cortex. **E** CD68 labels reactive microglia after CNS injury. **F** Expression of *Hexb* mRNA in microglia revealed by *in situ* hybridization. BAMs, border-associated macrophages; VZ/SVZ, (sub) ventricular zone; IZ, intermediate zone; CP, cortical plate. Scale bars, 50 μm.

responses to extracellular nucleotides [121]. Loss of *P2ry12* in microglia switches their morphology from a highly ramified resting state to an amoeboid reactive state. Moreover, P2RY12 is strongly expressed in resting microglia, but drastically reduced after microglial activation (Fig. 3D) [121]. CD68 is a 110-kDa transmembrane glycoprotein present in monocytes and tissue macrophages [122]. It has been reported that CD68 participates in the pathological activation of macrophages by low density lipoproteins, and functions as an inhibitor of immune reactions [123]. The expression of CD68 is not conspicuous in resting microglia, but is strongly upregulated when they are activated (Fig. 3E). As a marker for reactive microglia, one disadvantage of CD68 protein is its "dotted" distribution in the membrane.

### CD206 and Hexb

Thanks to the advent of single-cell RNA sequencing technology, scientists are now able to distinguish microglia from BAMs and monocytes at the molecular level. While CD206 detects BAMs and monocytes, *Hexb* specifically labels microglia. This is an important milestone for the diagnosis of BBB damage under pathological conditions. CD206, encoded by the *Mrc1* gene, is a well-defined marker for BAMs and other macrophages without cross-labeling microglial cells (Fig. 3B) [124]. In contrast, the *Hexb* gene is a stable marker for microglia in both health and pathological conditions without discernable expression in BAMs (Fig. 3F) [125]. Thus, *Hexb* is an important marker for distinguishing microglia from surrounding macrophages. In addition, other molecular markers for CNS microglia and macrophages have also been suggested in some recent studies. For instance, TMEM119 and Siglec-H appear to be specific to microglia [126, 127], and LYVE1 preferentially labels BAMs and macrophages [128]. Unfortunately, there are no high-quality commercial antibodies for many of the aforementioned microglial markers.

## Conclusions

In general, an ideal cellular marker should be specific enough to label only one type of cell or a particular stage of cell development with high affinity. For cells of oligodendrocyte lineage, abundant stage-specific markers have been identified and high-quality antibodies have been developed accordingly. However, for astrocytes, the situation is quite different. To date, there is a lack of definitive staging for astrocyte development, partly due to the lack of stage-specific markers. Many widely-used astrocyte markers are not specific or effective enough to distinguish among RGCs, APCs, and mature astrocytes. It is now becoming clear that astrocytes, radial glial cells, and even cerebellar Bergmann cells share a similar gene expression profile, including *Glast*, *Fabp7*, *Aldh1l1*, *GFAP*, *Hopx*, *Tnc*, *Fgfr3*, *Acsbg1*, *Dbi*, and *Qk*, as well as the transcriptional factors *Sox2*, *Sox9*, *Nfia*, *Zeb1*, and *Zbtb20* [20, 40]. Although a few markers *(e.g.* S100B, GS, and NFIA) are capable of detecting most cells of astrocyte lineage, they also label other neuronal cell types such as oligodendrocytes or neurons. Therefore, a combinatory expression of multiple markers should be considered for accurate identification of astrocyte subpopulations or developmental stages.

Intriguingly, mature astrocytes share some of the same molecular marker genes (*e.g.* S100B and GS) with mature oligodendrocytes in the CNS [95, 107]. When these markers are chosen to label astrocytes, the oligodendrocyte markers SOX10, CC1, and MYRF can be used in combination for more definitive identification. This suggests that these two types of macroglia may share some common metabolic pathways. Thus, part of supporting functions of astrocytes could be taken over by myelin structures. Conceivably, myelinated axons, especially those in the white matter, are remote from their cell bodies and some metabolic support may be provided by myelin.

Another important point is that some of the markers (e.g. *Sox2*, *Fabp7*, *Sox9*, *Nfia*, *Qk*, *Zeb1*, and *Zbtb20*) expressed in RGCs and astrocytes are also maintained in OPCs, in keeping with the suggestion that RGCs are the common precursors of OPCs and astrocytes. As a matter of fact, several recent studies have identified a special class of intermediate progenitor cells (IPCs) in cortical regions that express EGFR, ASCL1, and OLIG2 during gliogenesis in both mice and primates [40, 44]. These EGFR+/ASCL1+/OLIG2+ multipotent progenitors are described as "pre-OPCs", "pri-OPCs", "mGPCs (multipotent glial progenitor cells)", and "MIPCs (multipotent IPCs)" in different studies [42, 43, 129, 130]. Unexpectedly, these MIPCs are tripotent precursors as they produce not only OPCs, but also astrocytes and olfactory bulb interneurons [40]. Since the expression of OLIG2 does not restrict the fate of cells to OPCs, it seems to be more appropriate to name this type of cell "MPICs". In light of this new finding, SOX10, rather than OLIG2, would be the best choice for labeling OPCs among glial populations in brain tissue.

Based on the similar gene expression profiling among RGCs, APCs, and astrocytes, we recently hypothesized that astrocytes are the resting neural precursor cells without undergoing terminal differentiation and cell-cycle exit, providing structural and metabolic support for local neurons [22]. Functional adaptions to the local neuronal milieu may explain the heterogeneity of astrocytes in the CNS. In support of this hypothesis, astrocytes retain some degree of stemness and reactive astrocytes are capable of differentiating into neurons or OPCs *in vitro* or *in vivo* under certain pathological conditions [131, 132]. Also, astrocytes in

different regions retain a radial migration property and fail to migrate into adjacent regions after injury. Thus, astrocytes in different regions cannot be replaced by each other after lesions, contrary to the functional redundancy displayed by OPCs from different embryonic origins [19, 133].

Another issue to be aware of is the species differences in the expression of molecular markers. Although the fundamental principles are roughly the same, primates and rodents do exhibit some species-diversity in their cellular and molecular organization. For instance, cortical regions in human but not mouse develop an enlarged germinal zone called the outer subventricular zone, populated by multipotent outer radial glial cells [134, 135]. It has also been reported that GFAP in human brain and spinal cord is expressed by almost all of the astrocyte populations, while it only labels a subset of astrocytes in the murine CNS [40, 44]. Again, the classical OPC marker PDGFRA is weakly expressed in HOPX+/OLIG2+ MIPCs in the developing human cortex, but not mouse cortex before they differentiate into OPCs [44]. Therefore, it is not recommended to define a cell type with only one single marker, especially during early embryonic stages.

Methodologically, there are usually two major experimental procedures for *in situ* detection of a particular cell type in a tissue. One is through RNA *in situ* hybridization (ISH), which detects target mRNAs, and the other is by immunostaining based on specific antibody-antigen biochemical interactions. There are several advantages for ISH detection of molecular markers. First, the RNA probes for ISH are easier to prepare under laboratory conditions, especially when antibodies are not available. Second, mRNAs are usually localized to the cell body, so it is convenient for counting the number of cells of interest. Third, the difference in the nucleic acid sequence of homologous genes is greater than that of the amino-acid sequence, so the specificity of ISH is in general very high. However, its drawbacks are also apparent, as ISH is somewhat time-consuming and unsuitable for multiple-channel labeling and morphological observations. Immunostaining, including immunofluorescence, immunohistochemistry, and immunocytochemistry, is the most commonly used cell labeling technique. Immunostaining relies on specific antibodies. There are several means to validate the specificity of a particular antibody: (1) the expression pattern of a target protein detected by immunostaining should be consistent with that of its mRNA labeled by ISH; (2) knockout verification has now become a standard approach, especially when it is performed in tissue sections; (3) examination of cross-recognition between homologous proteins is recommended, considering that knockout verification of antibodies is mostly done in cell cultures, and homologous proteins that can be recognized non-specifically may not appear *in vitro*.

To date, the cellular markers for astrocytes and microglia are still relatively lacking, making it difficult to define their developmental stages, cell subtypes, or distinct functional states. At present, with the aid of single-cell RNA sequencing, it is more operable to identify novel cell type-specific markers. However, before being used as a marker, these candidate genes must be verified by both RNA *in situ* analysis and immunostaining.

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REVIEW

# The Oncogenesis of Glial Cells in Diffuse Gliomas and Clinical Opportunities

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**Abstract** Glioma is the most common and lethal intrinsic primary tumor of the brain. Its controversial origins may contribute to its heterogeneity, creating challenges and difficulties in the development of therapies. Among the components constituting tumors, glioma stem cells are highly plastic subpopulations that are thought to be the site of tumor initiation. Neural stem cells/progenitor cells and oligodendrocyte progenitor cells are possible lineage groups populating the bulk of the tumor, in which gene mutations related to cell-cycle or metabolic enzymes dramatically affect this transformation. Novel approaches have revealed the tumor-promoting properties of distinct tumor cell states, glial, neural, and immune cell populations in the tumor microenvironment. Communication between tumor cells and other normal cells manipulate tumor progression and influence sensitivity to therapy. Here, we discuss the heterogeneity and relevant functions of tumor cell state, microglia, monocyte-derived macrophages, and neurons in glioma, highlighting their bilateral effects on tumors. Finally, we describe potential therapeutic approaches and targets beyond standard treatments.

**Keywords** Glioma origin · Stem/progenitor cell · Oncometabolite · Immune heterogeneity · Neuron-tumor interaction

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## Introduction

Gliomas, traditionally named due to their close resemblance to glial cells, are the most frequent intrinsic primary tumors of the brain [1–3]. Different from other oncological diseases that benefit from multimodal therapy, limited progress has been made in the management of gliomas [4, 5]. Therefore, ongoing efforts to understand their highly heterogeneous nature and complicated reciprocal microenvironmental communication have been undertaken [6, 7]. Among their forms, diffuse gliomas, which have an unfavorable prognosis and high morbidity in adult patients, have been historically diagnosed as one of three categories outlined in the 2016 WHO central nervous system (CNS) classification [8, 9]: oligodendroglioma, astrocytoma, or glioblastoma (GBM). These subtypes share several molecular features and functional characteristics with their normal counterparts. Recent profiling efforts have identified subclassifications of diffuse gliomas by integrating histopathological analysis and genetic events [10]. Importantly, isocitrate dehydrogenase (IDH) status and chromosome 1p/19q co-deletion [11], have been identified as predictive genetic landmarks of favorable outcomes and have had a profound impact on treatment strategies and the design

of clinical trials [12]. In particular, robust biomarkers have also been described by Eckel-Passow *et al.*, who classify gliomas into five principal groups with prognostic significance. Of these, the triple-negative gliomas (no mutations in IDH and TERT plus a 1p/19q non-codeletion) were the most prevalent in a Chinese cohort [13, 14]. Mutations of *TP53* and H3.3-K27M in triple-negative gliomas implicated an unfavorable prognosis [14]. Notably, the fifth edition of the WHO CNS (2021 WHO CNS5) grouped gliomas according to these genetic changes to enable a complete diagnosis [15]. Other molecular signatures, such as cell-cycle regulatory elements (CDKN2A/B) and epidermal growth factor receptor (EGFR), have also contributed to the illustration of oncogenic pathways. Progress in genomics has validated diverse genetic alterations harbored in diffuse gliomas, rendering glioma cells distinct from one another. The expression patterns of genetic mutations suggest that astrocytomas and oligodendrogliomas originate from abnormal glial progenitors or stem cells. These findings have led to the hypothesis that the cellular heterogeneity of gliomas is affected by the glial developmental process, intercellular signaling, and microenvironment stress. This review discusses new advances in oncogenic glial lineage, and reciprocal interactions in gliomas (i.e. with neurons and microglia), offering new insights into the potential development of effective treatments.

## Glioma Origin: From Neurogenesis to Oncogenesis

### Glioma Stem Cells

Among the components constituting tumors, glioma stem cells (GSCs) are highly plastic subpopulations bearing stemness properties and are thought of as the site of tumor initiation. Similar to neural stem cells (NSCs), GSCs have the ability to self-renew, differentiate and resist DNA damage [16–18]. A series of biomarkers have been identified in GSC populations: CD133 (PROM1), SOX2 (a transcription factor widely expressed in potent stem cells), OCT-4 (a transcription factor that plays an essential role in stem cell pluripotency), and Nestin (an intermediate filament protein). Several studies have shown that the expression of these molecular markers is closely associated with pluripotency and stemness in gliomas. By intracranial grafting as few as 100 CD133+ cells, tumors have been effectively produced and resembled the phenotype of the original tumor type, whereas no transplanted tumor was observed after injection of $10^5$ CD133– cells [19]. Ablation of *Nestin*+ stem-like cells was not able to halt tumor progression, indicating the involvement of other factors [20]. CD133+ Notch1+ GSCs have also been reported to be located at the frontier of invasive tissues, exhibiting white-matter-tract tropism. The positive-feedback loop involving Notch-SOX2 controls the invasive phenotype of GSCs along white matter tracts [21]. The stem-cell activity of CD133+ cells has also been found in medulloblastomas, pilocytic astrocytomas, and gangliogliomas. Higher tumor grade is correlated with an increased fraction of CD133+ cells in tumor cultures [20]. In addition, the non-GSC population is induced to a newly converted GSC-like state after treatment with chemotherapeutic agents (e.g., temozolomide), and has a more invasive phenotype with higher implantation efficacy [22]. These findings focus attention on the cellular state of GSCs in gliomas. Lin *et al.* described a single axis of gene signatures in proliferating GBM cells, ranging from proneural GSCs to mesenchymal GSCs. Lineage tracing *in silico* supports the idea that mGSCs, which correlate with poor predicted survival, are the progenitors of pGSCs in IDH wild-type GBM [23]. *Via* enriching GSCs from primary GBM specimens, Richards *et al.* found that GSCs exist in two cellular states from the perspective of transcriptional programs: developmental and injury-response programs [24]. The astrocyte maturation gradient in tumor cells has also been implicated in the transformation of GSCs, which comprise the bulk of the tumor. Thus, understanding the evolution and differentiation of GSCs is essential for developing effective targeting therapies and identifying the source of heterogeneity in gliomas.

### Neural Stem/Progenitor Cells

Different from abnormal glioma stem cells that populate GBM, neural stem cells/progenitor cells (NSCs/NPCs) are the natural starting point for neuron/glial lineage development, and are highly regulated in the brain. It is essential to understand the tumorigenesis process and decipher the mechanisms through which glial developmental programs are used by tumor cells to populate the tumor. The largest NSC niches are located along the remote region of the lateral ventricles, named the subventricular zone (SVZ). These NSCs are relatively quiescent, maintain their stemness properties, and generate NPCs independent of the specific microenvironment around the perivascular niches. This complex microenvironment is composed of NPCs, oligodendrocyte progenitor cells (OPCs), astrocytes, microglia, macrophages, neurons, associated vasculature, and extracellular matrix. Interestingly, some typical markers of NSCs have been identified in GSCs such as Nestin, Sox2, CD44, and CD133 (Fig. 1) [25–27]. The striking similarities between NSCs and GSCs support the hypothesis that SVZ NPSCs play the role of apex cells in the hierarchy of gliomas. Chen *et al.* used a fluorescent reporter to label quiescent NSCs in the adult SVZ, and revealed the presence of neural stem-like cells in glioma tissue [28]. Deep genomic sequencing of a GBM patient cohort provided direct evidence for the hypothesis that astrocyte-like NSCs in the SVZ are the origin of GBM. More than 80% of patients diagnosed with

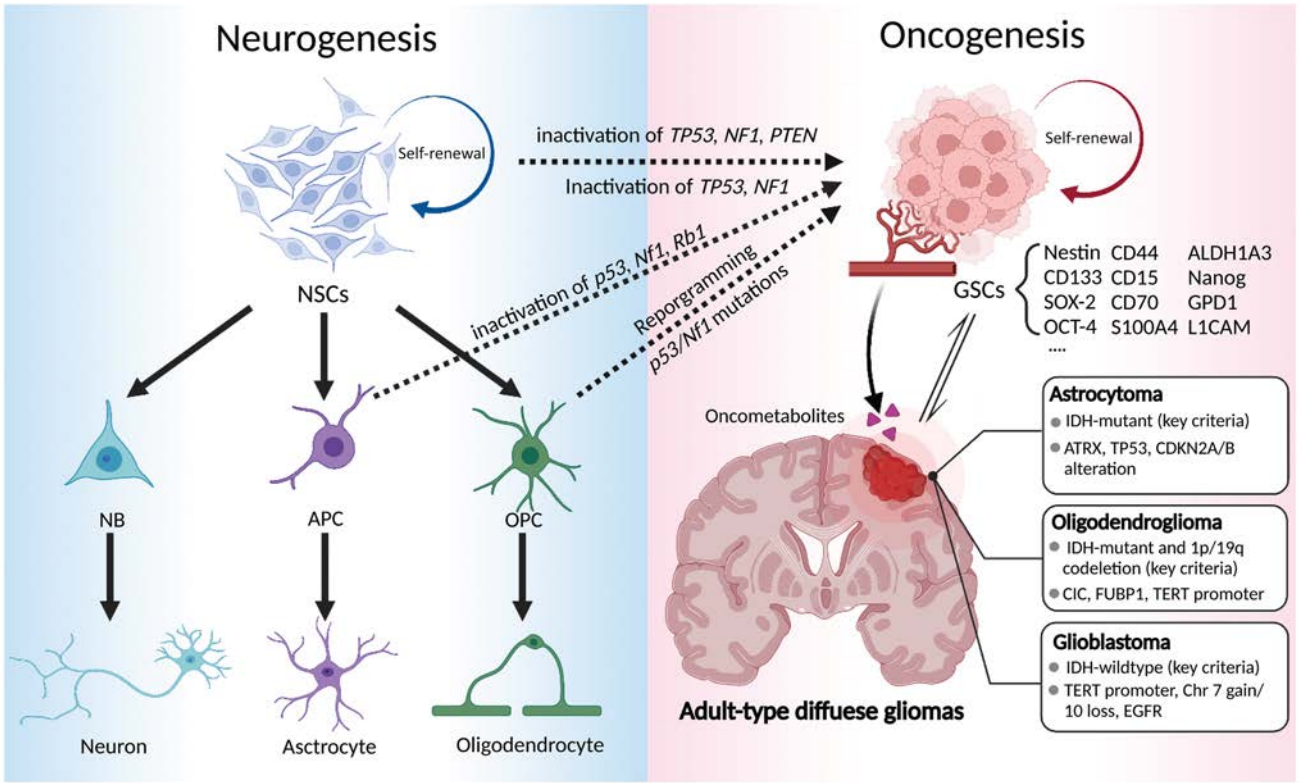


**Fig. 1** Glioma origin hypothesis. Left, schematic of the normal neurogenesis process in the brain. Neural stem cells differentiate into several types of progenitor cell, which can transform into neurons, astrocytes, and oligodendrocytes. Right, schematic of the potential oncogenesis process in the brain. Glioma-stem cells, which populate adult-type diffuse gliomas, are labeled with several reported biomarkers. Gliomas produce oncometabolites in the tumor microenvironment, which correspondingly stimulate their progression. The dashed line between neurogenesis and oncogenesis represents the reprogrammed molecular mechanisms that have been previously reported. NSCs, Neural stem cells; GSCs, Glioma stem cells, NBs, Neuroblasts; APCs, Astrocyte progenitor cells; OPCs, Oligodendrocyte progenitor cells.

GBM have tumor-free SVZ tissue that shares low-level driver mutations or cancer-driving genes with tumor samples [29]. Migration of astrocyte-like NSCs contributes to the generation of malignant gliomas in distinct regions of the brain [29]. Accordingly, genetically-engineered mouse models (GEMMs) are powerful tools for use in deciphering the lineage complexity of glial cells, and may reveal associations between progenitor cells and the broad spectrum of neoplasms throughout the brain. Parada *et al.* induced resultant malignant astrocytoma *via* early inactivation of *Tp53* and *Nf1* in mice [30], and demonstrated that manipulation of tumor suppressors (*Nf1*, *Tp53*, and *Pten*) in NSCs and NPCs *in vivo* is both necessary and sufficient for the formation of astrocytomas (Fig. 1). A recent study also showed that the histological and transcriptional heterogeneity of GBM is similar to genome-edited NSC-like cells such as sg*TP53/NF1/PTEN* or sg*TP53/NF1* in human pluripotent stem cells [31]. High-grade gliomas exhibit inactivation of $p16^{INK4a}/p19^{ARF}$ and activation of epidermal growth factor receptor (EGFR). Previous findings reported that co-deletion of $p16^{INK4a}/p19^{ARF}$ in NSCs with constitutive *EGFR* activation induce the phenotype of high-grade glioma [32]. However, no evidence of tumor formation was reported after targeting these GBM-relevant tumor suppressors in neuroblasts, late-stage neuronal progenitors, and differentiated neurons [33]. Jacques *et al.* reported that deletion of tumor suppressors genes (*TP53* and *PTEN*) in adult SVZ stem cells, but not astrocytes, gives rise to tumors. These studies imply that an increase in lineage restriction decreases the tumorigenic capacity of neuronal lineage cells [34]. Driving neuronal lineage differentiation is a potent antitumorigenic treatment strategy for GBM.

## Other Glial Lineage States

Combining single-cell sequencing (scRNA-seq) with advanced computational algorithms allows researchers to comprehensively analyze cellular states across tumors [35, 36]. Four cellular states, three of which are anchored in neurodevelopment, are found in diverse malignant cells of glioblastoma: OPC-like, NPC-like, astrocyte (AC)-like, and mesenchymal (MES)-like. These cellular states have the

potential for tumor plasticity and are influenced by genetic drivers, providing an understanding of the heterogeneity and therapeutic resistance of GBM. It is well-known that tumor phenotype transitions are derived from stage-specific fate-switches and transcriptional alterations in progenitor cells. Newly-generated cells including ACs and oligodendrocytes, which are considered to be the two broad categories of CNS glia, are continuously produced in the subgranular zone and ventricular SVZ [37]. Both oligodendrocytes and ACs perform a variety of functions for maintaining CNS homeostasis.

Although the precursors of ACs have not been clearly defined, some ACs can reenter the cell-cycle following traumatic brain injury [38]. A recent study reported that ASCL1+EGFR+ apical multipotent intermediate progenitor cells generated by cortical radial glial cells in the SVZ and VZ can differentiate into glial cells and olfactory bulb (OB) interneurons. Those progenitor cells transform into AC-lineage restricted progenitor cells in late embryogenesis in mice [39]. Injection of ACs carrying oncogenes leads to the genesis of malignant gliomas. Furthermore, all tumors expressed markers expected in astrocytomas, such as Gfap [32, 40–42]. Combinations of deletions of *Pten*, *Tp53*, and *Rb1* in ACs in mature mice result in the progress of astrocytomas from grade III to grade IV [43]. Applying fluorescence-activated cell sorting–based strategy, a recent study reported five distinct AC subtypes across the adult brain and identified the specific subpopulations correlated with tumor invasion in gliomas [44]. OPCs, the most abundant cycling population in the adult CNS, is the last potential progenitor source of glioma origin. The OPC markers OLIG2 and NG2 are concurrently expressed in the major cycle-related cell population of the hippocampus [45]. Their correlates in mitotic characteristics give rise to the possibility that OPCs play a key role in tumorigenesis. Moreover, there is a large population of OLIG2+ cells (high Ki76 and CD133) in human gliomas, suggesting that proliferative OPCs may act as tumor-propagating cells [46, 47]. Several studies have found that oncogene mutations in OPCs are involved in the development of high-grade gliomas [48–50]. Overexpression of PDGF in OPCs, along with evidence of specific inactivation of *Nf1* and *tp53* in OPCs, are involved in the formation of malignant gliomas [51, 52]. Intriguingly, lineage tracing based on mosaic analysis with double markers (MADMs) has revealed that introducing *p53*/*Nf1* mutations only in OPCs, but not NSCs, consistently leads to oncogenesis (Fig. 1). Phenotypic and transcriptomic analyses have identified the salient OPC features of these tumors [53]. By applying lineage-targeted scRNA-seq, Weng *et al.* elegantly identified a primitive OPC intermediate population in the neonatal cortex. Reprogrammed OPCs transformed into a stem-like state, resulting in the development of malignant tumors [54]. These studies revealed that OPCs can directly generate GBM *via* stepwise genetic and epigenetic reprogramming. We summarize reported gene-edited mouse models that mimic different types of gliomas in Table 1.

## Oncometabolites

Mutation at $Arg^{132}$ of IDH1 was thought to be an early initiating event driving the evolution of gliomas [55, 56]. Mutation of IDH enzymes results in the elevation of (R)-2-HG levels from 1 mmol/L to 3 mmol/L at the center of IDH mutant gliomas [57]. 2-HG, known to be an important oncometabolite, is a competitive substrate of α-ketoglutarate-dependent epigenetic enzymes [58–60]. A high concentration of 2-HG *in vivo* inhibits histone lysine demethylases and TET hydroxylases, leading to impairment of DNA demethylation and eventual hypermethylation in gliomas [61]. Intriguingly, accumulation of (R)-2-HG also causes impairment of collagen protein maturation, which is associated with the endoplasmic reticulum stress response and basement membrane aberrations, leading to a microenvironment favorable to gliomas [62]. Notably, expression of $IDH1^{R132H}$ cooperates with platelet-derived growth factor A expression and loss of *Cdkn2a*, *Atrx*, and *Pten* in glioma to mimic the proneural subtype of human GBM, which exhibits a stronger GBM formation ability *in vivo* [63]. By enhancing D-2-hydroxyglutarate-mediated DNA methylation, conditionally expressing $IDH1^{R132H}$ in the NPCs of the murine SVZ increases the number of NSCs and their progeny. Regulated stem cells exhibit invasive characteristics and uncontrolled expansion, which may explain the process of oncogenesis in the early phase [64]. Platten's research group conducted a phase I clinical trial in which 33 patients received treatment with an IDH1-specific peptide vaccine. The convincing clinical data showed that the vaccine is safe, and in terms of therapeutic effect, the IDH1-vaccine significantly prolongs the survival time of patients [65, 66]. These findings support the hypothesis that mutations in oncogenic metabolic enzymes dramatically affect the cellular status of gliomas, leading to mutations in other genes that collectively affect tumor transformation and promote tumorigenesis [67, 68]. Moreover, other groups have reported that tumor-derived kynurenine, IDO1, tryptophan 2,3-dioxygenase, and IL-4I1 mediate immunosuppressive activities in GBM [69–71]. Therefore, inhibitor therapy against these targets might be an alternative approach.

**Table 1** Gene-edited mouse models of gliomas.

| | Driver genes | Methods | Original cells | Reference |
|---|---|---|---|---|
| Oligodendroglioma models | *v-erbB*/*Ink4a*/*Arf*$^{+/-}$ | Transgenic | *S100Aβ*+ | Weiss *et al.* [153] |
| | *v-erbB*/*p53*$^{-/-}$ | Transgenic | *S100Aβ*+ | Persson *et al.* [154] |
| | *H-Ras*/*EGFRvIII* (embryonic) | Transgenic | *GFAP*+ | Ding *et al.* [155] |
| | PDGFB/*Ink4a-Arf*$^{-/-}$ | RCAS/*tv-a* | *Nestin*+ | Dai *et al.* [156]; E Tchougounova *et al.* [157] |
| | PDGFB/*Akt* | RCAS/*tv-a* | *Nestin*+ | Dai *et al.* [158] |
| | PDGFB | *In utero* intraventricular injections | Embronic neural precursors | Calzolari *et al.* [159] |
| | PDGFB | RCAS/*tv-a* | OPCs | Lindberg *et al.* [50] |
| | PDGFB-HA | RCAS/*tv-a* | *Nestin*+ | Shih *et al.* [173] |
| | PDGF-$A_L$ | Transgenic | *GFAP-Cre*+ | Nazarenko *et al.* [160] |
| Astrocytoma models | *c-Myc* | Transgenic | *GFAP*+ | Jensen *et al.* [161] |
| | *K-Ras*$^{G12D}$ | Transgenic | *GFAP*+ | Abel *et al.* [162] |
| | *H-Ras* | Transgenic | *GFAP*+ | Shannon *et al.* [163] |
| | *H-Ras*/*Pten*$^{fl/fl}$ | Transgenic | *GFAP-Cre*+ | Wei *et al.* [164] |
| | *Pten*$^{fl/fl}$/*Rb1*$^{fl/fl}$/*Tp53*$^{fl/fl}$ | Transgenic | *GFAP-Cre*+ | Chow *et al.* [43] |
| | *EGFR*/ *Ink4a-Arf*$^{-/-}$ | RCAS/*tv-a* | Nestin+/GFAP+ | Holland *et al.* [165] |
| | *cisNf1*$^{fl/+}$/*Trp53*$^{+/-}$ | Transgenic | *GFAP-Cre*+ | Zhu *et al.* [30] |
| Glioblastoma models | PDGFB/Trp53$^{-/-}$ | Transgenic | *GFAP*+ | Hede *et al.* [166] |
| | *K-Ras*/*Akt* | RCAS/*tv-a* | *Nestin*+ | Holland *et al.* [167] |
| | *K-Ras*/ *Ink4a-Arf*$^{-/-}$ | RCAS/*tv-a* | *Nestin*+/ *GFAP*+ | Uhrbom *et al.* [168] |
| | *K-Ras*/Akt/Pten$^{fl/fl}$ | RCAS/Cre | *Nestin-Cre*+ | Hu *et al.* [169] |
| | *Cdkn2a*$^{fl/fl}$/*Atrx*$^{fl/fl}$/*Pten*$^{fl/fl}$ | RCAS/Cre | *PDGFRA/IDH*$^{R132H}$*-Cre*+ | Philip *et al.* [63] |
| | *Pten*$^{fl/+}$/*cisNf1*$^{fl/+}$/*Trp53*$^{+/-}$ | Transgenic | *GFAP-Cre*+ | Kwon *et al.* [170] |
| | *P53*$^{fl/fl}$/*Pten*$^{fl/+}$ | Transgenic | *GFAP-Cre*+ | Zheng *et al.* [171] |
| | sg*TP53/NF1/PTEN* or sg*TP53/NF1* | CRISPER/Cas9 system | hNSCs | Wang *et al.* [31] |
| | sg*PTEN*/*NF1*, sg*TP53*/*PDGFRA*$^{\Delta 8-9}$ | CRISPER/Cas9 system | iPSCs | Koga *et al.* [172] |
| | *Rb*$^{fl/fl}$/*p53*$^{fl/fl}$, *Rb*$^{fl/fl}$/*p53*$^{fl/fl}$/*Pten*$^{fl/fl}$ | Transgenic | *Adeno-Cre*+/*Adeno GFAP-Cre*+ | Jacques *et al.* [34] |
| | Concurrent *p53*/*Nf1* mutations | Mosaic analysis with double markers | OPCs | Liu *et al.* [53] |
| | *p53*$^{-/fl}$/*NF1*$^{fl/fl}$ | Transgenic | *NG2*-Cre+ | Galvao *et al.* [52] |

## Interactions Between Glioma and Microglia/Macrophages

### Heterogeneity Between Resident Microglia and Monocyte-Derived Macrophages (MDMs)

The microenvironment of gliomas consists of multiple interacting networks among cells, in which brain-resident microglia and infiltrating monocytes/MDMs contribute to a large fraction of the glioma immune landscape [72]. Microglia, derived from hematopoietic precursor cells of the yolk sac in the early developmental period, are crucial residential innate immune cells of the brain [73, 74]. They have an important influence in supporting neurogenesis, scavenging apoptotic cells, and refining synapses [73, 74]. Notably, different stages of glioma lead to differential compositions of the myeloid cell landscape. GBM can lead to partial disruption of the blood-brain barrier, enabling monocytes/MDMS to infiltrate the tumor. These distinct populations, termed tumor-associated macrophages (TAMs), have been widely reported as an important factor impinging on the intrinsic characteristics of tumor progression [75]. Using the head-protected irradiation and fluorescently tagged cell lineage tracing technique, microglia expressing high CD45 represent an inherent part of a glioma, while infiltrated tagged TAMs constitute up to 25% of the myeloid cell fraction after 21 days of tumor implantation [76]. The heterogeneity of time-lapse

and spatial distribution in gliomas have been described through multiple timepoints and regional microdissection by scRNA-seq [77, 78]. Antunes *et al.* established the microglial fate-mapping system and revealed the similarities and differences in TAM distribution in newly-diagnosed GBM, recurrent GBM, and mouse GL261 models [79]. Moreover, microglia-derived TAMs or MDMs extracted from tumors are self-renewing populations that are unable to induce $CD4^+$ T-cells or $CD8^+$ T-cells, and compete for space in the tumor environment. Accordingly, the dominant myeloid population in glioma can progress from microglia-derived in the early phases to a mixture of microglia-derived TAMs and outnumbered MDMs in the later phase [89]. In addition, the aryl hydrocarbon receptor in monocytes boosts monocyte recruitment, and blocks antigen presentation expression in MDMs *via* the transcription factor KLF4 [69].

Considering the distinct biology of the two cell populations, it is essential to identify stable biomarkers to distinguish these two groups. In humans, microglia and macrophages can be classified *via* fluorescence-activated cell sorting using CD45 and CD49D (known as α4 integrin and ITGA4) [75]. Accurate separation in mice can be obtained by Ly6C, CD11b, F4/80, CD45, and Cx3cr1 [79]. As previously reported, the classical signature markers for microglia (P2ry12 and Sall1) and MDMs (Ly6c and Ccr2) are reduced during glioma-induced activation or differentiation and are insufficient for use in classifying the two populations [6]. After infiltration into the CNS, MDMs has a higher microglia signature gene pattern (Cx3cr1 and Tmem119) and lower CD45 (Fig. 2). Nevertheless, Qian *et al.* found that Crybb1 and Ldhb are specific and stable markers across different tumor stages in mice, and the cluster of MDMs consistently featured with Iqgap1 corresponded to other clinical datasets [80]. A variety of cytokines (IL-6 and IL-10) and several genes encoding chemokines associated with wound healing (Ccl22, Ccl17, Cxcl2, and Cxcl3) are upregulated in TAMs [81]. Of interest, a pro-inflammatory subset of microglia-derived TAMs was found to exhibit increased expression of Il1b (encoding IL-1β), Ifnb1 (encoding IFNβ1), Ccl4 (encoding C-C Motif Chemokine Ligand 4), Il12 (encoding IL-12), and Tnf (encoding TNF) [82, 83]. In addition, time-of-flight mass cytometry was also combined to reveal the heterogeneity of TAMs in gliomas [79]. Multiple subsets were identified exhibiting downregulation of classical microglial signature genes and, to the contrary, with upregulation of pro-inflammatory cytokines, responses to type I interferons, and hypoxia-associated molecules [79]. Collectively, these results describe novel glioma-associated microglia phenotypes and their diverging functions, which may provide new potential avenues for therapeutic interventions.

## Functional Characteristics of Microglia/Macrophages in Glioma

The role of microglia in glioma is controversial. In a model of organotypic slice cultures and *in vivo* implantation, the ablation of microglia impairs tumor growth and prolongs the survival of tumor-bearing mice. Further studies have revealed that glioma cells activate microglia but impair phagocytic activity [84–86]. Moreover, endogenous microglia derived from non-glioma subjects have a strong inhibitory effect on the expression of genes relevant to the cell cycle in tumor-initiating cells [87]. Microglia-activating substances such as GM-CSF and LPS can stimulate glioma cell migration cooperatively with endothelial cells, revealing that microglia do not merely react to tumor invasion but play a more complicated role in gliomas [88].

Compelling evidence underpins the perspective that genetic and molecular subtypes of GBM reflect distinct tumor microenvironment (TME), while secreted molecules or subsequently activated signaling from TAMs reciprocally remodels the cellular state of tumors. The functional interactions between GBM cells and components in the microenvironment play an important role in the modulation and infiltration of the brain. Liu *et al.* used scRNA-seq to characterize cell populations from IDH-WT and IDH-mutant samples and showed that the percentage of microglia and macrophages was higher in IDH-WT GBM [89]. Further, ~500 genes were found to be differently expressed in microglia isolated from IDH-WT and IDH-mutant samples [90]; but this cannot be exclusively considered as microglial heterogeneity. Using longitudinal scRNA-seq, Friedrich *et al.* examined myeloid cellular states in gliomas and demonstrated that differentiation of myeloid cells in IDH-mutant tumors is blocked by re-orchestration of tryptophan metabolism, leading to an immature phenotype [91]. Alteration of tryptophan metabolism in IDH-mutant gliomas reverses immunosuppression. It has also been reported that the mesenchymal subtype of GBM reported in The Cancer Genome Atlas is associated with an inferior prognosis and contains a higher proportion of TAMs compared to proneural or classical subtypes [36]. In addition, copy number amplifications such as *CDK4*, *EGFR*, and *PDGFRA* loci or mutation of the *NF1* locus are correlated with different cellular states in GBM [36]. Mutations of *NF1* result in a reduction of NF1 expression, which is predominantly found in the mesenchymal state of GBM, and are possibly responsible for the increase of TAM infiltration by NF1-regulated microglial chemotaxis [36]. Nevertheless, Hara *et al.* leveraged single-cell RNA sequencing and a mouse model to recapitulate mesenchymal GBM cellular states *in vivo*, exploring the crosstalk between macrophages and GBM subtype states. Analysis of ligand-receptor pairs suggests that oncostatin M in macrophages activates STAT3 signaling to induce a mesenchymal GBM

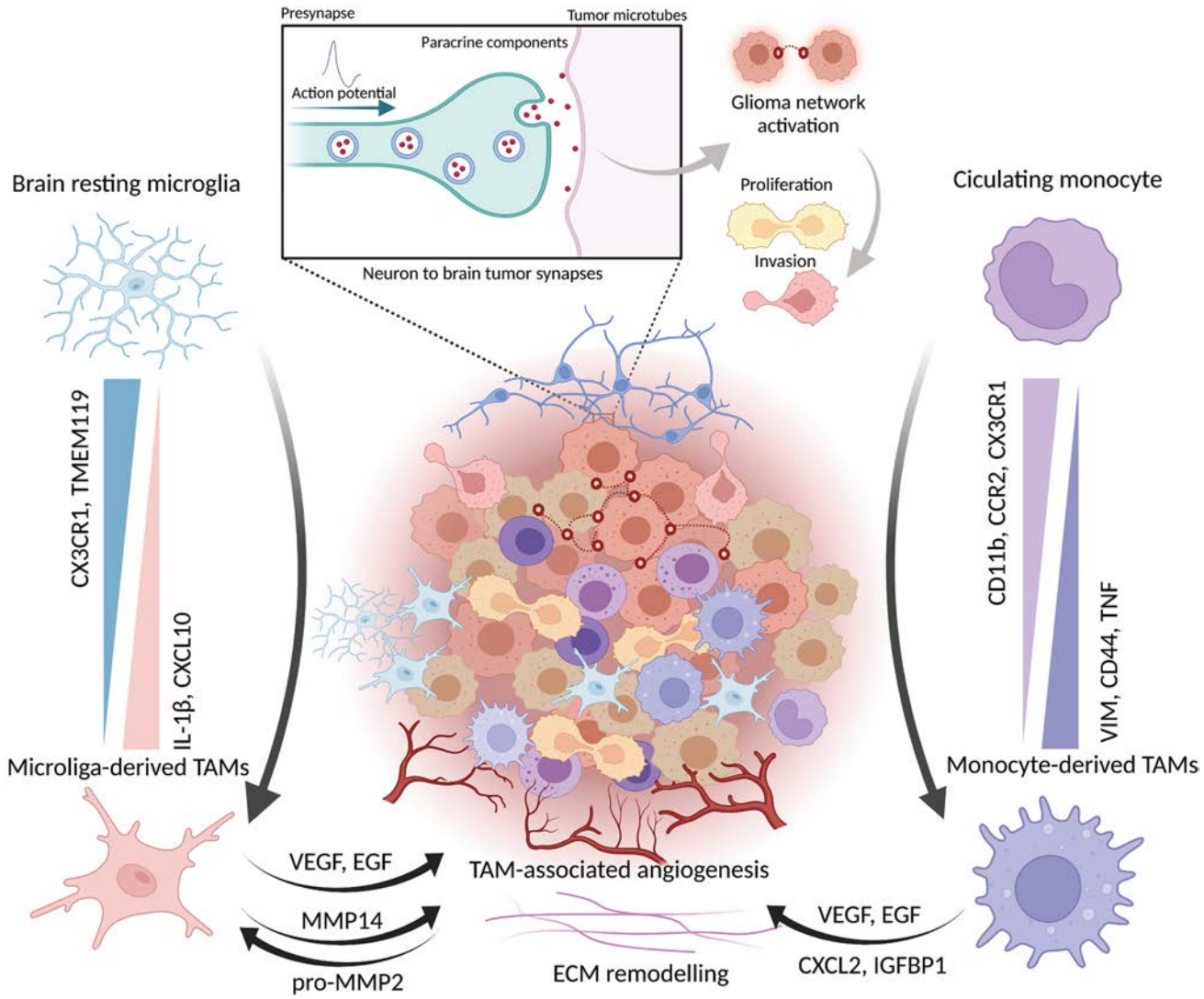


**Fig. 2** Reported communications in the glioma microenvironment. The tumor microenvironment of gliomas is complicated. Left, the relationship between brain resting microglia and microglia-derived TAMs. Wedges indicate differential biomarker expression between the two groups. The classical biomarkers of microglia (CX3CR1 and TMEM119) are reduced in microglia-derived TAMs, rather than other activated markers (IL-1β, CXCL10). Right, the relationship between circulating monocytes and monocyte-derived TAMs. Upper, schematic of neuron-to-brain tumor synapses. Lower, signaling pathways between TAMs and tumor cells. VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; TAM, tumor-associated macrophage; ECM, extracellular matrix.

cellular state *via* interaction with its receptors (or leukemia inhibitory factor receptor, in complex with GP130) in glioblastoma cells [92]. Moreover, in mouse GBM models, TAMs increase the levels of antigens presented, such as major histocompatibility complex type II expression, suggesting that TAMs in GBM can process antigens to T cells but are unable to activate the subsequent reaction [79]. Matrix metalloproteinases (MMPs) have been reported to be another group of proteins that are crucial to cells infiltration. MMP2 has been reported to be a marker of poor prognosis, facilitating the invasive and angiogenic properties of gliomas [93]. MMP2 is released in the form of pro-MMP2 and subsequently cleaved by MMP14 and converted into an active state which regulates the degradation of the extracellular matrix. In TME, the cleaved substrate pro-MMP2 is secreted by GBM cells, while microglia are the major source of MMP14 in TME. This reciprocal cooperation has been found to be regulated by its downstream signaling receptor TLR2 [94] and extracellular vesicles derived from GBM.

Furthermore, abundant and aberrant neovascularization is one of the defining characteristics of GBM. The process of angiogenesis and angiogenic factors have been extensively described. Resident microglia and peripheral macrophages collectively constitute perivascular niches, while a variety of pro-angiogenic molecules have been found to be upregulated. The CXCL2-CXCR2 signaling pathway is

significantly upregulated during angiogenesis and has been shown to have a stronger angiogenic effect than VEGF *in vitro* [95]. Inhibiting the CXCL2-CXCR2 signaling pathway or selectively reducing resident microglia after tumor implantation decreases the tumoral vasculature count and tumor volume. Nevertheless, insulin-like growth factor-binding protein 1 (IGFBP1), released by microglia, is a novel factor mediating macrophage colony-stimulating factor-induced angiogenesis in GBM *via* an SYK-PI3K-NFκB-dependent mechanism [96]. Of interest, STAT3 expression (known as IL6/JAK/STAT3 signaling) in the glioma cell affects a variety of targeting genes and can propagate tumorigenesis by facilitating proliferation and angiogenesis. STAT3 upregulation, closely associated with abundant microglia and macrophages, is preferentially enriched in MES-like GBM. Further, ablation of receptors for advanced glycation end products (RAGE) prolongs the overall survival in a GL261 mouse model, and reduces the angiogenic factors secreted by TAMs. It has also been recently reported that microglial neuropilin-1 regulates vascular morphogenesis and affects its receptor VEGFR2 [97]. Administration of the inhibitor EG00229, which impairs the binding of tuftsin (Thr-Lys-Pro-Arg) with Nrp1, reverses the anti-inflammatory state of microglia through transforming growth factor beta signaling [97, 98]. Thus, the interactions between TAMs and GBM are complicated and multifactorial, and understanding the subtypes of TAMs presented in different primary gliomas is important for screening and developing subtype-specific targets.

## Neurobiology of Gliomas

Epileptic seizures, memory disorders, and cognitive impairment are common manifestations of patients with gliomas. These clinical characteristics have long been thought of as the result of mechanical pressure caused by the occupying lesion, while little is known about the interactions between tumor cells and surrounding neurons. However, a close relationship between tumors and the CNS has been validated in that gliomas exhibit electrical activity and are integrated into neural networks [99], which is thought to be a milestone event in the rapidly emerging field termed cancer neuroscience [100]. Compared to other pro-tumor factors derived from adjacent normal cells, the supportive influence of neurons includes direct (electrical, synapses, or synapse-like structures) and indirect (chemical) effects. Using electron microscopy, Venkataramani *et al.* described subtypes of distinct synapses formed by gliomas [101]. There are three morphological categories of neuron-glioma synapses that are consistently formed in incurable human gliomas but hardly exist in oligodendrogliomas. A parallel study found broad expression of glutamate receptor genes in high-grade gliomas, including IDH-mutant glioma, IDH-wild-type glioma, and diffuse intrinsic pontine glioma. Targeted patch-clamp recordings showed the existence of spontaneous excitatory postsynaptic currents that are mediated by glutamate receptors of the AMPA subtype. Synchronized $Ca^{2+}$ transients are generated by neuronal firing, while genetic perturbations of AMPA receptors or the AMPA receptor antagonist perampanel reduce the invasiveness of gliomas [101]. These studies suggest direct, biologically relevant glutamatergic communication between neurons and glioma cells (Fig. 2). Excessive glutamate released by glioma cells may explain the recurrent seizures in patients. Intriguingly, it has been reported that the expression of glutamate transporters is increased in para-tumor cells, and performs a neuroprotective function in animal models [102]. In addition, excessive glutamate release may also lead to opening of the blood-brain barrier *via* the activation of N-methyl-D-aspartate receptors [103], and this is beneficial to the efficacy of drug delivery. In a recent impressive study, Chen *et al.* used an autochthonous mouse model to recapitulate adult OPC-originated gliomagenesis and found that olfaction can directly regulate gliomagenesis *via* insulin-like growth factor 1 (IGF1) signaling [104]. The activity of olfactory receptor neurons (ORNs) has significant effects on the progress of gliomas, while specific knockout IGF1 receptors in mutant OPCs abolishes the influence derived from ORNs. According to these groundbreaking studies, gliomas have the ability to form electrical and functional synapses with surrounding neurons, which drive tumor growth and resistance [105, 106].

Notably, tumor cells from incurable gliomas share several features with developing neurons (in the process of axonal and dendritic outgrowth) and extend long and thin microtubes [105]. Several reports have found that neurotransmitters in TME drive tumor growth and invasion. Nevertheless, Venkatesh and colleagues [107, 108] revealed a novel mechanism behind this reciprocal influence, showing that neuron paracrine secretion of neuroligin-3 (NLGN3) facilitates tumor progression and in turn induces a synaptic gene signature in the tumor cell. Researchers applied the optogenetic approach *in vivo* and *in vitro*, demonstrating that the firing activity of neurons promotes the proliferation and growth of glioma cells. Moreover, NLGN3, secreted by cortical projection neurons and oligodendrocyte precursor cells, is the leading candidate mitogen regulating this process. NLGN3 is broadly expressed in excitatory synapses and affects glioma proliferation through the phosphoinositide 3-kinase–mammalian target of rapamycin pathway [107]. Remarkably, the growth of GBM xenografts is significantly impaired in *Nlgn3*-knockout mice. In addition, brain-derived neurotrophic factor (BDNF) has also been validated to play a central role in classical synaptic functions and has a stimulating effect in TME [109].

Of interest, the interactions between glioma and neurons might involve the intimate interplay of neurons with precursor cells (NPCs and OPCs). Neuron-to-non-neuron synapses were first described by Bergles *et al.* in 2000. They reported that neurons form *bona fide* synapses with OPCs and regulate their proliferation [110]. Electrophysiological analyses revealed that these neuron-glial synapses are similar to normal neuron-neuron synapses, sharing features such as rapid activation, quantal responses, facilitation, depression, and presynaptic inhibition. Previous evidence showed that gliomas mainly originate from NPCs and/or OPCs, which may explain these structural similarities. Moreover, Elizabeth *et al.* reported that NPCs in the SVZ stimulate invasion of glioma cells through the secretion of chemoattractant signals. Inhibition of Rho/ROCK signaling reduces invasion of glioma cells induced by factors secreted by SVZ NPCs [111]. This novel framework provides new insights into understanding the progression of cancer and sheds light on therapeutic opportunities that can disrupt these communications.

## New Insights for Therapeutic Opportunities in High-Grade Glioma

### Immune Checkpoint Therapy

The treatment of high-grade glioma is still mainly based on surgery with postoperative radiotherapy and chemotherapy [112]. It is promising that some novel treatment strategies have shown high promise. Tumor immunotherapy has attracted much attention, but owing to the lack of specificity of brain immunity, current immunotherapy strategies require further improvement before application in high-grade gliomas [113, 114]. A series of clinical trials that tested the safety and efficacy of targeting immune checkpoints showed no improved survival benefit in GBM patients [115–117]. In 2017, a phase III clinical trial comparing nivolumab (PD1 monoclonal antibody) with bevacizumab (VEGFA monoclonal antibody) showed that patients with recurrent glioblastoma did not benefit from nivolumab treatment (CheckMate-143) [117]. A phase III clinical trial comparing nivolumab plus radiotherapy with standard chemoradiotherapy further confirmed that patients with *de novo* O-6-methylguanine-DNA methyltransferase (MGMT) unmethylated glioblastoma did not benefit from nivolumab therapy (CheckMate-498) [118]. In addition, CheckMate-548 yielded similar negative results in a phase III trial which compared nivolumab plus standard chemoradiation *versus* standard chemoradiation in patients with MGMT-methylated glioblastoma [119]. Alternatively, a recent study demonstrated that changing the dosing strategy and administering PD1 antibodies using neoadjuvant therapy can prolong the median survival of patients with relapsed glioblastoma [120]. However, immune checkpoint inhibitors are unable to reverse immune exhaustion in GBM [121]. Mass cytometry time-of-flight analysis revealed that macrophages contributed to 72.6% of the leukocytes in the TME [122], most of which expressed multiple immunosuppressive markers. These data indicate that immune suppressive macrophages are an important confounder for attenuation of the T-cell response. Further understanding of the immune microenvironment within brain tumors is needed to improve the clinical efficacy of immune checkpoint therapy.

### Cell-Based and Oncolytic Virus Therapy

Cell therapy based on chimeric antigen receptors (CARs), which involves grafting a specific designed receptor onto an effector cell, is also a research frontier in the treatment of high-grade gliomas [123]. Clinical trials targeting three antigens, EGFRvIII, HER2, and IL-13R alpha2, have confirmed that the application of CAR-T is safe, feasible, and potentially effective [124–127]. However, the application of CARs to brain tumors still faces challenges due to tumor heterogeneity and antigen loss. Antigen loss in recurrent tumors has been reported in both CAR-T therapy targeting EGFRvIII and IL-13R alpha2 [125, 127]. Interestingly, the major toxicity of CAR-T cells is cytokine release syndrome (CRS). Myeloid-derived macrophages have been found to contribute to the pathogenesis of CRS, mainly mediating the production of core cytokines including IL-6, IL-1, and interferon-γ [128]. In addition, Rodriguez-Garcia *et al.* demonstrated that CAR-T cell-mediated selective elimination of folate receptor β TAMs resulted in an increase in endogenous activated CD8+ T cells, decreased tumor burden, and prolonged survival [129]. Several reports have highlighted that engineering CAR macrophages is a valuable strategy in GBM. CAR-macrophages have been adapted and designed to produce pro-inflammatory cytokines, which convert subtypes of macrophages from M2 to M1. The polarization of macrophages increases T cell anti-tumor activity and further modulates the pro-inflammatory characteristics of TME [130, 131]. Thus, it is essential to develop new techniques to screen out suitable antigen sites of tumors or immunosuppressive cells, reduce antigen loss, and retard immune cell exhaustion.

Notably, therapeutic vaccination for brain tumors may be a promising treatment strategy. The EGFRvIII-based vaccine was successful in phase II clinical trials for glioblastoma, but failed to achieve positive results in phase III clinical trials. Tumor samples from relapsed patients showed immune escape, which is also a pressing problem during the vaccination treatment period and similar to the challenges of cell therapy [132–135]. Developing individualized vaccines based on specialized patient gene mutation patterns and expression profiles that collectively target the multiple

glioma antigens is a potential future direction. Moreover, oncolytic virus therapy can activate antitumor immune responses, which are an important active immune therapy. A clinical trial using recombinant poliovirus in the treatment of recurrent glioblastoma suggested that this technique is effective and safe [136]. Therefore, improving the targeting of oncolytic viruses, slowing the clearance of oncolytic viruses by the immune system, and reducing the side-effects of oncolytic viruses are important methods for improving the clinical application value of oncolytic viruses.

### Transdifferentiation Induction and Glioma Reprogramming

Owing to the similarity between GSCs and NSCs researchers have proposed that inducing GSCs to differentiate into terminally-differentiated cells, especially neurons, might be a supportive strategy to inhibit the progression of brain tumors. It has been reported that inhibition of the Notch pathway can significantly induce a subset of patient-derived GSCs with high *ASCL1* expression to differentiate into neuron-like cells [137]. In addition, previous studies have shown that the pression of glioma cells can be retarded by inducing glial differentiation *via* activation of microRNA or BMP signaling [138, 139]. Overexpression of three neurogenic transcription factors (*ASCL1*, *BRN2*, and *NGN2*) reprogrammed 20%–40% of human glioma cells into TUBB3-positive neurons *in vitro* [140]. Cooperating with *NGN2* and *SOX11*, intravenous injection of overexpressing viruses has been shown to improve the reprogramming efficiency of human glioma cells into terminally-differentiated neuron-like cells, thus delaying tumor progression and significantly prolonging the survival of tumor-bearing mice [141]. A similar result was found *via* overexpression of *NGN2*, *ASCL1*, and *NeuroD1* in glioma cells. Unfortunately, gene regulation as a treatment for GBM are bound to face great challenges, and certain risks exist in the clinical application of transgenic technology and virus transfection such as off-target effects and neurotoxicity. However, these results suggest the potential for reprogramming of GBM cells into neurons [142–144].

### Disruption of Neuron-Glioma Communication

Glutamatergic synaptic structures and gap junctions have been identified in diffuse gliomas and can evoke long-lasting depolarizing currents, $Ca^{2+}$ flux, and subsequent electrical network reactions. This cascade of electrical responses in glioma subpopulations ultimately promotes cell invasion and mitosis [99, 101]. Noninvasive brain stimulation (NiBS) is a group of techniques applied to the scalp that are broadly used in clinical practice to modulate neural activity *via* transcranial electrical or magnetic fields (transcranial magnetic stimulation, TMS; tumor-treating fields, TTFields). Interestingly, NiBS can increase or decrease neural activity depending on different stimulation patterns, of which the mechanisms are considered to involve the regulation of synaptic plasticity [145]. Long-lasting effects across multiple regions of the brain have been reported after stimulation with magnetic or electrical fields [145, 146]. NiBS has also been reported to induce effects such as the modulation of glutamatergic transmission, BDNF-dependent plasticity, and the regulation of pathway activity [147, 148]. Thus, disruption of communication between glioma and neurons is a promising area of study. Considering the efficacy and safety of NiBS, both TMS and TTFields have been approved for the treatment of several psychiatric diseases [145]. As for neoplasms, the landmark EF-14 trial, showed that TTFields plus maintenance Temozolomide (TMZ) resulted in an increase in overall survival in patients with newly diagnosed GBM compared to TMZ alone (20.9 *vs* 16.0 months with TMZ alone). Furthermore, no systemic adverse events are associated with the addition of TTFields (48% *vs* 44% with TMZ alone) [149, 150]. Indeed, TTFields deliver a low intensity (1–3 V/cm) at medium frequency (100–300 kHz) to the tumor region, alternating extra physiological currents which do not affect neural activity but rather impede cancer cell mitosis. The formation of microtubules, which are essential structures for neuron-glioma communications [101, 151], is disrupted by TTFields. Recent reports also revealed that TTFields induce an increased release of micronuclei from tumor cells, leading to activation of cGAS/STING and AIM2/Caspase-1 [152]. After treatment with TTFields, T-cell activation and clonal expansion have been reported in samples and derived from the upregulation of adaptive immunity. Taylor *et al.* genetically or pharmacologically blocked BDNF-TrkB signaling in a xenograft model of pediatric glioblastoma, abrogating the tumor-promoting effects of BDNF on synapses and prolonging survival [109]. Thus, growing evidence suggests that the application of NiBS may be used to suppress glioma progression and tumor-promoting neuronal communication.

## Discussion

Understanding of biology and immunology in gliomas has advanced at an impressive pace in recent years. The brain TME comprises heterogeneous populations of cells exhibiting differences in genetic characteristics and performing various modes of reciprocal interaction to mediate tumor initiation, progression, and therapeutic response. Combining advancing technologies in genetic engineering and sequencing enables promising capabilities in diagnosis and personalized treatment, and deciphering the origins of tumor-supporting cells at the single cell level as well.

Studying developmental programs is a promising strategy for understanding the process of oncogenesis, and essential targets for disrupting disease progression or remission may be found. We here reviewed advances in gene-edited mouse models that mirror human disease and discussed potential glioma-initiated progenitor cells that may be used for further investigations. In addition, interactions between tumor subtypes, microglia, MDMs, and neurons in the brain TME play an important role in tumor progression. In light of many mechanisms of tumor/non-tumor cell crosstalk and their accompanying outgrowths, these implications in complex TME caused by these interactions are important components of the major driver in glioma biology. We here highlight a nascent but fast-growing field termed cancer neuroscience, which mainly focuses on the tumor-neuron network and its role in the progress of cancer growth. Interesting questions that remain to be answered include: (1) how to specifically target tumor cells in the tumor-neuron axis and integrate neural regulation methods into existing clinical strategy, (2) why and how tumors communicate with neurons in the brain, and (3) whether histopathological subtypes of glioma have a neuron communication preference. Lessons learned from TME suggest that disruption of the tumor/non-tumor cell dialogue could be helpful in inventing potential novel therapeutic approaches beyond standard treatment such as immune checkpoint inhibitors, cell-based biotechniques, and noninvasive brain stimulation. These therapies could potentially become the keystones of clinical practice in the future.

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REVIEW

# The Memory Orchestra: Contribution of Astrocytes

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**Abstract** For decades, memory research has centered on the role of neurons, which do not function in isolation. However, astrocytes play important roles in regulating neuronal recruitment and function at the local and network levels, forming the basis for information processing as well as memory formation and storage. In this review, we discuss the role of astrocytes in memory functions and their cellular underpinnings at multiple time points. We summarize important breakthroughs and controversies in the field as well as potential avenues to further illuminate the role of astrocytes in memory processes.

**Keywords** Astrocytes · Learning and memory · GPCR · Calcium signal · Plasticity

## Introduction

Memory, the ability to retain learned information, is necessary for survival. However, the cellular mechanisms of learning and memory remain obscure even though these functions are two of the most intensely studied processes of the nervous system. Learning is highly dependent on states of arousal, attention, motivation, and emotion as well as the sleep cycle [1] and prior experience [2]. In addition, new events must be coupled with multimodal aspects of an experience and integrated with existing memories [3]. Thus, interactions within local neuron populations and across distant brain regions are essential for all but the most rudimentary forms of learning [4]. However, neuronal electrical signaling occurs on the time scale of milliseconds to seconds, far narrower than the wide time scales (hours, days, and months) required for extensive spatial integration; in contrast, the temporal dynamics of astrocytic communication and plasticity are well suited for these time scales [5]. Although molecular and cellular investigations of learning and memory have mainly focused on neuronal mechanisms thus far, recent research has begun to investigate the roles of astrocytes in learning and memory.

Astrocytes are the largest class of glial cells in the mammalian CNS [6]. Astrocytes are morphologically complex and characterized by intricate processes and anatomical specializations that mediate local interactions with other cells in the CNS. They express a variety of receptors, transporters, and other molecules that allow them to sense external signals and respond to the signals with intracellular $Ca^{2+}$ elevations [7]. In addition, astrocytes are equipped with the machinery to secrete various molecules, including γ-aminobutyric acid (GABA), adenosine triphosphate, D-serine, L-lactate, brain-derived neurotrophic factor, and other factors, in response to $Ca^{2+}$ elevations and other stimuli [8]. Accordingly, astrocytes are involved in multiple structural, metabolic, and homeostatic functions and regulate higher cognitive functions, including emotion and memory [9]. The contributions of astrocytes seem to be target-, circuit- and task-specific [10–13], and their outputs are consistent with how they are

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activated [14]. Moreover, the spatiotemporal diversity of astrocytic $Ca^{2+}$ signals revealed by advanced $Ca^{2+}$ imaging methods may underlie their ability to encode and process different patterns of activation, explaining the above properties [15].

In this review, we specifically discuss the critical contribution of astrocytes to short-term memory, which lasts from seconds to minutes, and long-term memory, which lasts for hours to years and potentially throughout the entire lifetime. We first summarize recent studies showing the involvement of astrocytes in memory processing and then discuss future challenges in the field.

## Astrocytes and Short-Term Memory

Short-term memory is the ability to retain newly acquired information for short durations. The immediacy of short-term memory demands rapid cellular alterations and relies on post-translational protein modifications. Recent studies demonstrating the involvement of astrocytes in short-term memory have shed new light on the underlying mechanisms. For a summary, see Table 1 and Fig. 1.

Astrocytes are involved in working memory and can temporarily store and manipulate information (on the order of seconds). Much attention has been given to astrocytic G-protein-coupled receptors (GPCRs). A

**Table 1** Astrocyte-specific molecules involved in short-term memory.

| Approach | Molecule/mechanism | Effects | Test | References |
|---|---|---|---|---|
| Astrocyte-specific deletion of the CB1 receptor | LTD | Abolished exogenous cannabinoid-induced impairments of spatial working memory | Delayed nonmatching-to-sample task (T-maze) | [21] |
| Astrocyte-specific deletion of the prefrontal $GABA_B$ receptor<br>Optogenetic activation of prefrontal astrocytes with melanopsin | Gamma oscillation | Impaired working memory<br>Improved working memory | Delayed nonmatching-to-place task (T-maze)<br>The object in place novelty preference test | [19] |
| Inhibition of astrocytic Gq-GPCR signaling with iβARK | Gq-GPCR signaling | Impaired spatial working memory<br>Impaired short-term spatial memory | Y-maze<br>The novel object placement task | [17] |
| Astrocyte-specific deletion of the $A_{2A}$ receptor | Glutamate homeostasis | Impaired spatial working memory | Y-maze<br>Baited 8-radial arm maze | [22] |
| Genetic deletion of the IP3R2 | LTD | Impaired spatial working memory<br>(Impaired remote fear memory, recognition, and spatial memory) | Y-maze<br>(audFC; NOR test; Barns maze) | [18] |
| Inhibition of glycogen phosphorylase in the hippocampus | Lactate | Impaired working memory | Spontaneous alternation in an elevated plus-maze | [23] |
| Activation of astrocytic Gq-GPCR signaling in the CA1 with hM3DGq | Neuronal potentiation | Improved short-term memory<br>(Improved recent fear memory) | T-maze<br>(cFC) | [13] |
| Optogenetic activation of hippocampal astrocytes with melanopsin | LTP | Improved short-term spatial memory | The object in place novelty preference test | [24] |
| Ablation of astrocytic Bmal1 | GABA signaling | Disrupted short-term recognition and spatial memory<br>(Disrupted recent recognition) | NOR test<br>The spatial object-location task | [26] |
| Decreased mitochondrial ROS in astrocytes | Brain metabolism and redox reactions | Disrupted short-term recognition | NOR task | [27] |
| Genetic deletion of S100β | LTP | Enhanced short-term spatial memory<br>Enhanced recent fear memory | MWM test;<br>(cFC) | [25] |
| Astrocytic expression of SNARE | Hippocampal-prefrontal theta synchronization | Impaired short-term memory<br>(Impaired recent spatial memory and recognition) | 2TPR<br>(MWM test, hole board task; NOR task) | [28] |

2TPR, two-trial place recognition task; audFC, auditory fear conditioning test; cFC, contextual fear conditioning test; MWM, Morris water maze; NOR, novel object recognition.

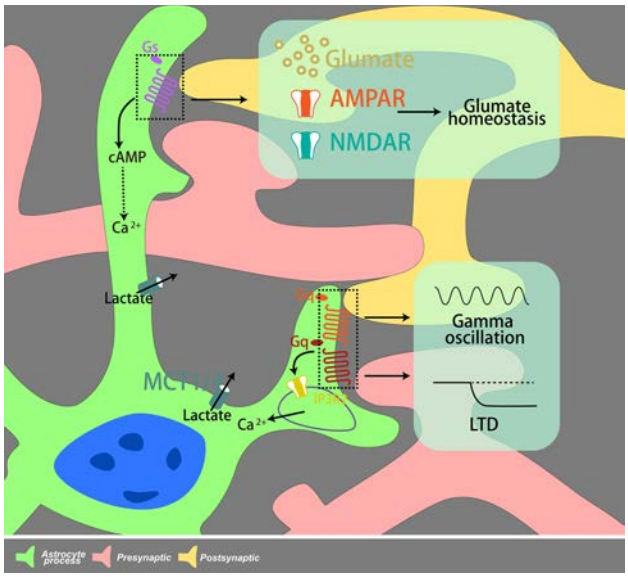


**Fig. 1** Involvement of astrocytes in working memory. Inhibition of endogenous Gq-GPCR signaling in astrocytes with iβARK impairs working memory, while stimulation of opto-Gq-GPCR (melanopsin) has the opposite effect; astrocytic knockout of $GABA_BRs$, $CB_1Rs$, and $A_{2A}Rs$ impairs working memory and disrupts gamma oscillation, LTD, and glutamate homeostasis, respectively. Astrocytes also provide metabolic support for memory processes. A2AR, adenosine A2A receptor; AMPAR-2B, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor 2B subunit; CB1R, cannabinoid receptor 1; $GABA_BR$, $GABA_B$ receptor; IP3R, inositol 1,4,5- trisphosphate receptor; LTD, long-term depression; NMDA-R, N-methyl-D-aspartate receptor.

major route of inducing $Ca^{2+}$ elevations is by activating Gq-GPCRs, resulting in the release of $Ca^{2+}$ from internal stores through the activation of phospholipase C and the inositol 1,4,5-trisphosphate receptor (IP3R) [16]. Specifically, inhibition of astrocytic Gq-GPCRs with the inhibitory peptide β-adrenergic receptor kinase 1 results in reduced spontaneous alternation in the Y-maze test, suggesting deficits in spatial working memory [17]. Consistent with this finding, transgenic mice lacking IP3R2 (IP3R2 $^{-/-}$ mice), the major subtype of IP3R in astrocytes, display disruption of GPCR-mediated $Ca^{2+}$ signaling and impaired performance in the Y-maze [18]. In contrast, activation of prefrontal astrocytes by Gq-coupled melanopsin improves working memory [19]. In addition, mice with genetic deletion of Gq-coupled $GABA_B$ receptors in prefrontal astrocytes show poor performance on a delayed nonmatching-to-sample task (T-maze) and reduced gamma oscillatory activity [19], providing additional support for the idea that astrocytic Gq-GPCRs are involved in working memory. These studies indicate that stimulation of Gq-GPCRs facilitates working memory, while inhibition has the opposite effect. Interestingly, a study showed that disruption of vesicular release in astrocytes reduces cortical gamma oscillations but has no effect on spatial working memory [20]. While the above two studies showed that astrocytes are associated with gamma oscillations, further studies are required to clarify their contribution to working memory. Astrocytic Gq-GPCRs play an important role in the deficits of spatial working memory induced by the application of exogenous cannabinoids. However, selective deletion of Gq-coupled CB1 receptors in astrocytes does not affect the deficits of spatial working memory [21]. Studies have conducted preliminary explorations of astrocytic Gs-GPCRs. Ablation of the Gs-coupled adenosine $A_{2A}$ receptor on astrocytes results in impaired spatial working memory and disruption of glutamate homeostasis [22]. Furthermore, astrocytes provide metabolic support for working memory. Pharmacological inhibition of glycogenolysis impairs working memory, while administration of exogenous lactate rescues memory deficiency [23].

We next focus on the form of short-term memory that lasts for minutes (up to 300 min). Chemogenetic and optogenetic activation of the Gq signaling pathway in hippocampal astrocytes enhances short-term memory [13, 24]. While these studies suggest that astrocytic activation is critical for regulating memory performance, they did not demonstrate astrocyte activation during short-term memory. However, studies that have suppressed specific signaling pathways in astrocytes have provided such evidence. For instance, mutant knockout mice lacking the $Ca^{2+}$-binding protein S100β exhibit enhanced short-term spatial memory and enhanced hippocampal long-term potentiation (LTP) [25]. Another study showed that deletion of the clock gene Bmal1 in astrocytes disrupts short-term recognition and spatial memory, while pharmacological regulation of $GABA_A$-receptor signaling completely reverses behavioral deficits [26]. In addition, *in vivo* decreases in endogenous mitochondrial reactive oxygen species in astrocytes lead to impaired short-term recognition [27], indicating that brain energy and redox metabolism are necessary for short-term memory. Selective expression of the SNARE domain of synaptobrevin II (dnSNARE) in astrocytes [28] prevents vesicular release and causes a deficiency in short-term spatial memory. Although progress has been made in this field, chronic expression (or deletion) of genes suffers from multiple disadvantages, particularly the activation of compensatory mechanisms. As short-term memory (including working memory) occurs on the order of seconds to hours, the mechanism through which permanent deletion or overexpression of astrocytic genes contributes to short-term memory formation needs further investigation. Combining the above results from optogenetic, chemogenetic, and transgenic methods, we argue that astrocytic Gq-GPCRs and their downstream $Ca^{2+}$ signals and energy metabolism are critical for the formation of short-term memory.

## Astrocytes and Long-term Memory

After initial encoding, memories undergo consolidation for long-term storage. Consolidation of recent memories is achieved by changes in gene expression and protein synthesis at the relevant synapses, and long-term memories are gradually reorganized among various brain areas and stored and retained as remote memories. In this section, we describe astrocytic involvement in long-term memory from different aspects. Unless otherwise specified, the term "long-term memory" includes both recent memory and remote memory. For a summary, see Table 2 and Figs. 2 and 3.

### Information Detection and Modulation of Synaptic Plasticity

By monitoring astrocyte $Ca^{2+}$ activity at the single-cell level (over several days with *in vivo* two-photon $Ca^{2+}$ imaging) and at the level of small clusters (with optic fiber-based recordings) in freely-moving mice, a recent study revealed learning-induced changes in astrocytic activity [29]. These changes were found in a subset of astrocytes during fear learning, persisted for the duration of the fear memory, and vanished upon extinction of the learned behavior [29]. These authors further found that this astrocytic responsiveness requires the activation of α7-nAChRs (acetylcholine receptors) by afferent cholinergic neuronal inputs [29]. Knockout of α7-nAChRs in astrocytes of the auditory cortex in mice significantly impairs persistent fear memory [29]. Thus, determining which gliotransmitter is released from astrocytes and how astrocytes modulate neurons during this process is intriguing. Interestingly, in the hippocampus, astrocytes receive cholinergic inputs through m1-AChRs and modulate BDNF secretion, which is essential for adult hippocampal neurogenesis and fear memory, as deletion of astrocytic m1-AChRs in the DG impairs recent contextual fear memory and the development of newborn neurons [30]. Consistent with these findings, artificial activation of astrocytes by optogenetic and chemogenetic activation of Gq signaling in hippocampal astrocytes enhances recent fear memory and induces LTP through the release of D-serine [13]. Another study suggested that the Gq-D-serine pathway also plays a role in recent memory. Deletion of astrocytic CB1 receptors reduces D-serine-mediated N-methyl-D-aspartate receptor (NMDAR) activation, suppresses LTP in CA1 neurons, and eventually impairs recent recognition memory [31]. However, optogenetic activation of hippocampal astrocytes with ChR2, activation of which induces increased $[Ca^{2+}]_i$ influx into astrocytes from the extracellular space (in contrast to the release of $Ca^{2+}$ from internal stores through activation of Gq-GPCRs), leads to the activation of $A_1$Rs through the release of adenosine and impairs recent fear memory [32]. In addition, Gq activation of astrocytes in the medial central amygdala disrupts recent fear memory, mediated by astrocyte-derived adenosine that increases $A_1$R-mediated inhibition from the lateral central amygdala and decreased $A_2$R-mediated excitation from the basolateral amygdala [11]. These contradictory results indicate that (1) $[Ca^{2+}]_i$ elevation from internal stores *versus* extracellular space may induce astrocytes to release different substances (i.e., D-serine *vs* adenosine), which then activate their associated receptors on nearby neurons, leading to different forms of synaptic regulation [14]; and (2) hippocampal and amygdala astrocytes may be different subpopulations with substantial molecular diversity and different functions, as astrocytes are highly heterogeneous across and within brain regions [33].

In addition to Gq signaling, astrocyte-specific Gi-GPCR activation enhances astrocyte-specific $Ca^{2+}$ changes *in vivo* [34, 35]. In line with this idea, stimulation of CA1 astrocytes with DREADD-Gi or Gi-coupled μ-opioid receptors enhances recent contextual memory for conditioned place preference (CPP) [36]. Activation of astrocytic Gi-GPCRs, especially Gi-coupled μ-opioid receptors, induces glutamate release through K2P channels, increased the probability of glutamate release by activating presynaptic mGluR1s, and potentiates early-phase LTP, thus potentially accounting for the acquisition of memory associated with CPP [36]. However, another study that applied the same DREADD-Gi manipulation in CA1 found impaired remote but not recent fear memory, accompanied by the activation of anterior cingulate cortex (ACC) neurons [12]. These studies indicate that astrocytes exhibit task-specific effects; that is, astrocytic Gi-coupled stimulation selectively facilitates recent pleasant memories [36] but diminishes remote salient memories [12]. Although there have been no studies that directly activated the astrocytic Gq pathway using DREADD-Gq to explore remote memory, conditional silencing of the astrocytic IP3R2 pathway in adults impairs remote but not recent fear memory and spatial memory and disrupts late-phase LTP (L-LTP) through changes in astrocyte-derived BDNF [37]. Another study ablating IP3R2s also demonstrated impairments in remote fear memory and spatial memory, in addition to remote recognition memory [18]. The possible explanations for these discrepancies are as follows. (1) Gq-GPCRs and Gi-GPCRs regulate $Ca^{2+}$ activity differently in the long term. Studies have reported that the initial increase in astrocytic $Ca^{2+}$ activity following Gi pathway activation [12, 34, 35] is accompanied by a later decrease in $Ca^{2+}$ dynamics [12] as opposed to Gq-mediated astrocytic activation, which results in both acute and minute-scale increases in $Ca^{2+}$ activity [13]. The difference in $Ca^{2+}$ characteristics might thus lead to different effects on remote memory; that is, Gi stimulation [12] and Gq blockade [18, 37] in the hippocampus results in impaired remote fear memory. (2) Although activation of both Gi- and Gq-GPCRs initially elevate $Ca^{2+}$

**Table 2** Astrocytic-specific molecules involved in long-term memory.

| Approach | Molecule/mechanism | Effects | Test | References |
|---|---|---|---|---|
| Astrocyte-specific deletion of CB1 receptors in the hippocampus | D-serine and LTP | Impaired recent recognition | NOR test | [31] |
| Astrocyte-specific knockdown of β2AR in the hippocampus | Lactate and LTP | Impaired recent memory (Impaired remote memory) | Inhibitory avoidance task | [65] |
| Astrocyte-specific deletion of $A_{2A}$ receptor<br>Chemogenetic activation of astrocytic Gs-coupled signaling | Gs-GPCR signaling | Improved recent spatial memory/remote contextual memory<br>Impaired recent spatial memory | MWM test; cFC | [38] |
| Astrocyte-specific knockdown of the α7-nAChR in the auditory cortex | $Ca^{2+}$ transients | Impaired recent fear memory (Impaired remote fear memory) | audFC | [29] |
| Astrocyte-specific deletion of m1-AchRs in the DG | BDNF and neurogenesis | Impaired recent fear memory | cFC | [30] |
| Activation of Gq-GPCR signaling in astrocytes in CA1 with hM3DGq or opto-α-1AR | Neuronal potentiation | Improved recent fear memory (Improved short-term memory) | cFC (T-maze) | [13] |
| Activation of Gq-GPCR signaling in astrocytes in the amygdala with hM3DGq | Synaptic transmission | Impaired recent fear memory | audFC | [11] |
| Optogenetic activation of astrocytes in the hippocampus | Adenosine | Impaired recent fear memory (Impaired remote fear memory) | audFC | [32] |
| Activation of μ-opioid receptors in astrocytes in the hippocampus<br>Activation of Gi-GPCR signaling in astrocytes in the hippocampus with hM4DGi | LTP | Improved recent contextual memory for CPP | CPP test | [36] |
| Activation of Gi-GPCR signaling in astrocytes in the hippocampus with hM4DGi | Hippocampal-prefrontal communication | Impaired remote fear memory | cFC | [12] |
| Inhibition of glycogen phosphorylase in the hippocampus<br>Disrupted expression of astrocytic MCT4 or MCT1 in the hippocampus | LTP | Impaired recent memory (Impaired remote memory) | Inhibitory avoidance task | [62] |
| Inhibition of glycogen phosphorylase in the hippocampus<br>Disrupted expression of astrocytic MCT4 or MCT1 in the hippocampus | -- | Impaired recent memory (Impaired remote memory) | Inhibitory avoidance task | [63] |
| Inhibition of glycogen phosphorylase in the hippocampus | -- | Impaired recent recognition | NOR test | [64] |
| MCT1 knockout mice | -- | Impaired recent memory | Inhibitory avoidance task | [121] |
| Inhibition of glycogen phosphorylase in the amygdala | -- | Impaired recent contextual memory for CPP (Impaired remote contextual memory for CPP) | CPP test | [69] |
| Inhibition of glycogen phosphorylase in the amygdala | -- | Impaired recent contextual memory for CPP (Impaired remote contextual memory for CPP) | CPP test | [70] |
| Astrocytic expression of SNARE | Sleep regulation | Prevented sleep-induced deficits in recent recognition memory | NOR test | [74] |
| Astrocytic expression of SNARE | LTP | Prevented sleep-induced deficits in recent spatial memory | The spatial object recognition task | [75] |

**Table 2** (continued)

| Approach | Molecule/mechanism | Effects | Test | References |
|---|---|---|---|---|
| Astrocytic expression of SNARE | Hippocampal-prefrontal theta synchronization | Impaired recent spatial memory/recognition memory (Impaired short-term memory) | 2TPR (MWM test, hole board task; NOR test) | [28] |
| Genetic deletion of S100β | LTD in hippocampus | Enhanced recent fear memory (Enhanced short-term spatial memory) | cFC (MWM test) | [25] |
| Genetic deletion of GFAP | LTD in cerebellum | Impaired recent memory | Eye-blink conditioning task | [122] |
| Blockade of astrocytic CX43 hemichannels in the amygdala | -- | Impaired recent fear memory | cFC | [123 |
| Astrocyte-specific deletion of glucocorticoid receptors | -- | Impaired recent fear memory (Impaired remote fear memory) | cFC | [68] |
| Astrocyte-specific deletion of IL-6 and IL-6R | -- | Impaired recent spatial memory | MWM test | [78] |
| Astrocyte-specific deletion of the carrier receptor for proBDNF uptake | LTP | Impaired recent recognition memory | NOR test | [43] |
| Astrocyte-specific deletion of Bmal1 | GABA signaling | Disrupted recent recognition memory (Disrupted short-term recognition memory/spatial memory) | NOR test; the spatial object-location task | [26] |
| Astrocyte-specific deletion of MEGF10 | astrocytic phagocytosis, LTP, LTD | disrupted recent recognition memory | NOR test; the spatial object-location task | [56] |
| Astrocyte-specific deletion of ephrin-B1<br>Astrocyte-specific overexpression of ephrin-B1 | Synapse remodeling | Improved recent fear memory<br>Impaired recent fear memory | cFC | [57] |
| Astrocyte-specific deletion of the IP3 receptor<br>Astrocyte-specific deletion of BDNF | LTP | Impaired remote fear memory/ spatial memory | cFC; MWM test | [37] |
| Genetic deletion of the IP3 receptor | LTD | Impaired remote fear memory/ spatial memory/recognition memory | audFC; NOR test; Barns maze test | [18] |
| Disruption of astrocytic $Ca^{2+}$-dependent vesicular release | Gamma oscillation | Impaired remote recognition memory | NOR test | [20] |
| Inhibition of astrocyte cholesterol synthesis | -- | Impaired remote memory | Stone T-maze test | [124] |

audFC, auditory fear conditioning test; cFC, contextual fear conditioning test; CPP, conditioned place preference; MWM, Morris water maze; NOR, novel object recognition.

concentrations, the characteristics of $Ca^{2+}$ activity may be different; thus, Gq stimulation in the hippocampus facilitates recent fear memory, while Gi stimulation has no effect. The above studies reported an overall increase or decrease in intracellular $Ca^{2+}$ activity; however, studies have shown that there is a rich variety of $Ca^{2+}$ transients among different astrocyte compartments [15], and it is not known whether the subcellular $Ca^{2+}$ microdomains in astrocytes have different functions within distinct neural circuits associated with different aspects of memory.

Deletion of Gs-coupled adenosine $A_{2A}$ receptors in astrocytes leads to an increase in recent spatial memory and remote contextual memory [38], whereas activation of the Gs-coupled DREADD Rs1 in astrocytes impairs recent spatial memory [38]. The caveat was that Rs1 is constitutively active independent of its ligand, thus chronic expression of its own ligand impairs memory [38]. Notably, the knockout of adenosine $A_{2A}$ receptors in astrocytes leads to impaired spatial working memory and disruption of glutamate homeostasis [22]. This finding suggests a reverse function of astrocytic $A_{2A}$ receptors, enabling fine-tuning of working memory and long-term memory. Previous reports have shown that $A_{2A}$ receptors modulate behavior in different ways depending on the brain area and cell compartment in which

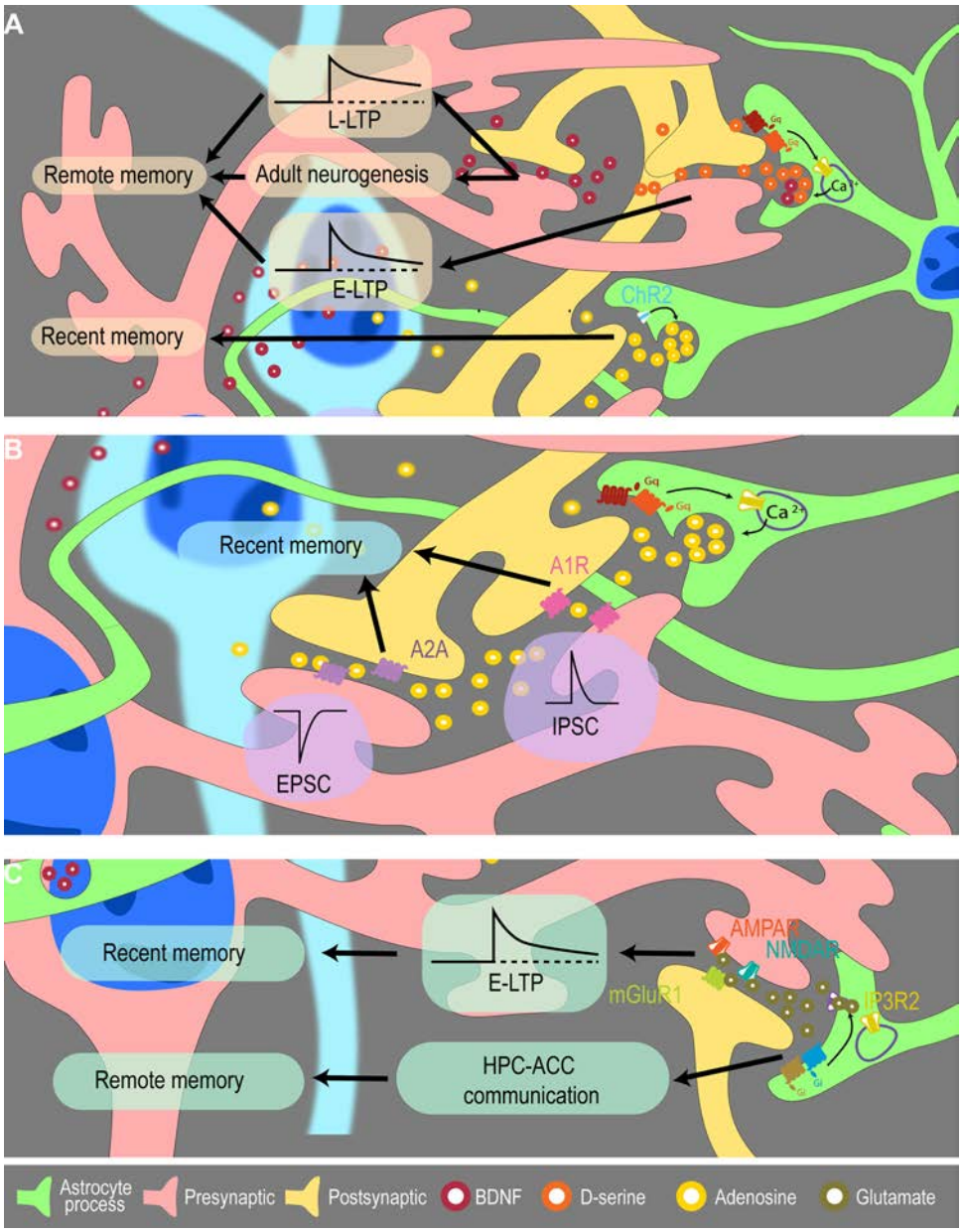


**Fig. 2** Involvement of astrocytic GPCRs in long-term memory. **A** Astrocytic Gq-GPCRs in the hippocampus are involved in long-term memory: IP3R2-dependent $Ca^{2+}$ signaling, mediated by BDNF, is critical for L-LTP and remote memory; m1-AChRs in the DG regulate adult neurogenesis and recent fear memory through BDNF; Gq-GPCR activation in the hippocampus increases D-serine release, facilitates E-LTP, and eventually promotes recent memory formation; optogenetic activation of ChR2, which induces $Ca^{2+}$ influx from extracellular space in the hippocampus, leads to impaired recent fear memory through the release of adenosine. **B** Astrocytic Gq-GPCRs in the amygdala are involved in recent memory: Gq-GPCR activation in the central medial amygdala promotes adenosine release, increases inhibitory inputs from the lateral central amygdala *via* A1Rs, and inhibits excitatory inputs from the basolateral amygdala *via* $A_{2A}$Rs, thus eventually impairing recent fear memory. **C** Gi-GPCRs are involved in long-term memory: activation of astrocytic Gi-GPCRs, especially MORs, induce- glutamate release through K2P channels, increases the probability of glutamate release through presynaptic mGluR1 activation, and potentiates NMDAR-dependent synaptic plasticity, thus explaining the acquisition of CPP-associated memory; while activation of astrocytic Gi-GPCRs impairs fear memory and activates anterior cingulate cortex (ACC) neurons. A1R, adenosine A1 receptor; A2AR, adenosine A2A receptor; BDNF, brain-derived neurotrophic factor; $CB_1R$, cannabinoid receptor 1; CPP, conditioned place preference; E-LTP, early-phase long-term potentiation; EPSC, excitatory postsynaptic current; $GABA_BR$, $GABA_B$ receptor; Glut, glutamate; HPC, hippocampus; IPSC, inhibitory postsynaptic current; IP3R, inositol 1,4,5-trisphosphate receptor; K2P, K2P channel; L-LTP, late-phase long-term potentiation; MOR, μ-opioid receptor.

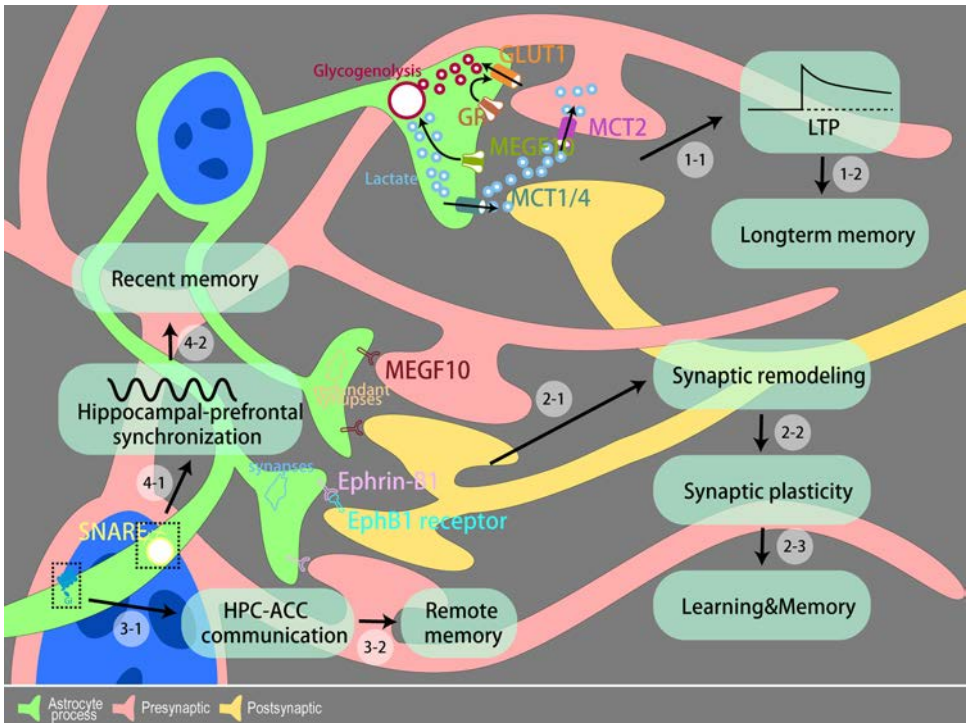


**Fig. 3** Astrocytic involvement in synaptic remodeling, neuronal network communication, and metabolic support for memory. **1.** Astrocyte-neuron lactate coupling is involved in long-term memory formation: glucose is taken up by glucose transporters (GLUT1) and then stored as glycogen or undergoes glycolysis to become lactate, which is exported out of the astrocyte by monocarboxylate transporter 1 or 4 (MCT1/4) and transported into neurons *via* MCT2, providing metabolic support for synaptic plasticity and long-term memory. Glucocorticoid receptors (GRs) promote glucose uptake, and β2 adrenaline receptors (β2Ars) promote glycolysis in astrocytes. **2.** Astrocytes are involved in synaptic remodeling and memory: astrocytes eliminate unnecessary excitatory synapses from neurons by phagocytosis through MEGF10, and astrocyte-specific deletion of ephrin-B1 results in increased formation of immature synapses, while overexpression of ephrin-B1 triggers synapse loss. The above processes have important implications for synaptic plasticity and memory formation. **3, 4.** Astrocytes modulate neuronal network communication and contribute to memory performance: (3) astrocytic Gi activation in the CA1 during memory acquisition impairs remote but not recent memory retrieval and decreases activity in the ACC during retrieval; (4) blockade of exocytosis in astrocytes disrupts hippocampal–prefrontal synchronization and impairs recent memory. ACC, anterior cingulate cortex; HPC, hippocampus; LTP, long-term potentiation.

they are expressed [39, 40], likely related to how different sources of adenosine fulfill different roles as modulators and homeostatic regulators of synaptic and brain function [41]. Further studies should explore how astrocytic $A_{2A}$ receptors bidirectionally regulate working memory and long-term memory. Taken together, these studies indicate that GPCRs are important for long-term memory and that the mechanisms underlying the influences of astrocytic GPCR-coupled signaling on memory need to be clarified.

Astrocytes are key elements of BDNF signaling in the brain: proBDNF, produced and released by neurons, accumulates in astrocytes where it is converted into its mature form (BDNF) and is secreted by vesicle-associated membrane protein-mediated exocytosis [42]. Astrocyte-specific deletion of carrier receptor $p75^{NTR}$, which is responsible for proBDNF uptake, leads to the disruption of memory processes such as recent object recognition as well as impaired L-LTP [43]. In addition, BDNF is synthesized in and released from astrocytes in an IP3-dependent manner [44–46]. Conditional knockout of astrocytic BDNF in adults disrupts remote but not recent fear memory; this deficit is rescued by astrocytic overexpression of BDNF in the hippocampus before fear training [37]. BDNF is involved in LTP [47–49]. Notably, a study suggested that early-phase LTP is mediated mainly by neuron-derived BDNF, whereas astrocyte-derived BDNF is critical for L-LTP [37]. When L-LTP is divided into L-LTP1 (~120 min) and L-LTP2 (~180 min), astrocytic IP3-dependent $Ca^{2+}$ signaling has been found to be essential for both L-LTP1 and L-LTP2 [37], whereas astrocyte-produced BDNF is essential only for L-LTP2 [37]; L-LTP1 may be mediated by astrocytic recycling of BDNF secreted by neurons as proBDNF [43]. However, the spatial and temporal characteristics of astrocytic involvement have yet to be determined.

### Regulation of Synaptic Remodeling

Adult synapses constantly undergo synaptic turnover [50, 51] during experience-dependent plasticity and cognitive function [52, 53]. Astrocytes control the development, maintenance, and elimination of synapses during postnatal development [54, 55].In addition, recent studies have shown that astrocytes play a critical role in synaptic remodeling in adults. Researchers found that astrocytes eliminate redundant excitatory synapses in the adult hippocampus through the phagocytic receptor MEGF10, as astrocytic deletion of Megf10 results in significant increases in the number of excitatory synapses and abnormal synaptic plasticity [56]. Further study showed that mice with astrocyte-specific conditional Mefg10 knockout in the hippocampus display deficits in recent recognition [56]. In addition, astrocyte-specific deletion of ephrin-B1, a regulator of synaptogenesis, increases the density of glutamatergic synapses and immature dendritic spines but enhances recent fear memory, whereas overexpression of ephrin-B1 leads to a loss of dendritic spines associated with impaired contextual memory [57]. One explanation for this phenomenon is that the deletion of astrocytic ephrin-B1 triggers the excessive formation of immature (and potentially α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor-silent) synapses; when these silent synapses become mature, they may be recruited to hippocampal circuits following contextual learning. These studies propose possible mechanisms by which astrocytes control synaptic turnover and re-pattern connectivity, which may contribute to the memory trace in the adult hippocampus, yet several follow-up questions remain. For example, how does learning induce the astrocytic recruitment of unnecessary excitatory synapses; alternatively, how is the elimination of excitatory synapses necessary for learning prevented? Elucidation of the internal and external factors controlling these processes is needed.

### Coordination of Distributed Brain Circuits for System Consolidation

Ongoing interactions between the hippocampus and frontal cortical regions occur during the transition from recent to remote memory [4, 58]. Recent studies have demonstrated that astrocytes are involved in the coordination of activity across brain areas [12, 28]. Specifically, astrocytic Gi activation in CA1 during memory acquisition impairs remote but not recent memory retrieval and decreases activity in the ACC during retrieval [12]. The study further demonstrated massive recruitment of CA1 cells projecting to the ACC during learning; specific inhibition of these projections at this time point through astrocyte manipulation prevents the engagement of the ACC during memory acquisition, resulting in impaired remote memory [12]. Similarly, the astrocytic expression of dnSNARE reduces exocytosis and impairs hippocampal–prefrontal synchronization as well as recent spatial and reference memory [28]. The leading hypothesis in the memory field is that the hippocampus plays a time-limited role in memory: it is required for acquisition and recent recall but becomes redundant for remote recall, being replaced by the frontal cortices [58]. However, this temporal separation between the hippocampus and frontal cortex is not rigid. The above manipulation of astrocytic Gi-coupled signaling [12, 37] and findings from other studies [59, 60] suggest that the hippocampus is still critically involved in the consolidation and retrieval of remote memory. Notably, astrocytes play a key role in these processes [12, 37].

### Metabolic Support

A model proposed by Pellerin and Magistretti, known as the astrocyte-neuron lactate shuttle (ANLS) model, suggests that astrocytic glycolysis and neuronal oxidation play coordinated roles in long-term memory formation *via* the transport of lactate [61]. Advances in genetic and pharmacological approaches allow the selective inhibition of specific signaling pathways in astrocytes. One study showed that L-lactate is required for long-term memory [62]. Reducing the production of L-lactate in hippocampal astrocytes suppresses LTP and impairs long-term memory in an inhibitory avoidance task, and these effects are reversed by the application of exogenous L-lactate [62]. Similar impairments have been reported from the knockdown of monocarboxylate transporters (MCTs), which transfer lactate from astrocytes (MCT1 and MCT4) to neurons (MCT2) [62]. A further study showed that the Krebs cycle substrates pyruvate and ketone body B3HB can functionally replace lactate in rescuing the memory impairment caused by inhibition of glycogenolysis or knockdown of astrocytic MCT1 and MCT4 [63]. However, neither metabolite is able to rescue the memory impairment induced by the knockdown of neuronal MCT2, indicating that a critical role of astrocytic lactate is to provide energy for the neuronal mRNA translation required for long-term memory [63]. Another study demonstrated that pharmacological inhibition of glycogenolysis in the hippocampus impaired recent recognition; recognition was restored by exogenous L-lactate [64]. In addition, noradrenaline increased L-lactate production during memory processing by acting on β2ARs [65, 66]. Knockdown of the receptors specifically in astrocytes impaired long-term memory consolidation [66], and this impairment was rescued by the local application of L-lactate in the hippocampus [65]. These data are important since they show that the enhancement of memory due to arousal (i.e., noradrenaline release) is partially mediated through astrocytes. Stress elicits the release of glucocorticoids that regulate energy metabolism

and play a role in emotional memory [67]. Consistent with these findings, mice with astrocytic deletion of glucocorticoid receptors present impaired long-term fear memory and altered brain glucose metabolism [68]. Furthermore, astrocyte signaling *via* L-lactate appears to be involved in maladaptive memory formation as well as recovery from drug addiction. For example, cocaine-induced CPP is disrupted by pharmacological inhibition of L-lactate release from astrocytes [69, 70]. These findings suggest that astrocytic glycogenolysis and/or glycolysis in conjunction with astrocytic–neuronal lactate shuttling provide a mechanistic explanation for the critical role of astrocytes in memory formation and storage [71, 72]. In addition to the role of lactate as an energy substrate, several studies have focused on other types of lactate signaling mechanisms [72, 73]. In the context of memory research, many questions have yet to be answered. For example, does lactate simply provide metabolic fuel or does it also serve as a cellular signal? We cannot exclude the possibility that lactate plays multiple roles in this process. Moreover, which target mechanisms and cellular functions does lactate support?

### Other Aspects

Astrocytes also play a role in the beneficial effects of sleep on memory function. No adenosine accumulation in hippocampal slices has been reported in sleep-deprived dnSNARE mice. Furthermore, these mice display much milder impairments than control mice and fewer alterations of both slow-wave cortical oscillations and recent spatial memory [74, 75]. The adverse effects of human sleep disruption on declarative memory are well documented [76]. A recent study uncovered the astrocytic mechanism underlying this phenomenon. Deletion of astrocytic Bmal1 alters circadian rhythms and recent recognition through GABA signaling [26]. In addition, the expression of chemokines and cytokines is crucial for cognitive processes [77]; thus, it is not surprising that disruption of astrocytic IL-6 and its receptors induce deficits in recent spatial memory [78].

Almost everything we know about astrocytes and their contribution to memory is derived from studies of astrocyte physiology in rodents. Compared to rodents, in humans, astrocytes are larger, more structurally complex, and cellularly diverse, and exhibit faster propagation of $Ca^{2+}$ signals [79]. Interestingly, in a recent study, human glial progenitor cells were grafted into neonatal immunodeficient mice; these mice then developed large human glial progenitors and astrocytes [80]. The mice with human glial grafts, but not those with murine glial grafts, showed enhanced learning in the Barnes maze, object-location test, and contextual and tone fear conditioning as well as enhanced LTP [80], suggesting that astrocytes may have played an important role in cognitive development throughout evolution.

## Possible Involvement of Astrocyte-Oligodendrocyte and Astrocyte-Microglia Interaction in Memory Processing

We have discussed several potential ways through which astrocytes interact with neurons to modulate memory. Notably, other glial cells, namely oligodendrocytes and microglia, also play important roles in regulating memory. Oligodendrocyte lineage cells, including oligodendrocyte progenitor cells (OPCs) and mature oligodendrocytes, have numerous important functions, which include forming myelin sheaths that enwrap central nervous system axons, supporting axons metabolically, and mediating certain forms of neuroplasticity [81]. Studies have shown that normal myelin architecture is required for fear learning [82] and motor learning [83]. In addition, oligodendrogenesis is required for motor learning [84, 85], recent and remote memory retrieval but not initial spatial learning [86, 87] as well as remote memory recall but not recent recall or initial fear learning [88]. For reviews, see reference [89]. Astrocytes share their lineage with oligodendrocytes and interact with these myelin-forming cells by sharing gap junctions which allow passage of small metabolites and molecules for communication [90], and is important in oligodendrocyte maturation and myelination [91, 92]. In addition, astrocytes guide OPC migration by releasing chemokines and modulating Sonic hedgehog signaling [93, 94] and promote OPC maturation through releasing platelet-derived growth factor and bone morphogenic protein [95, 96], which are critical processes for oligodendrogenesis. Furthermore, both astrocytes and oligodendrocytes secrete and respond to many immune factors [97] i.e., tumor necrosis factor, interleukin, and interferon, which are critical for cognitive processes [77]. Overall, cross-talk between astrocytes and oligodendrocytes is important for steps regulating learning and memory; future studies need to directly explore the causal role of astrocyte-oligodendrocyte interactions in memory processing.

Microglia are the main immune cells that permanently reside in the brain alongside astrocytes and other types of cells. They are crucial for neuronal survival and neurogenesis, the formation and elimination of dendritic spines, and the clearance of apoptotic cells [98]. Accordingly, microglia are important effectors of plasticity, contributing to synaptic integrity and plasticity, as well as neuronal circuit maintenance and remodeling [99, 100], which are believed to be the cellular and neuronal mechanisms of learning and memory. Indeed, several studies have shown that microglia regulate neural ensemble connectivity and the strength of remote memory [101], synaptic elimination, neurogenesis, and forgetting in adults [102], as well as short-term recognition memory [103]. In addition, there is a balance between the pro-inflammatory and anti-inflammatory cytokines released by microglia that, if disrupted, can affect synaptic plasticity

[104] and spatial memory [105]. For review, see reference [106]. Emerging evidence demonstrates a bidirectional communication between microglia and astrocytes *via* molecules secreted by them, including cytokines, chemokines, ATP, and growth factors [107–109]. Microglia play a crucial role in reactive astrocyte induction and the regulation of astrocytic immune reactions; in parallel, astrocytes modulate microglial phenotypes and functions including neuroinflammation through numerous astrocyte-derived factors [110]. Notably, microglia-astrocyte crosstalk plays a regulatory role in synaptic transmission. For example, inflammation-induced ATP release from microglia, which in turn, recruits astrocytes through the ATP receptor P2Y1. The astrocytes subsequently release glutamate, which acts on neuronal glutamate receptors to modulate synaptic activity [111]. It remains unknown whether microglia-astrocyte interactions are involved in physiological functions such as learning and memory.

## Considerations and Perspectives

We have presented evidence that astrocytes participate in normal memory functions. From this evidence, we conclude- the following. (1) Astrocytic metabolic support is highly important for both short-term memory and long-term memory; this support includes sequential processes with high energy demands, such as post-translational modifications, gene transcription, and protein synthesis. (2) Astrocyte $[Ca^{2+}]_i$ is regulated by Gq-coupled signaling (i.e., CB1Rs, $GABA_BRs$, and IP3R2s) or Gs-coupled signaling (i.e., $A_{2A}Rs$); these signaling pathways are critical for both short-term memory and long-term memory. Specifically, Gi-coupled signaling (i.e., μ-opioid receptors) is important for long-term memory. (3) Astrocytic modulation of neuronal functions at the network level is critical for long-term memory. We, therefore, propose the following model for the formation of long-term memory: astrocytes detect environmental information (the what, when, and where of memory), actively regulate synaptic plasticity through the bidirectional exchange of regulatory signals with neurons, and undergo adaptive changes in coordination with the pre- and post-synaptic regions, further enabling memory consolidation by coordinating distributed brain circuits (system plasticity). Moreover, astrocytes provide metabolic support *via* astrocyte-neuron lactate coupling through all these processes (Fig. 4). Intuitively, the multiscale spatiotemporal properties of astrocytes are well suited to long-term memory, which requires broad spatial integration and long-term temporal regulation [5, 112]. Notably, new techniques with high temporal resolution (within 2 ms) and with a linear correlation with $[Ca^{2+}]$ have been developed [113]; these techniques provide better estimates of $Ca^{2+}$ transients at the timescale

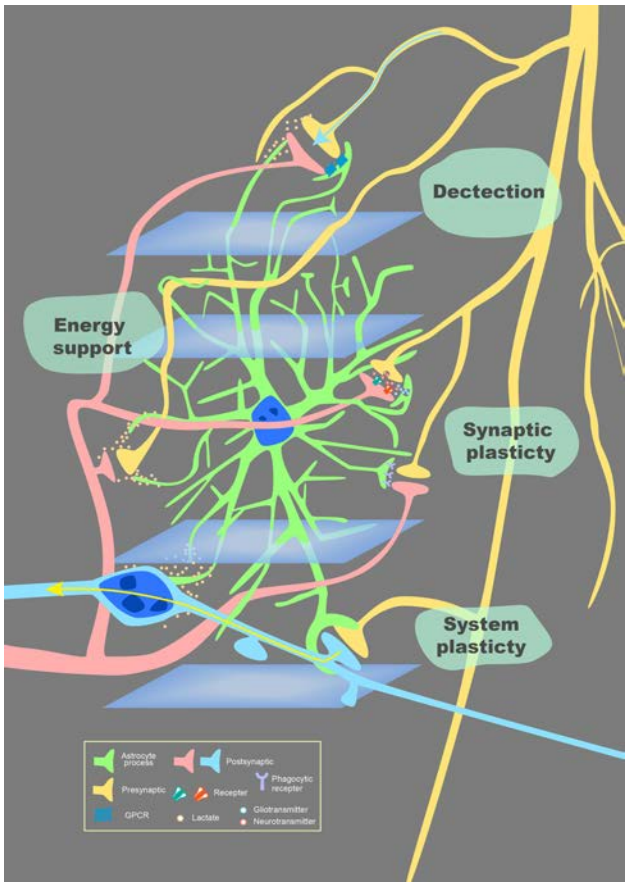


**Fig. 4** Integration of astrocytes with synapses and neuronal networks for memory. We propose a model in which astrocytes detect environmental information, modulate synaptic plasticity, coordinate system plasticity, and provide metabolic support for neuronal changes, thus contributing to memory formation.

of synaptic events, achieving temporal resolution similar to processes involved in working memory. Future studies need to investigate and manipulate astrocytes to explore their effects on working memory and the underlying mechanisms.

The current understanding of astrocytes, their dynamic changes, and their role in learning and memory are rudimentary compared with that of neurons; moreover, little effort has been made to include astrocytes in realistic computational models. A potential avenue to accelerate progress in this field is the inclusion of approaches that directly assess the chemical signals of astrocytes to ascertain astrocyte function, rather than relying on indirect measurements in neurons. Future investigations using astrocytic $Ca^{2+}$ imaging combined with genetically encoded fluorescent indicators for gliotransmitters as well as neuronal recordings combined with astrocyte manipulation in memory-relevant brain regions of awake rodents during different phases of learning and memory will shed new light on these unanswered questions. In addition, studies should examine the real-time

crosstalk between astrocytes and neurons and develop computational models that consider activity-dependent changes in the timing of intercellular communication and incorporate the broad spatial and temporal dynamics of interactions between networks of astrocytes and neurons. While this program for future research outlines both conceptual and technical advances, there are several caveats that need to be considered, as described below.

(1) *What constitutes astrocyte excitation or inhibition?* Currently, changes in astrocytic $Ca^{2+}$ levels are generally considered a proxy for astrocyte excitation. However, this definition is controversial as different compartments of astrocytes (e.g., soma *versus* proximal processes) display differential $Ca^{2+}$ dynamics (in terms of speed and duration) that are mediated by different mechanisms [15]. The variety in $Ca^{2+}$ events suggests that different information is processed in astrocytes. It is even harder to define astrocytic inhibition, as both increases and decreases in astrocyte $Ca^{2+}$ have been reported to induce cFos expression in astrocytes [114]. Therefore, it is important to use as many approaches as possible to demonstrate astrocytic activity; hopefully, future research will enable better quantification of astrocytic activity.

(2) *Genetic specificity in a heterogeneous population* To elucidate the role of astrocytes in memory regulation *in vivo*, many transgenic mouse lines have been constructed to achieve astrocyte-specific expression of inducible Cre recombinase. However, many astrocyte-CreER$^{T2}$ lines, such as hGfap-Cre ER$^{T2}$, Glast-Cre ER$^{T2}$, and Cx30-Cre ER$^{T2}$, do not meet the requirement of astrocyte-specific expression of Cre recombinase, in part because these astrocyte markers are also expressed in non-astrocytes, especially neurons, adult stem cells, and the cells of some peripheral organs [115]. Thus, it is important to note that the astrocyte-specific transgenic lines for functional studies should be carefully selected. Furthermore, astrocytes are morphologically and functionally heterogeneous among different brain regions [33, 115, 116]. Importantly, this heterogeneity may influence how astrocytes interact with each other and with neurons (e.g., [10, 11]). This heterogeneity further emphasizes the need to identify and manipulate subpopulations of astrocytes. Modern molecular studies, such as single-cell sequencing, will hopefully isolate unique markers for astrocytic subpopulations [117, 118] and thus enable the genetic identification and functional modulation of subpopulations to determine their functions in the brain.

(3) *Astrocyte-based computation* In the past decades, astrocytes have been found to play "active" roles in many aspects of brain functions: a recent study found that astrocytes are a specific computational element in a circuit that mediates an adaptive behavioral response [119]. We posit that variables used in brain encoding may be partially embedded in the biophysical substrates of astrocytes, such that the incorporation of astrocytes as computational building blocks in neural circuits may help systems neuroscience to attain a comprehensive understanding of how to regulate learning, memory, and other brain functions. However, there are substantial knowledge gaps. First, there is no evidence that astrocytes gate, transform, store, or reroute information in the brain by carrying out processes that can be described in abstract mathematical terms. Second, if astrocytes carry out computations, are $Ca^{2+}$ transients the biophysical substrate of astrocyte-based computations? Although astrocytes can encode extracellular cues into variations in $Ca^{2+}$ transients [120], the statistical methods currently used to encode and decode neuronal action potentials have not been applied to astrocyte data obtained *in vivo*. A prototypical study in systems neuroscience includes three components: (a) recording electrical activity in multiple neurons, (b) computational analysis to decode information embedded in action potentials, and (c) simultaneous assessment of cognitive or behavioral function. To the best of our knowledge, there are no studies recording the $Ca^{2+}$ activity of multiple astrocytes, followed by analysis with the General Linear Model or decoders in the context of a behavioral paradigm defined by distinct features that can be correlated with patterns of astrocytic $Ca^{2+}$ activity. This approach may provide the best avenue to advance our knowledge in this area.

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REVIEW

# Astrocytes in Chronic Pain: Cellular and Molecular Mechanisms

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**Abstract** Chronic pain is challenging to treat due to the limited therapeutic options and adverse side-effects of therapies. Astrocytes are the most abundant glial cells in the central nervous system and play important roles in different pathological conditions, including chronic pain. Astrocytes regulate nociceptive synaptic transmission and network function *via* neuron–glia and glia–glia interactions to exaggerate pain signals under chronic pain conditions. It is also becoming clear that astrocytes play active roles in brain regions important for the emotional and memory-related aspects of chronic pain. Therefore, this review presents our current understanding of the roles of astrocytes in chronic pain, how they regulate nociceptive responses, and their cellular and molecular mechanisms of action.

**Keywords** Astrocyte · Microglia · Neuron-glia interaction · Spinal cord · Chronic pain

## Introduction

Pain, as defined by the International Association for the Study of Pain, is an unpleasant sensory and emotional experience associated with, or resembling that associated with actual or potential tissue damage. Under physiological conditions, pain plays a protective role to warn the organism to evade noxious stimuli (such as heat, chemical irritants, and cold) and avoid them in future. However, under injury or disease conditions, pain can persist for months to years, and this type of pain is called pathological or chronic pain. Chronic pain is characterized by spontaneous pain, allodynia (pain evoked by a normally innocuous stimulus), and hyperalgesia (enhanced pain evoked by a noxious stimulus). Changes in neuronal plasticity are the major mechanisms of chronic pain [1]. Thus, several neuron-targeting drugs such as NMDA receptor antagonists, opioids (such as morphine, oxycodone, and codeine), and $Na^+$ channel blockers (such as lidocaine, oxcarbazepine, and carbamazepine) are used for the treatment of chronic pain. Although these drugs have therapeutic effects, they also have different degrees of side-effects [2]. Therefore, the development of new types of analgesic with few adverse reactions and better targeting are urgently needed. In the last two decades, non-neuronal cells, especially glial cells, have attracted increasing attention. Targeting the function of glial cells is likely to be a new direction for chronic pain treatment.

In the central nervous system (CNS), more than half of the cells are glia (including astrocytes, microglia, and oligodendrocytes), ~20%–40% of which are astrocytes [3, 4]. Astrocytes not only provide structural and nutritional support for neurons, they also play important roles in many neural processes [5]. Under normal conditions, astrocytes are mostly in a resting state; however, when tissue injury or disease occurs, they transform into a reactive state and contribute to the development of neurological disorders. One of the important features of astrocytes, different from other glial cells, is that they directly communicate with each other by forming gap-junction protein complexes, which allow adjoining cells to freely exchange ions and small cytosolic components [6]. In addition, when CNS neurons are activated, astrocytes regulate blood flow through their extensive contact with cerebral blood vessels [7]. Astrocytes also widely connect with neuronal synapses: a single cortical

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astrocyte can contact 4–6 neuronal somata, almost 140,000 synapses, and 300–600 neuronal dendrites [3, 4]. Close contact with neurons and synapses makes it possible for astrocytes to support neurons and regulate the physiological/pathological environment during synaptic transmission. These features show that astrocytes play important roles in signal transmission and processing.

In this review, we provide an overview of the roles of astrocytes in the pathogenesis of chronic pain and the interactions between astrocytes and microglia/neurons. We discuss recent neurobiological mechanisms and possible downstream molecular pathways of the astrocytic control of chronic pain. Finally, we discuss how they can be targeted as an alternative strategy for the treatment of chronic pain.

## Spinal Astrocytes in Chronic Pain

Classically, chronic pain is classified into two main categories: nociceptive and neuropathic. Nociceptive pain is associated with an ongoing input from real or threatened tissue injury, such as arthritis, trauma, and visceral inflammation. Neuropathic pain is caused by a direct consequence of a lesion or disease affecting the somatosensory system, such as nerve or nerve root compression, toxins, and ischemia. In 2016, the term nociplastic pain was proposed to describe pain that arises from the abnormal processing of pain signals without any clear evidence of tissue damage or discrete pathology involving the somatosensory system, such as fibromyalgia, irritable bowel syndrome, and temporomandibular disorder [8]. In the past two decades, the role of astrocytes in nociceptive pain and neuropathic pain has been widely studied. Under various disease conditions, astrocytes are activated and change to a reactive state characterized by morphological, molecular, and functional changes. Reactive astrocytes are identified by glial fibrillary acidic protein (GFAP) upregulation and hypertrophy after nerve injuries such as spinal nerve ligation (SNL) [9–12], chronic constriction injury (CCI) [13–15], and spinal cord injury (SCI) [16–18]. In addition, this process has also been reported in other pain models like tissue inflammation [19, 20], chemotherapy-induced pain [21, 22], arthritic pain [23, 24], and chronic post-cast pain [25, 26].

Reactive astrocytes can display various states. Some reactive states happen within minutes, such as a change in the phosphorylation of signaling molecules or an increase in intracellular $Ca^{2+}$. Others occur after hours or days (e.g., astrocyte hypertrophy or translational regulation). When a peripheral nerve is injured, astrocyte hypertrophy occurs 3 days later and lasts for several months [27]. Different from astrocytes, microglia immediately respond to a stimulus and proliferate [28], and this process grows to maximal levels in the first week following nerve injury. Raghavendra *et al.* reported that in a neuropathic pain model, when minocycline (a microglia activation inhibitor) administration is started at the time of nerve transection (pre-emptive treatment), it reduces allodynia and hyperalgesia, which is associated with its ability to suppress microgliosis. However, administration on day 5 after surgery (treatment of existing hypersensitivity) fails to attenuate the behavioral hyperalgesia and allodynia, although it inhibits microglial activation [29]. Meanwhile, astrocyte inhibitors work in both the early and late phases of neuropathic pain [30], indicating that microglia and astrocytes play different roles in the induction and persistence of chronic pain. Evidence shows that microglial reaction may lead to astrocyte reaction [31–34]; however, astrocyte reaction can also cause microglial reaction [33, 35]. A recent study showed that direct activation of astrocytes using an optogenetic approach induces microglial reactivity and pain hypersensitivity [36]. In addition, inhibition of astrocyte reaction by deletion of astrocyte-expressing CXCR5 reduces the microglial reaction under neuropathic pain conditions [37]. These reports indicate that there is a bilateral interaction between microglia and astrocytes in pain states. Thus, we discuss some of the signaling molecules and related signaling pathways in astrocyte-mediated chronic pain, as well as how astrocytes contribute to chronic pain *via* communicating with microglia and neurons.

### Signaling Molecules Related to Astrocyte-mediated Chronic Pain

As noted above, reactive astrocytes not only change in shape, size, and number, but also change in the expression of various molecules. An increasing list of signaling molecules in astrocytes have been implicated in persistent pain, such as cytokines, chemokines, ion channels, enzymes, and structural proteins. Meanwhile, astrocyte reactivity can be triggered by various molecules. Here, we introduce some classical and important signaling molecules in astrocyte-mediated chronic pain (Table 1).

#### *Inflammatory Cytokines and Chemokines*

Cytokines and chemokines are secreted proteins that regulate the immune responses and control immune cell trafficking. It is well known that cytokines and chemokines in peripheral tissues, dorsal root ganglia (DRG), spinal cord, and even the brain play a certain role in the pathogenesis of chronic pain.

Tumor necrosis factor-α (TNF-α) belongs to a superfamily of ligand/receptor proteins called TNF superfamily proteins. It is a cytokine that is usually produced by activated microglia and astrocytes and has pleiotropic effects on normal and malignant cells [38]. When a peripheral nerve is injured, TNF-α is released from activated microglia and

**Table 1** Astrocyte-selective molecules in chronic pain models

| Molecule | Model | Species | Effect of manipulation in the spinal cord | Refs. |
|---|---|---|---|---|
| *Cytokines, chemokines* | | | | |
| TNF-α | 2′,3′-dideoxycytidine-induced neuropathic pain; SNL; Formalin; CFA | Mouse Rat | Inhibitor or recombinant soluble receptor suppresses mechanical allodynia or thermal hyperalgesia; Inhibitor prevents TNF-α-induced phosphorylation of NR1 unit. | 44, 45, 50 |
| IL-1β | CFA; SNL | Mouse | Antagonist attenuates inflammatory hyperalgesia; Deletion of related receptor suppresses neuropathic pain. | 49, 51, 53, 54 |
| CCL1 | pSNL; SNL | Mouse Rat | i.t injection of CCL1 induces phosphorylation of NR1 and NR2B units; Knockdown of related receptor prevents mechanical allodynia and thermal hyperalgesia; Inhibitor of receptor or neutralizing antibody suppresses the development of neuropathic pain. | 57, 58 |
| CCL2 | SNL; CFA | Mouse Rat | Inhibitor modulates inhibitory synaptic current and suppresses neuropathic pain; CCL2-overexpressing mice show greater edema and hyperalgesia. | 62, 63 |
| CXCL10 | SNL; ischemia-reperfusion-induced pain | Mouse Rat | Knockdown of related receptor suppresses the development of neuropathic pain; Inhibitor or antagonist of receptor attenuates nociceptive response. | 64, 65 |
| *Channel protein* | | | | |
| AQP4 | SCI | Rat | After spinal cord injury; Knockout mice show less pain sensitivity and dorsal horn sensitivity to noxious stimulation. | 72, 74 |
| SUR1 | PNI | Mouse | Antagonist or knockout suppresses the development of neuropathic pain. | 76 |
| P2X3 | CCI-ION | Rat | Inhibitor attenuates reactive astrogliosis, release of downstream inflammatory factors, and pain hypersensitivity. | 82 |
| *Enzymes* | | | | |
| MMP-2 | SNL | Mouse | Inhibitor suppresses the late phase of neuropathic pain; Knockdown reduces mechanical allodynia and pJNK1/2. | 83, 84 |
| MMP-9 | SNL | Mouse | Inhibitor suppresses the early phase of neuropathic pain. | 83, 84 |
| TAK-1 | CCI | Rat | Antisense oligodeoxynucleotide prevents and reverses allodynia but not hyperalgesia and inhibits JNK1 activation. | 86, 87 |
| TPA | SNL; SNI | Rat | Inhibitor suppresses mechanical allodynia. | 90 |
| *Other molecules* | | | | |
| NDRG2 | Diabetic neuropathic pain | Rat | Blocking its upstream glucocorticoid receptor reverses tactile allodynia. | 92 |
| FGFR3 | SNI | Mouse | Inhibitor reduces expression of GFAP, TNF-α, and pain hypersensitivity. | 95 |
| TRAF6 | CFA; Visceral inflammatory pain | Mouse | Knockdown of TRAF6 reduces excitatory postsynaptic currents and inflammatory pain. | 96, 98 |
| *Long non-coding RNA* | | | | |
| PVT1 | SCI | Rat | Knockdown down-regulates CXCL13/CXCR5 and alleviates neuropathic pain | 99 |
| MEG3 | CCI | Rat | Inhibitor weakens MEG3-induced pro-inflammatory effects and relieves pain | 101 |
| *Structure-related protein* | | | | |
| Cx43 | CCI; pION | Mouse Rat | Inhibitor suppresses spontaneous excitatory postsynaptic currents and late-phase neuropathic pain; Inhibitor reduces mechanical hypersensitivity and central sensitization. | 30, 165 |
| Panx1 | CFA | Mouse | Deletion of astrocytic Panx1 prevents hypersensitivity completely; Deletion of neuronal Panx1 reduces baseline sensitivity and duration of hypersensitivity | 167 |

CCI, chronic constriction injury; CCI-ION, constriction injury of infraorbital nerve; CFA, complete Freund's adjuvant; pION, partial transection of the infraorbital nerve; pSNL, partial sciatic nerve ligation; PNI, peripheral nerve injury; SCI, spinal cord injury; SNI, spared nerve injury; SNL, spinal nerve ligation.

stimulates astrocytes [39–42]. Accordingly, inhibition of TNF-α signaling by neutralizing antibodies or inhibitors alleviates chronic pain [43–45]. Our previous study indicated that spinal injection of TNF-α-activated astrocytes induce persistent pain by releasing CCL2 (C-C Motif Chemokine Ligand 2) [46]. Similar to TNF-α, IL-1β is also expressed in microglia and astrocytes. IL-1β is upregulated in astrocytes in different types of chronic pain such as inflammatory pain [47], neuropathic pain [48], and bone cancer pain [49]. IL-1β activates the IL-1 receptor, which is expressed on nociceptive neurons and activates the mitogen-activated protein kinase (MAPK) pathway and sensitizes the neurons. IL-1β and TNF-α regulate the phosphorylation of the NR2B and NR1 subunit of the NMDA (N-methyl D-aspartate) receptor [50] and enhance NMDA-induced currents [51, 52], which suggest a potentiation of glutamatergic synaptic transmission. TNF-α and IL-1β also increase the frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) [53]. IL-1 receptor-knockout mice show decreased nociceptive responses after SNL [54]. In addition, optogenetic activation of astrocytes causes an increase of IL-1β and TNF-α secretion [36]. In human studies, TNF-α and IL-1β are also increased in the spinal astrocytes of patients with HIV-associated chronic pain [55]. These results suggest the important role of TNF-α and IL-1β in regulating neuropathic pain.

In addition to cytokines, several chemokines such as CCL1, CCL2, CCL3, CCL4, CCL7, CXCL10, CXCL12, CXCL13, and CX3CL1 and their receptors contribute to the pathogenesis of neuropathic pain [56]. Here, we focus on the chemokines or chemokine receptors associated with astrocytes. CCL1 was initially identified in T cells and stimulates the migration of human monocytes through binding to its receptor CCR8. In the SNL model, CCL1 is mainly produced in the DRG and transported to the spinal cord [57]. Meanwhile, CCR8 is increased in astrocytes of the ipsilateral superficial dorsal horn. Inhibition of CCL1 by intrathecal injection of a neutralizing antibody reduces nerve ligation-induced tactile allodynia [57]. Also, oral administration of RAP-103, a peptide inhibitor of CCR8, fully prevents mechanical allodynia and inhibits the development of thermal hyperalgesia after SNL, suggesting the involvement of CCR8 in the initiation and maintenance of nerve injury-induced neuropathic pain [58, 59].

CCL2 is highly expressed by spinal astrocytes and is upregulated in the SNL model [46]. Meanwhile, CCL2 is produced in cultured astrocytes after stimulation with lipopolysaccharide (LPS), TNF-α, or IL-1β [60]. CCR2 is the major receptor of CCL2 and is expressed in primary afferents and neurons in the spinal cord [61]. Mice overexpressing CCL2 in astrocytes display enhanced nociceptive responses in the CFA (complete Freund's adjuvant) model [62]. Our previous study demonstrated that CCL2 induces rapid phosphorylation of ERK (extracellular signal-activated kinase) in spinal cord neurons. In addition, when lamina II neurons in the spinal cord slice are recorded, the application of CCL2 immediately enhances NMDA- and AMPA-induced inward currents and causes an increase in the frequency and amplitude of sEPSCs [46]. CCL2 also modulates inhibitory synaptic transmission since it inhibits GABA-induced currents in spinal neurons [63].

CXCL10 belongs to the CXC chemokine family and is also known as keratinocyte-derived chemokine-10. CXCL10 is the major ligand of CXCR3 and is increased in neurons and astrocytes of the spinal cord after SNL or spinal cord ischemia reperfusion [64, 65]. Inhibition of CXCL10 by spinal injection of shRNA lentivirus attenuates SNL-induced mechanical allodynia and heat hyperalgesia [64, 66]. CXCL9 and CXCL11 belong to the same subfamily as CXCL10 [67]. However, the roles of these chemokines in pain hypersensitivity are different from CXCL10. Intrathecal injection of CXCL9 or CXCL11 does not induce hyperalgesia or allodynia behaviors, and their inhibition does not inhibit neuropathic pain either [68], suggesting different roles of these chemokines in pain regulation. Other chemokines and their receptors such as CX3CL1/CX3CR1, CXCL1/CXCR2, and CXCL12/CXCR4 are also involved in chronic pain and have been introduced in our previous and others' reviews [56, 69, 70], so they are not discussed in detail here.

### *Channel Proteins*

Different types of cationic or anionic channels are located on astrocytic membranes to regulate ions for the resting membrane potential or conductance and intracellular signaling. The ion channels on astrocytes are also involved in regulating the release of various gliotransmitters associated with several physiological processes. Here, we introduce some typical water and ion channels that are expressed on astrocytes involved in chronic pain regulation.

Aquaporin-4 (AQP4) is a major water channel expressed in the central nervous system, primarily in astrocytes. The role of AQP4 has been widely studied in a range of pathological conditions [71]. In the spinal cord, AQP4 exhibits a graded decline in distribution from the dorsal to the ventral horn, with abundant expression in laminae I and II [72]. The function of AQP4 is to regulate water influx or efflux driven by osmotic pressure to maintain water homeostasis. AQP4 is increased in spinal cord astrocytes after SCI and nerve injury in humans [73]. In addition, AQP4-knock-out mice show reduced pain sensitivity and dorsal horn sensitivity to noxious stimulation [74].

Sulfonylurea receptor 1 (SUR1), encoded by the *Abcc8* gene, is a regulatory subunit that co-assembles with the inward rectifier $K^+$-selective channel to form the $K_{ATP}$ channel [75]. SUR1 also co-assembles with the non-selective

cation channel, transient receptor potential melastatin 4 (TRPM4) to form the SUR1-TRPM4 complex. SUR1-TRPM4 is upregulated in dorsal horn astrocytes, and global or astrocytes-targeted deletion of SUR1-TRPM4 relieves mechanical allodynia and thermal hyperalgesia in a sciatic nerve cuffing mouse model [76]. Meanwhile, chronic administration of glibenclamide (an SUR1 antagonist) to mice with neuropathic pain causes a reduction of pain behaviors and the expression of IL-6, CCL2, and CXCL1 in astrocytes. Thus, glibenclamide may be an astrocyte-targeted candidate drug for the treatment of some kinds of neuropathic pain.

P2X3 is a non-selective ligand-gated ion channel that belongs to the purinergic receptor family [77]. P2X3 is activated by adenosine triphosphate (ATP) and is selectively permeable to $Na^+$, $K^+$, and $Ca^{2+}$, especially $Ca^{2+}$, which plays an important role in the generation and transmission of nociceptive information [78]. Nerve injury causes the release of a large amount of ATP, which activates the P2X3 receptor in the presynaptic membrane and causes $Ca^{2+}$ influx, resulting in phosphorylation of PKA and PKC and the release of glutamate. This process further activates the NMDA receptors on neurons and causes EPSC generation and central sensitization [79]. It is well known that activation of P2X3 in the DRG causes abnormal nerve discharge, strengthens the transmission of sensory information, and induces visceral hyperalgesia [80, 81]. P2X3 is also expressed on astrocytes in the spinal cord and is increased in a rat model of neuropathic pain [82]. Inhibition of P2X3 in the spinal cord reduces hypersensitivity after nerve injury. In addition, administration of MPEP (2-methyl-6-(phenylethynyl) pyridine; an mGluR5 antagonist) reduces the mechanical allodynia and abolishes the increase in the density of P2X3 in astrocytes induced by nerve injury [82].

*Enzymes*

Metalloproteases (MMPs) have been suggested to act in the cleavage of extracellular matrix proteins, cytokines, and chemokines to control the inflammation and tissue remodeling associated with various neurodegenerative diseases [83]. MMP-2 and MMP-9 are members of the MMP family involved in IL-1β cleavage [83, 84]. Spinal astrocytes continuously secrete MMP2 after SNL. Downregulation of MMP-2 through intrathecal injection of MMP-2 siRNA reduces mechanical allodynia and the level of spinal GFAP and phosphorylated c-Jun N-terminal kinase 1/2 (JNK1/2), an astrocyte-expressing kinase, in a neuropathic pain model. Local inhibition of MMP-9 inhibits the early phase of neuropathic pain, whereas inhibition of MMP-2 suppresses the late phase of neuropathic pain [83].

Transforming growth factor-β-activated kinase 1 (TAK-1), also known as MAPK kinase kinase 7, is an enzyme regulating innate immunity and pro-inflammatory signaling [85]. TAK-1 mediates the activation of the nuclear factor-kB (NF-kB), JNK, and p38 pathways. Soto-Diaz *et al.* found that both the production of chemokines and neutrophil migration caused by astrocyte reaction are dependent on TAK1 signaling [86]. Another study reported by Katura *et al.* showed that peripheral nerve injury induces an increase in TAK1 expression in astrocytes in the spinal dorsal horn, and this TAK1 upregulation increases JNK1 phosphorylation in spinal astrocytes and contributes to the development and maintenance of mechanical hypersensitivity [87].

Tissue type plasminogen activator (tPA) is a well-known extracellular serine protease that converts zymogen plasminogen into an active serine protease. tPA is found on the endothelial cells of blood vessels and is involved in the degradation of blood clots [88]. In addition, tPA participates in modification of the extracellular matrix that leads to long-term potentiation in the hippocampus [89]. Kozai *et al.* reported that tPA is upregulated in spinal astrocytes following root injury [90]. Moreover, continuous intrathecal administration of a tPA inhibitor suppresses root ligation-induced mechanical allodynia. These data suggest that astrocyte-derived tPA in the dorsal horn is essential for the mechanical hypersensitivity following root injury.

*Other Molecules*

N-myc downstream-regulated gene 2 (NDRG2) is a member of the NDRG family and is widely distributed in the CNS but only expressed in astrocytes. NDRG2 members have different functions in cell differentiation, proliferation, and maintenance of cell morphology [91]. Ma *et al.* found that down-regulation of NDRG2 in spinal astrocytes inhibits their reactivity and reduces nociceptive behaviors in a rat model of spared nerve injury (SNI) [92]. Another study reported by Li *et al.* also indicated that inhibition of NDRG2 contributes to astrocyte-specific neuroprotection [93].

FGFR3 is a member of the fibroblast growth factor receptor (FGFR) family, which contains four members (FGFR1–4) that mediate FGF signal transduction. FGF/FGFR signaling plays an important role in cell differentiation, neuronal survival, and cell development [94]. Previous studies have shown that activated FGFR3 promotes the proliferation and development of astrocytes [95]. Xie *et al.* showed that FGFR3 upregulates GFAP and TNF-α expression in astrocytes *in vivo* and *in vitro*. Inhibition of FGFR3 leads to reduced GFAP and TNF-α and increases the withdrawal threshold in SNI model [95]. These results suggest that FGFR3 induces production of the inflammatory mediator TNF-α and astrocyte reactivity to cause hyperpathia in neuropathic pain.

TRAF6 belongs to the TNF receptor-associated factor (TRAF) protein family which has 6 members (TRAF1–TRAF6). A growing body of literature has

established the important role of TRAF6 in the development and maintenance of chronic pain. Our previous study showed that TNF-α, IL-1β, and TLR4 mediate TRAF6 upregulation in spinal astrocytes after SNL in mice [96]. However, TRAF6 is increased in spinal microglia in CFA-induced chronic inflammatory pain [97]. Direct inhibition of TRAF6 by siRNA or indirect inhibition by docosahexaenoic acid has therapeutic effects on neuropathic pain and inflammatory pain [97]. Another study showed that TRAF6 is increased in spinal astrocytes in the chronic visceral pain model [98]. Knockdown of TRAF6 remarkably reduces the amplitude of EPSCs of spinal dorsal horn neurons and relieves visceral hypersensitivity [98].

Long non-coding RNAs (lncRNAs) have hundreds of nucleotides with no protein-coding potential. Currently, there is growing evidence that lncRNAs play an important role in regulating chronic pain. For example, lncRNA PVT1 is up-regulated in spinal astrocytes after SCI [99]. Depletion of PVT1 in the spinal cord reduces nociceptive responses such as thermal hyperalgesia and mechanical allodia as well as the expression of neuroinflammatory factors and proteins. Furthermore, it has been confirmed that PVT1 is a competitive endogenous RNA of miR-186-5p, while miR-186-5p targets CXCL13 [99]. Inhibition of lncRNA PVT1 alleviates neuropathic pain in SCI rats by upregulating miR-186-5p and down-regulating CXCL13/CXCR5 [99, 100]. Another similar study showed that lncRNA MEG3 aggravates neuropathic pain and astrocyte reactivity by mediating the miR-130a-5p/CXCL12/CXCR4 axis [101].

## Interaction of Astrocytes, Microglia, and Neurons Under Chronic Pain Conditions

It is well-accepted that neuronal plasticity is a key mechanism for the development and maintenance of chronic pain. Astrocytes and microglia are important players in the regulation of neuronal functions [102]. The communication between astrocytes, microglia, and neurons in the spinal cord and brain facilitates central sensitization, which is manifested as an increased responsiveness of neurons to normal or subthreshold afferent inputs.

Microglia are the resident immune cells of the CNS. They are activated by tissue damage or nerve injury [28] and their morphology changes to an amoeboid shape, accompanied by enhanced secretion of numerous inflammatory factors and microglial phagocytosis [3]. Evidence has already demonstrated that microglia play an important role in the pathogenesis of chronic pain [28]. With different stimuli (usually extracellular/intracellular signals like inflammatory mediators or anti-inflammatory mediators), microglia display two phenotypes, M1 and M2 [33]. The M1-like phenotype is induced by TNF-α, IL-1β, or other inflammatory mediators, and then microglia secrete pro-inflammatory cytokines such as IL-6, IL-23, or chemokines (CCL2 and CCL5) to induce neuroinflammation, resulting in the maintenance of chronic pain [28]. The M2-like phenotype is induced by IL-4 or IL-13. Microglia with M2-like phenotypes have increased phagocytosis and produce growth factors such as insulin-like growth factor-1 and anti-inflammatory cytokines such as IL-10 [103]. The cross-talk between astrocytes and microglia is maintained in part *via* secreted mediators, such as growth factors, neurotransmitters, cytokines, chemokines, innate-immunity mediators, tissue damage molecules (e.g., ATP), mitogenic factors, NO, ROS, and metabolic mediators such as amino-acids, that can be used for cell metabolism and may also mediate tissue changes [104]. The production and release of these mediators are normally controlled by key intracellular signaling pathways, such as the MAPK pathway. Many chemokines, such as CXCL12, CXCL10, CXCL1, and CCL2 are mainly stored in astrocytes, and microglia express the corresponding chemokine receptors like CXCR4 or CCR2 [105]. This suggests a strong association between microglia and astrocytes [6]. IL-18, one member of the IL-1 family, is an important regulator of innate and acquired immune responses. Kan *et al.* reported that IL-18 and IL-18 receptors are expressed in microglia and astrocytes, respectively, mediate microglia-astrocyte interaction in the spinal cord, and enhance neuropathic pain processing after nerve injury [106]. These results suggest that activated microglia in the spinal dorsal horn are directly responsible for the induction of astrocyte reactivity after nerve injury. However, reactive astrocytes sometimes are not correlated with reactive microglia. Hald *et al.* found that bone cancer markedly induces spinal astrocyte reactivity, not microglial reactivity [107]. In addition, Robinson *et al.* reported that astrocyte but not microglial reactivity is induced in oxaliplatin- and bortezomib-induced peripheral neuropathy in rats [108].

In addition to the interaction with microglia, astrocytes also directly interact with neurons. We found that CXCL13-CXCR5 mediates neuron-astrocyte interaction in the spinal cord after SNL [37]. SNL increases the expression of CXCL13 and its receptor CXCR5 in neurons and astrocytes in mice. In *Cxcr5*-KO mice, the reactivity of astrocytes and microglia in the dorsal horn is remarkably reduced after nerve injury [37]. Furthermore, when astrocytes are activated, they release CCL2 and CXCL1 to act on their receptors CCR2 and CXCR2 on spinal neurons, thus causing an enhancement of excitatory synaptic transmission and chronic pain [46, 56, 109, 110]. Recent work has shown that CXCL2 secreted by astrocytes interacts with CXCR2 expressed on neurons in the spinal cord and this contributes to carrageenan-induced prostatitis pain [111]. Meanwhile, the CXCL1/CXCR2-mediated interaction between astrocytes and neurons in the periaqueductal gray (PAG)

also facilitates chronic pain [112]. A recent study showed that Wnt5a from neurons is crucial for reactive astrogliosis in an animal model of HIV-associated pain [113]. Wnt5a from neurons targets its receptor ROR2, which is expressed in astrocytes. Furthermore, conditional knockout of either Wnt5a in neurons or its receptor ROR2 abolishes not only gp120-induced astrocyte reactivity but also hyperalgesia. These results show that astrocytes, microglia, and neurons form a loop to interact with each other and regulate chronic pain.

## The Intracellular Signaling Pathway in Reactive Astrocytes in Chronic Pain

Several intricate roles are played by astrocytes in the pathogenesis of chronic pain, not only in the means of intercellular communication but also in the alteration of intracellular downstream signaling pathways as well as the variation in metabolic patterns. Nerve injury or pathogen invasion can be the initial factor for astrocyte activation in chronic pain. Here, we introduce astrocytic intracellular signaling and the actions of astrocyte-released neuromodulators in chronic pain. Some typical pathways such as the p38 and JNK pathways have been described in previous reviews [4, 6], thus we do not discuss them in detail.

Janus kinase (JAK) signal transducers and activators of the transcription 3 (STAT3) signaling pathway is involved in the restricted proliferation of dorsal horn astrocytes after peripheral nerve injury [114]. Inhibition of JAK-STAT3 suppresses both the proliferation of dorsal horn astrocytes and the maintenance of tactile allodynia. It has been shown that the dimerization of gp130 participates in the conduction pathway of activated JAK1 and JAK2, followed by phosphorylating STAT3 [115]. Gp130 cooperates with other receptors such as IL-6, IL-11, and IL-27 to constitute receptor complexes [116–118]. Considering the pleiotropic effects and widespread expression of these cytokines, the gp130-JAK-STAT3 signaling pathway may provide new directions in the research on reactive astrogliosis concerned with chronic pain.

NF-κB plays important roles in many aspects of cell regulation such as proliferation, apoptosis, and differentiation [119]. NF-κB can regulate neuropathic pain by mediating neuroinflammation, neuron apoptosis, and synaptic plasticity [120]. NF-κB is also strongly activated in reactive astrocytes and contributes to the development of neuropathic pain. During the progression of SCI, the inactivation of NF-κB in transgenic GFAP-IκBα-dn mice causes a reduction of lesion volume, an increase of white matter preservation, and a reduction in the expression of proteoglycan and chemokines CXCL10 and CCL2, and improves functional recovery [121]. During the inflammatory pain process, NF-κB plays an important role in gene transcription induced by cytokines. IL-1β and TNF-α activate NF-κB and lead to the transcription of several genes including pro-inflammatory factors and chemokines. This is a positive feedback process that further activates NF-κB and leads to more expression of downstream genes associated with neuroinflammation. In addition, Toll-like receptors (TLRs) are key regulators of the NF-κB signaling pathway. TLR4 regulates neuropathic pain through its activation of microglia and astrocytes in the spinal cord. When TLR4 is activated by LPS or other stimuli, NF-κB acting as its main downstream pathway is activated to begin the next process to develop chronic pain [122]. Using different methods including blockers or siRNA to inhibit the NF-κB signaling pathway can relieve different types of chronic pain. Thus, regulation of the NF-κB signaling pathway could be a potential therapeutic strategy [121, 123, 124].

The transcription factor Olig2 is considered to play an essential role in the differentiation of oligodendrocytes and motor neurons in the embryonic spinal cord. However, recent findings suggest that Olig2-lineage astrocytes are a different subgroup from GFAP-lineage astrocytes, both of which frequently occupy mutually exclusive territories and have distinct mRNA expression patterns [125–127]. In addition, Olig2-lineage astrocytes tend to express GABA transporter-3 and are involved in inhibitory neuronal transmission [126]. Ablation of Olig2 decreases the proliferation of reactive astrocytes in response to injury [128]. Olig2 is a direct target of Notch signaling [129], which upregulates Olig2 expression and promotes Olig2 localization in the nucleus in reactive astrogliogenesis in the brain. Inhibitor of the Notch-activating enzyme reduces the number of reactive astrocytes [130]. Given the fact that Olig2 influences Wnt signaling in gliomas and neural stem cells and participates in the interaction between Wnt signaling and Notch signaling, further research on the Notch-Olig2-Wnt pathway is important in the area of astrogliogenesis and chronic pain [131, 132].

TGF-β is rapidly and chronically elevated in response to CNS injury. Intrathecal injection of TGF-β inhibits neuropathy-induced hyperalgesia as well as spinal microglial and astrocytic reactivity [133, 134]. TGF-β attenuates the upregulation of pp38 and pERK in spinal microglia and astrocytes of mice with CCI. Despite the neuroprotective role of TGF-β, it also stimulates astrocytes to a reactive state with up-regulated GFAP [135]. Schachtrup *et al.* emphasized that fibrinogen is a carrier of latent TGF-β and induces phosphorylation of Smad2 in astrocytes after leakage through the disrupted blood–brain barrier or vascular damage [136]. Nuclear Smads interact with a large number of transcription factors to activate target genes [137]. TGF-β regulates adenine nucleotide translocator 1 gene expression, which is responsible for the removal of extracellular glutamate through a cooperative interaction of both Smad and

Sp1 binding elements located immediately upstream of the transcriptional start site [138, 139].

## Supraspinal Astrocytes in Chronic Pain

Chronic pain results from peripheral and central sensitization [1]. Central sensitization occurs not only in the spinal cord but also in supraspinal areas. Noxious stimuli are detected and transduced into electrical signals and further transmitted from the DRG to the dorsal horn. The signal is then sent up through the spinothalamic tract to the thalamus and then to the primary somatosensory cortex (S1) [140]. Several areas such as the PAG, parabrachial nucleus, nucleus accumbens, and anterior cingulate cortex (ACC) are important for pain regulation [141]. Under nerve injury or inflammatory conditions, astrocytes undergo varied changes in the brainstem, thalamus, ACC, and S1 [142, 143], and these changes contribute to central sensitization and chronic pain.

The spinal trigeminal nucleus is similar to the spinal dorsal horn and plays an essential role in trigeminal pain transmission. Reactive astrocytes in the spinal trigeminal nucleus contribute to the pathogenesis of chronic orofacial pain [144]. In reactive astrocytes, the TNF signaling pathway is activated and increases the expression of GFAP, CX43, and IL-1β, leading to neuropathic pain in the trigeminal system [48, 145]. This activation process of astrocytes is initiated by upregulation of several marker genes, including complement 3, complement factor B, MX dynamin-like GTPase 1, and S100a10 [32]. Neurons and glial cells regulate this astrogliosis process through various signaling pathways, including the JAK-STAT3, Notch-OLIG2, and TGFβ-SMAD pathways [114, 128, 146–148]. In addition, after infraorbital nerve injury, reactive astrocytes are detected in the rostral ventromedial medulla (RVM), a major component of the brainstem for descending pain modulatory circuitry [48]. The expression levels of the pro-inflammatory factors TNF-α and IL-1β are increased in RVM astrocytes. Intra-RVM administration of astrocytic inhibitors attenuates mechanical hyperalgesia and allodynia behaviors. Moreover, TNFR1 and IL-1R are expressed in RVM neurons that express the NMDA receptor subunit NR1. Injection of recombinant TNF-α or IL-1β upregulates NR1 phosphorylation and causes an NMDAR-dependent allodynia [48]. Supraspinal astrocytes also participate in descending nociceptive modulation in a cancer-induced bone pain model. Ni *et al.* showed that activation of astrocytes in the ventrolateral PAG is implicated in facilitating bone cancer pain in rats *via* the JNK signaling pathway [149]. Intrathecal administration of astrocytic cytotoxin or a JNK inhibitor reduces the expression of GFAP and mechanical allodynia. All the above suggest a contribution of supraspinal astrocytes and central glia-neuronal interactions to the descending facilitation of chronic pain.

Astrocytes in other regions of the brain have also been reported to be involved in the pain matrix such as the ACC and S1. Astrocytes in the ACC play a role in the affective component of pain, including unpleasantness or aversion [142]. SNL activates astrocytes in S1 and increases mGluR5 expression [143]. This leads to an increase in S1 astrocytic $Ca^{2+}$ transients and, thus, the release of thrombospondin 1 from astrocytes, which promotes synapse formation as well as mechanical allodynia [143]. In addition, Wahis *et al.* reported a new function of astrocytes in the central nucleus of the amygdala (CeA) under neuropathic pain conditions [150]. They found that activation of oxytocin receptors (OTRs) in the CeA reduces neuropathic pain-induced anxiety behavior. Meanwhile, the deletion of OTRs from the astrocytes in the lateral part of the CeA abolishes the anxiolytic effects of OTRs agonists [150]. These data highlight the central role of astrocyte-mediated oxytocin signaling in the regulation of emotional states under chronic pain conditions.

It is important to note that the research above was mostly based on animal studies. Human astrocytes have several distinct properties that are quite different from those of rodents. The human brain contains subtypes of GFAP-positive astrocytes that are not expressed in rodents [135]. Also, the size of human cortical astrocytes is almost double that in rodents [151]. Real-time imagining studies on the brains of patients with chronic lower back pain have demonstrated glial activation in multiple regions including the thalamus and S1 [152]. This research suggests possible astrocyte activation in higher brain regions in humans. Thus, determining whether there is concomitant neuronal activation with astrocytes activation in chronic pain may be of major importance in future research.

## Targeting Astrocytes as an Alternative Strategy for the Treatment of Chronic Pain

Long-term unendurable and severe chronic pain impairs patients' quality of life and imposes a heavy economic burden on society. Non-steroidal anti-inflammatory drugs, opioids, and adjuvant analgesics like antidepressants and antiepileptics are common pharmacological treatments for managing chronic pain. However, most patients face the problem of receiving inadequate analgesic therapy and the prescription is limited by the side-effects including gastrointestinal hemorrhage, thrombotic cardiovascular events, and addiction [153]. Hence, there is an urgent need for developing new therapeutic strategies for chronic pain.

With the improving understanding of the roles of astrocytes in chronic pain, therapeutic strategies may target their underlying mechanisms in order to reduce pain or enhance the recovery process. For example, matrix

metalloproteinases (MMPs), particularly MMP-2, are known to participate in neuropathic pain after nerve injury [83, 84]. MMP-2 is released by astrocytes after the injury and induces activation of IL-1β [83]. Therefore, it would be ideal to target MMP-2 and reduce inflammation after injury. Unfortunately, most available drugs have a non-specific affinity for MMPs and thus induce various side-effects or minimal desired effects. MMP-1 and MMP-9 inhibitors have recently been developed, and yet not much progress has been made in the development of MMP inhibitors [154]. TNF-α is a cytokine that is usually produced by activated microglia and astrocytes. Administration of TNF-α antibody effectively alleviates hyperalgesia [155], indicating the prospect of anti-TNFα therapy in the treatment of chronic pain. An ongoing phase III clinical trial is aimed to test the efficacy of infliximab (a TNFα antagonist) in treating lower-back pain in patients (NCT03704363). Multiple compounds and medicines targeting astrocytes alleviate chronic pain by regulating pro-inflammatory mediators such as TNFα, IL-1β, and CCL2 [156–160]. Of note, besides the neuronal expression of TNFR1 and IL-1R, they are also expressed in astrocytes and microglia and contribute to glial activation [45, 96, 161]. Pharmacological inhibition of TNF-α also attenuates glial activation then relieves chronic pain. However, since a certain amount of TNFR1 or IL-1R is expressed on neurons, blocking TNF-α also affects neuronal function.

In addition, pharmacological inhibition of P2X3 in rats following CCI of the trigeminal infraorbital nerve attenuates facial pain [82]. The purinergic receptor P2X3 is found in astrocytes in the spinal trigeminal nucleus, and blocking P2X3 inhibits reactive astrogliosis and the release of downstream inflammatory factors [82]. Thus, it might also be possible to target downstream molecules of reactive astrogliosis to reduce its effect on pain behaviors. For example, patients with neuromyelitis optica have been treated with tocilizumab, an IL-6 antibody, which was found to be safe and effective [162]. Although some of these compounds have antinociceptive effects in animal models and inhibit the reactivity of astrocytes, more designs for clinical trials to test their analgesic efficacy on humans are needed.

Another potential approach is to block gap junction proteins, such as connexin-43 (Cx43) and pannexin 1 (Panx1). Cx43 is specifically upregulated in spinal astrocytes after CCI [30]. Once upregulated, Cx43 enhances ATP release from astrocytes and finally leads to microglial activation and allodynia [163, 164]. Besides, Cx43 also contributes to the release of glutamate and chemokines, and blocking this protein remarkably attenuates neuropathic pain sensitization [30, 165]. The main mechanism is that, in chronic pain states, Cx43 modulates hemichannel function, leading to an increase in the permeability to various cytokines and chemokines [30, 166]. In addition, glial Panx1 contributes to the tactile hypersensitivity in chronic orofacial pain by inducing hyper-responsiveness to ATP [167]. Some Panx1 blockers (including mefloquine and probenecid) have been reported to improve morphine withdrawal syndrome [168]. However, further studies are needed to clarify their effects on humans.

## Conclusion and Future Perspectives

In summary, we have reviewed different kinds of evidence to demonstrate the necessity and sufficiency of astrocytes in chronic pain. We also explain how astrocytes promote chronic pain through astrocyte-microglia or astrocyte-neuron interactions (Fig. 1). When peripheral nerve injury or tissue damage occurs, astrocytes change to a reactive state in response to different neurotransmitters or neuromodulators in the spinal cord or brain. Reactive astrocytes are usually accompanied by the activation of a variety of intracellular signaling pathways. Therefore, the molecular mechanisms of astrocyte-microglia-neuron crosstalk in the spinal cord and brain under chronic pain conditions deserve further study.

Given the important role of astrocytes in the facilitation of chronic pain, targeting them may provide novel prevention and treatment strategies. However, because astrocytes play an essential supportive and protective role in the CNS, it is important to target specific signaling events in astrocytes without disrupting their overall well-being. The recent identification of astrocyte-expressing genes by transcriptome analyses suggests that astrocytes display inter- or intra-regional heterogeneity and act as a gate for descending noradrenergic control of mechanosensory behavior, which indicates the diverse functions and phenotypes of astrocytes for chronic pain regulation [169]. Akdemir *et al.* also found that subpopulations of Lfng-labeled astrocytes in laminae III and IV of the dorsal horn are involved in the regulation of neuronal activity and maintaining sensory-processing circuity associated with light touch [170]. Ablation of Lfng+ astrocytes reduces glutamatergic synapses and mechanosensory responses. In addition, compared to the classical neuronal gate control theory of pain, Xu *et al.* reported a new function of astrocytes in the gating of nociceptive signals in the spinal cord [171]. Spinal astrocytes are activated by electrical stimulation of peripheral Aβ fibers, which induces long-term depression in NK1R+ neurons and antinociception. Meanwhile, suppression of reactive astrocytes by different methods blocks such processes. Their results demonstrate astrocytes as a new and important component of pain gating by activation of Aβ fibers that exert non-neuronal control of pain. Thus, these recent discoveries may provide a new research direction for astrocyte regulation of chronic pain.

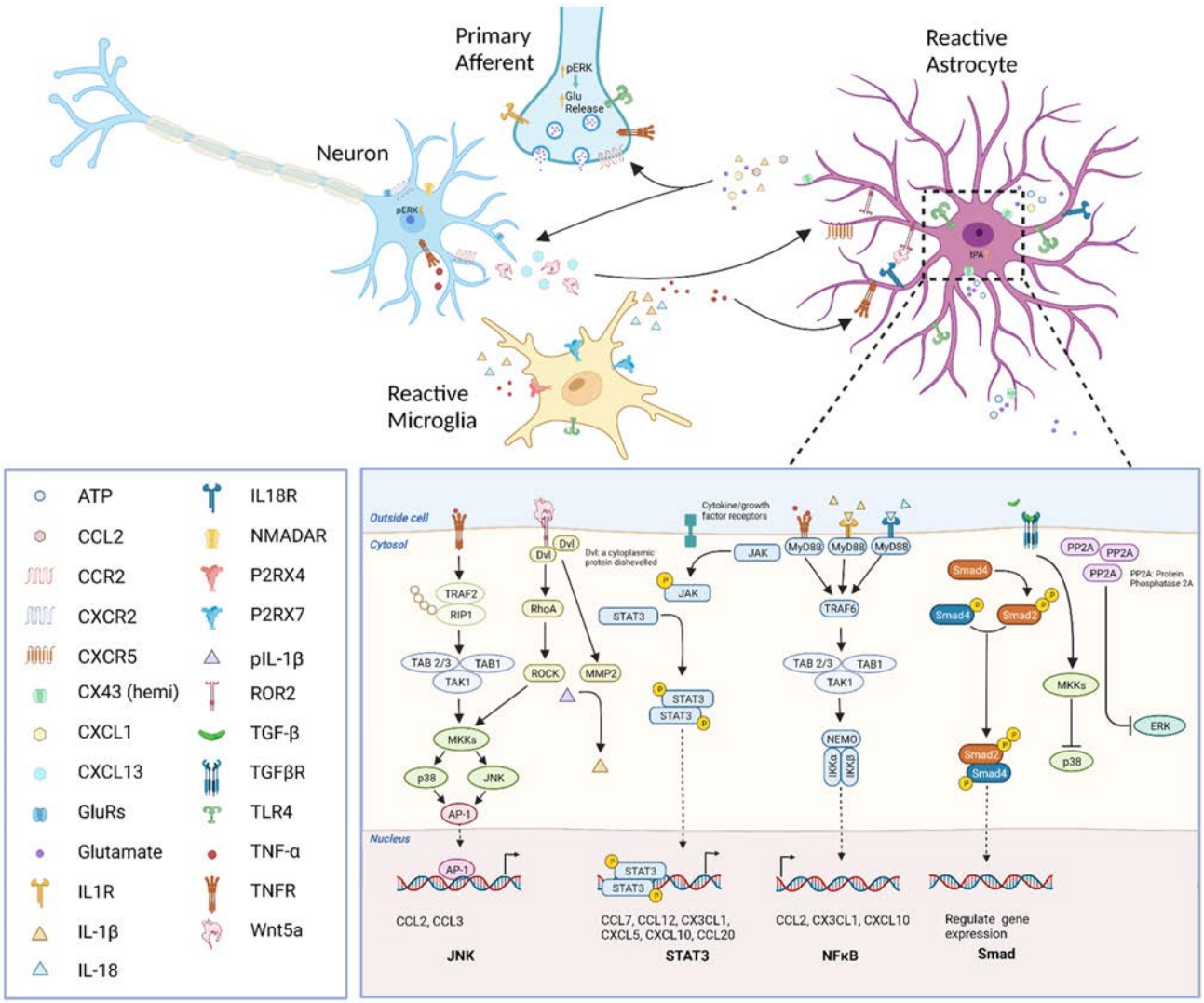


**Fig. 1** Astrocytic, microglial, and neuronal interaction in chronic pain. Nerve injury induces the release of CXCL13, which activates astrocytes *via* the CXCR5 receptor. The activation of astrocytes results in the upregulation of CX43 expression and a switch in CX43-mediated function from gap-junction communication to CX43 hemichannel-mediated paracrine signaling, resulting in the increased release of pro-inflammatory cytokines, chemokines, glutamate, and ATP, which activate microglia through P2RX4, P2RX7, and other receptors. The activation of these microglial receptors induces the release of pro-inflammatory cytokines (including TNF-α and IL-1β) and further amplifies neuronal excitability. These cytokines also result in the upregulation of the transcriptional regulators TRAF6, STAT3, and subsequent activation of the JNK and ERK pathways in astrocytes, further increasing their production and release of chemokines and facilitating neuropathic pain. The figure was created with BioRender.com.

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REVIEW

# Regulation of Glial Function by Noncoding RNA in Central Nervous System Disease

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**Abstract** Non-coding RNAs (ncRNAs) are a class of functional RNAs that play critical roles in different diseases. NcRNAs include microRNAs, long ncRNAs, and circular RNAs. They are highly expressed in the brain and are involved in the regulation of physiological and pathophysiological processes of central nervous system (CNS) diseases. Mounting evidence indicates that ncRNAs play key roles in CNS diseases. Further elucidating the mechanisms of ncRNA underlying the process of regulating glial function that may lead to the identification of novel therapeutic targets for CNS diseases.

**Keywords** Noncoding RNA · MicroRNA · Long noncoding RNA · Circular RNA · Astrocytes · Microglia · Oligodendrocytes

## Introduction

The mammalian genome contains sequences for both protein-coding RNAs (mRNAs) and non-coding RNAs (ncRNAs). Recently, the expression, function, and mechanisms of ncRNAs have drawn wide attention. NcRNAs, a class of epigenetic and translational regulators, consist of microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), each of which plays important physiological roles by controlling transcription and translation [1, 2]. Increasing numbers of studies of ncRNAs have revealed their unsuspected functions (Fig. 1) [3–11]. MiRNAs are small 21–22 nucleotide noncoding RNAs, which are known regulators of essential biological processes in animals and plants. They are involved in the regulation of many processes, including proliferation, differentiation, apoptosis, and development [3, 12]. LncRNAs are a heterogeneous group of noncoding transcripts >200 nucleotide in length and comprise the largest proportion of the non-coding transcriptome. In recent years, overwhelming evidence has demonstrated that lncRNAs are key regulators of many cellular processes, especially gene expression [9, 13]. Another type of ncRNA, circRNAs are biologically active nucleic acid molecules that are specifically expressed in large amounts in brain tissue. They exist in closed-loop RNA forms and are thought to regulate biogenesis, degradation, translation, transport, and interaction [10, 14]. In general, a better understanding of ncRNA functions will help in the development of novel therapeutic strategies to treat central nervous system (CNS) disease.

Glial cells are abundant in the CNS and are essential for brain development and homeostasis [15]. Emerging evidence has shown that classes of glial cells, including astrocytes, microglia, and oligodendrocytes (OLs), play important roles in the CNS [16]. Astrocytes are widely distributed in the brain. They undertake the tasks of signal transmission and transmitter metabolism. In addition, they have been shown to bind tightly to capillary endothelial cells to form the blood-brain barrier. Increasing evidence indicates that astrocytes

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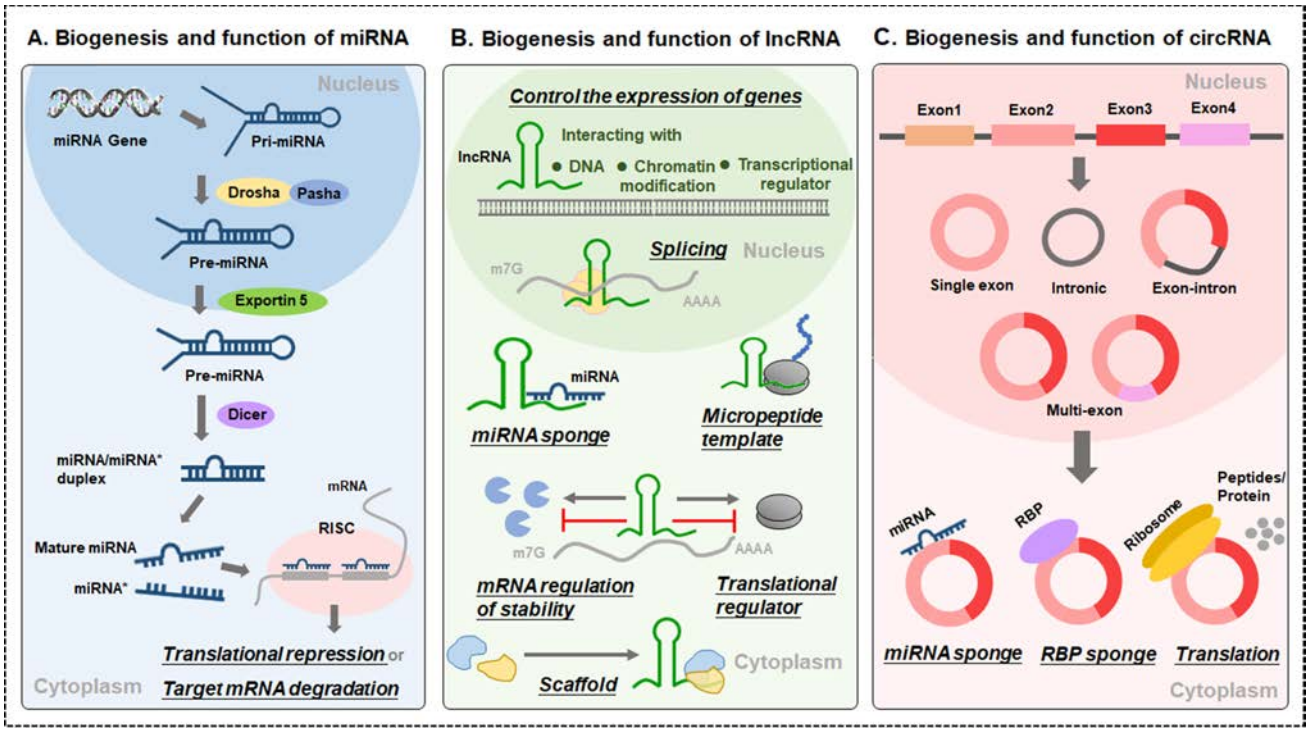


**Fig. 1** Biogenesis and functions of ncRNAs. **A** miRNAs. In the nucleus, pre-miRNA is formed after the activity of Drosha and Pasha enzymes on pre-miRNA. Subsequently, pre-miRNA is exported to the cytoplasm by RNA-GTP-associated exportin 5, where it is acted upon by Dicer to generate an miRNA:miRNA* duplex structure. Mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) and regulate translational repression or target mRNA degradation. **B** lncRNAs. LncRNAs control the expression of genes by interacting with DNA, transcriptional regulators, and/or chromatin-modifying complexes in the nucleus. Cytoplasmic lncRNAs act as scaffolds to bring two or more proteins into a complex. In addition, they act as sponges for other transcripts or proteins, serve as protein templates, or regulate mRNA degradation and translation. **C** circRNAs. CircRNAs are generated *via* back-splicing. Based on different combinations of exons and introns in the final circRNA sequence, circRNA can be divided into three categories: exonic circRNA, circular intronic, and exon-intron circRNA. Notably, exonic circRNA can be formed as a single- or multi-exon molecule. CircRNAs have been suggested to have multiple functions: miRNA sponge, RNA binding protein (RBP) sponge, and translation.

are involved in mediating the development of CNS diseases. As the brain's resident macrophages, microglia are classified as both glial and immune cells, interacting with neurons and exhibiting a wide array of functions under physiological and pathological conditions [17, 18]. OLs, as intrinsic components of the CNS, are involved in the formation of myelin sheaths, support axonal metabolism, and play an important role in the conduction of action potentials [19].

In this review, we briefly describe the roles of ncRNAs by regulating glia function in CNS diseases, including acute CNS injuries (e.g., stroke, spinal cord injury [SCI], and traumatic brain injury [TBI]), chronic neurodegenerative diseases (e.g., Alzheimer's disease [AD], Parkinson's disease [PD], multiple sclerosis [MS], and amyotrophic lateral sclerosis [ALS]), and other CNS diseases (e.g., epilepsy and depression). We also discuss the mechanisms and recent advances in the study of the roles of ncRNAs in glial function to help us better understand the role of ncRNAs in mechanisms of CNS pathology.

## NcRNA and Astrocytes

Astrocytes are the most abundant glial cell population in the CNS and are closely associated with CNS diseases [20]. Based on the functions of ncRNAs, researchers have investigated their roles in glial function and CNS diseases [21]. The available evidence shows that ncRNAs are associated with astrocyte proliferation, activation, apoptosis, and autophagy [22–26], indicating that ncRNAs function in various CNS diseases *via* different mechanisms (Fig. 2). In conclusion, ncRNAs have the potential to serve as clinical diagnostic markers and therapeutic targets.

### MiRNAs and Astrocytes

At present, the effects of miRNAs on astrocytes are mostly focused on regulating astrocyte activation [27, 28], apoptosis [29], and the expression of inflammatory factors [30]. In addition, by modulating inflammatory responses and proliferative effects, astrocytes also influence the release of myelin

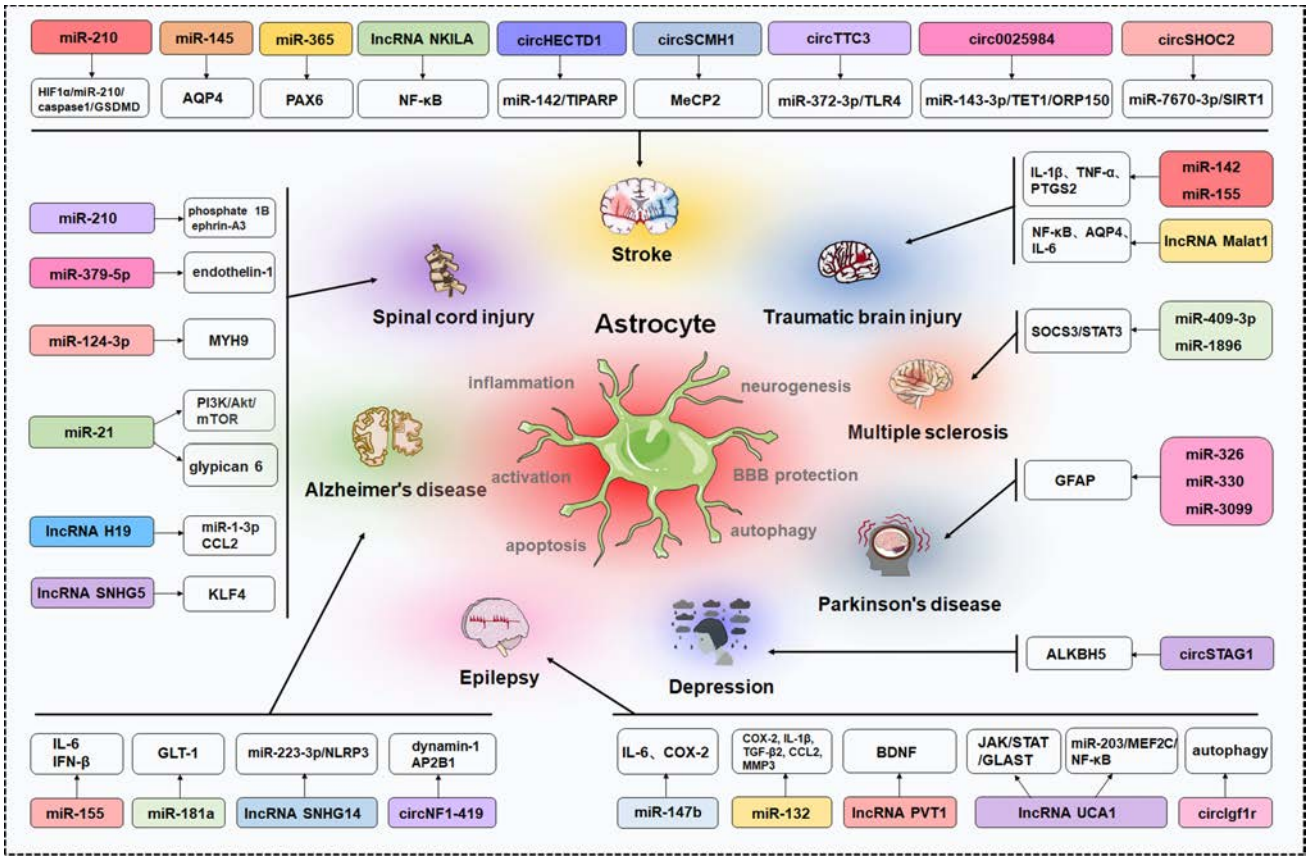


**Fig. 2** Regulation of astrocyte function by ncRNAs in CNS disease. The mechanism of action of ncRNAs on astrocyte function and CNS disease [CNS injuries (stroke, spinal cord injury, and traumatic brain injury), chronic neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, and multiple sclerosis), and other CNS diseases (epilepsy and depression)].

debris and scar tissue formation [31, 32]. Considering the various roles of miRNAs in astrocyte function, they might be important contributors to physiological and pathological processes in CNS diseases.

*Acute CNS Injuries*. The miRNA expression profiles are known to be altered after acute CNS injuries including stroke, SCI, and TBI. Mounting evidence demonstrates that miRNAs are involved in the regulation of astrocyte function in acute CNS injuries. Researchers have found that miR-210 and miR-142 promote the anti-inflammatory effect of astrocyte in ischemic stroke [22, 23, 33]. MiR-210 has also been proposed as a potential therapeutic target in SCI because it promotes neovascularization, astrogliosis, axon growth, and myelination in the injured spinal cord *via* the inhibition of protein-tyrosine phosphate 1B and ephrin-A3 [34]. Furthermore, miRNAs can regulate the activation of astrocytes in acute CNS injuries. For example, miR-379-5p attenuates the activation of astrocytes and significantly suppresses the expression of nerve growth inhibitors after SCI [35]. MiR-155 and miR-142 promote brain inflammation *via* astrocyte activation and may be involved in secondary brain injury after TBI [36]. MiR-124-3p, a biological messenger between neurons and microglia, suppresses the activation of A1 astrocytes [37]. In addition, Zheng *et al.* investigated how miR-145 ameliorates astrocyte injury by targeting aquaporin 4 (AQP4), highlighting a novel potential therapeutic target in ischemic stroke [38]. In another study of astrocyte injury, miR-21 was found to regulate astrocyte proliferation and significantly promote astrocyte apoptosis post-SCI through the transforming growth factor-β-mediated phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin pathway, thus promoting recovery from SCI [39]. In addition, miR-21 regulates the polarization of astrocytes. For example, miR-21 is a switch to regulate the polarization of reactive astrocytes and improves the formation of synapses by targeting glypican 6 *via* the signal transducer and activator of transcription-3 pathway after acute ischemic SCI [40]. More notably, miRNA regulates the reprogramming of reactive astrocytes and conversion into neurons in the adult brain by targeting their specific transcription factors. Mo *et al.* found that miR-365 modulates astrocyte conversion into neurons in the adult rat brain after stroke

by targeting Pax6, which may help to find new therapeutic targets for cerebral ischemic stroke [41].

*Chronic Neurodegenerative Diseases.* Chronic neurodegenerative diseases are caused by the chronic and progressive degeneration of neural tissue. There is increasing recognition that ncRNAs are implicated in both the onset and pathogenesis of chronic neurodegenerative diseases. MiR-155, an inflammatory miRNA, is upregulated in Aβ-activated astrocytes and may be a promising target for controlling neuroinflammation in AD [42]. Another study suggested that miR-181a controls rapid modifications of glial glutamate transporter 1 (GLT-1) levels in astrocytes and is involved in the pathological consequences in AD. These data suggest that the pathogenesis of AD is characterized by distinct neuroinflammatory events involving dysregulation of miRNA expression in astrocytes [43]. Besides AD, miRNAs are also involved in the pathogenesis of PD by regulating the function of astrocytes. PTEN-induced putative kinase 1 (PINK1) is a PD-related gene [44]. MiR-326, miR-330, and miR-3099 have all been reported to positively regulate glial fibrillary acidic protein (GFAP) expression in astrocytes. PINK1 deficiency reduces the expression levels of miR-326, miR-330, and miR-3099, which may regulate GFAP expression during brain development [45]. Recent studies have shown that experimental autoimmune encephalomyelitis (EAE), the animal model of MS, rapidly alters the expression profiles of ncRNAs [46]. In an EAE model, cytokine-injected EAE mice showed a dysregulated miRNA profile [47]. Liu *et al.* found that miR-409-3p and miR-1896 coordinately promote the production of inflammatory cytokines in reactive astrocytes through the SOCS3/STAT3 pathway, aggravating the pathogenesis in EAE mice [48].

*Other CNS Diseases.* To explore the relationship between miRNAs and epilepsy. Scheppingen *et al.* applied small RNA-sequencing analysis to investigate the expression of miRNAs in astrocytes treated with IL-1β. Of these, only miR-146a and miR-147b were differentially expressed. Combining their results, these researchers concluded that miR-147b deserves further investigation as a potential therapeutic target in neurological disorders associated with inflammation, such as epilepsy [49]. In another study, Korotkov *et al.* found that transfection of miR-132 in human primary astrocytes reduces the expression of pro-epileptogenic COX-2, IL-1β, TGF-β2, CCL2, and MMP3. This suggests that miR-132, particularly in astrocytes, is a potential therapeutic target that warrants further *in vivo* investigation [50].

## LncRNAs and Astrocytes

Similar to the effects of miRNAs on astrocyte function, lncRNAs also affect the proliferation, activation, and dysfunction of astrocytes [51–53]. Many of the functions of lncRNAs *via* different regulatory modes have been elucidated in different CNS diseases.

*Acute CNS Injuries.* Several studies have indicated that lncRNAs function in acute CNS injuries *via* astrocyte proliferation, activation, and viability. Gao *et al.* found that the lncRNA NKILA is a key booster of the NF-κB pathway; NKILA blocks the activation of the NF-κB pathway and inhibits astrocyte proliferation and neuron apoptosis as well as inflammation and oxidative stress [52]. Li *et al.* suggested that lncRNA H19 expression is increased by lipopolysaccharide (LPS) in normal human astrocytes and modulates astrocyte proliferation and activation by the lncRNA (lncRNA H19)–miRNA (miR-1-3p)–mRNA (CCL2) axis [27]. In another study, the expression of the lncRNA SNHG5 was significantly increased in the spinal cord tissue of SCI rats compared with controls, indicating that a cellular lncRNA SNHG5/KLF4/eNOS signaling axis in inducible of astrocytes and microglia viability in response to SCI [54]. In addition, lncRNAs can also regulate astrocyte swelling in TBI. Zhang *et al.* demonstrated the impact of the lncRNA Malat1 on TBI-induced astrocyte swelling, indicating that Malat1 is downregulated in both a rat model of TBI and the astrocyte fluid percussion injury model, which concurs with brain edema and astrocyte swelling [55].

*Chronic Neurodegenerative Diseases.* LncRNAs have also been reported to be involved in regulating inflammatory responses, and astrocytes play a key role in AD-related neuroinflammation. Duan *et al.* isolated astrocytes from APP/PS1 mice for high-throughput lncRNA sequencing. Among them, lncRNA SNHG14 was the most significantly down-regulated lncRNA after AVE0991 treatment. They demonstrated that the angiotensin-(1-7) analog AVE0991 modulates astrocyte-mediated neuroinflammation through the lncRNA SNHG14/miR-223-3p/NLRP3 pathway and provides neuroprotection in a transgenic mouse model of AD [56].

*Other CNS Diseases.* Several lncRNAs regulate astrocyte activation *via* different mechanisms in other CNS diseases. Zhao *et al.* demonstrated that the expression of the lncRNA PVT1 is increased in the hippocampus of epileptic rats. Inhibition of PVT1 inhibits astrocyte activation, increases hippocampal BDNF expression, and decreases pro-inflammatory cytokine expression [57]. Similarly, the lncRNA UCA1 inhibits hippocampal astrocyte activation and JAK/STAT/GLAST expression in TLE rats and improves the adverse reactions caused by epilepsy [51]. In another study, the lncRNA UCA1 also inhibited the inflammation of astrocytes by regulating the miR-203-mediated regulation of MEF2C/NF-κB signaling in epilepsy [58]. Based on these results, the studies of lncRNA have elucidated pathological mechanisms and provided potential therapeutic targets for CNS disease.

### CircRNAs and Astrocytes

Although it is known that circRNAs are derived from precursor mRNAs, their biogenesis remains elusive [21]. Many of the function of circRNAs have been elucidated over the past years. They interact with RNA-binding proteins [59], act as an miRNA sponge [60], and translate proteins [61] in physiological and pathological processes. Based on the functions of circRNAs, researchers have investigated the roles of circRNAs in astrocyte function.

*Acute CNS Injuries.* The available evidence shows that circRNAs are involved in acute CNS injuries by regulating astrocyte activation, inflammation, and apoptosis. Han *et al.* elucidated that circHECTD1 inhibits the expression of TIPARP and reduces astrocyte activation by regulating autophagy. They also pointed out that circHECTD1 can serve as a novel biomarker for and therapeutic target of ischemia-induced astrocyte activation [23]. Another study also confirmed the role of circRNA in astrocyte activation. Knocking down the expression of circCDC14A in peripheral blood cells relieves astrocyte activation in the peri-infarct cortex, thereby relieving brain damage in the acute phase of ischemic stroke [62]. In addition, Yang *et al.* found that circSCMH1 promotes neural remodeling and inhibits glial activation in ischemic stroke [63]. In addition to astrocyte activation, some circRNAs also regulate the process of astrocyte apoptosis. Yang *et al.* concluded that depletion of circTTC3 reduces apoptosis in astrocytes and cerebral ischemia/reperfusion injury by the miR-372-3p/TLR4 axis in cerebral infarction [64]. Moreover, circ0025984 induces promoter demethylation and decreases ORP expression levels by targeting the miR-143-3p/TET1 signaling pathway, effectively preventing the autophagy and apoptosis of astrocytes [65]. Recently, exosome-mediated transport has been shown to be involved in several physiological and pathological processes and emerging data indicate that exosomes released by astrocytes improve neuronal survival under hypoxic and ischemic conditions. Chen *et al.* demonstrated that circSHOC2 in ischemic-preconditioned astrocyte-derived exosomes suppresses neuronal apoptosis and ameliorates neuronal damage by regulating autophagy and acting on the miR-7670-3p/SIRT1 axis, which might contribute to a therapeutic strategy for ischemic stroke [66].

*Chronic Neurodegenerative Diseases.* To explore the relationship between circRNA and AD. Chen *et al.* performed RNA sequencing of circRNAs in D-galactose-induced aging astrocytes and found that circNF1-419 enhances autophagy to ameliorate senile dementia by binding Dynamin-1 and Adaptor protein 2 B1 in AD-like mice [67].

*Other CNS Diseases.* Mounting evidence reveals that astrocyte dysfunction is associated with the pathogenesis of major depressive disorder [68, 69]. However, the underlying etiological mechanism remains unclear. Recent evidence suggests that circRNAs regulate the process of astrocyte dysfunction in depression. Huang *et al.* found that overexpression of circSTAG1 notably attenuated the astrocyte dysfunction and depressive-like behaviors induced by chronic unpredictable stress (CUS) [69]. To explore the relationship between circRNAs and epilepsy, Shao *et al.* screened circIgf1r in the status epilepticus model through circRNA sequencing and found that it was upregulated. Further analysis demonstrated that circIgf1r promoted the polarization of astrocytes to phenotype A1 by inhibiting autophagy and may serve as a potential target for the prevention and treatment of neuron damage after epilepsy [70].

## NcRNAs and Microglia

Microglia are the resident immune cells in the CNS and play an essential role in brain homeostasis and neuroprotection in brain diseases [71]. Microglial cells have both pro-inflammatory and anti-inflammatory phenotypes, called M1 and M2. M1 microglia produce pro-inflammatory molecules, such as TNF-α, IL-6, and IL-12, which exacerbate neurotoxicity and myelin damage. M2 microglia secrete the anti-inflammatory factors IL-10, IGF-1, and TGF-β that play a neuroprotective role in tissue repair and homeostasis maintenance. Mounting evidence indicates that ncRNAs can ameliorate CNS disorders by microglia polarization [72, 73], activation [74, 75], neuroinflammation [76], pyroptosis [77], and apoptosis [78] *via* different mechanisms (Fig. 3).

### MiRNAs and Microglia

MiRNAs can control the degree of neuroinflammation and influence microglial activation by regulating the expression of different genes [37, 74]. Freilich *et al.* demonstrated that miR-689, miR-124, and miR-155 are the most closely related predictors of mediating the pro-inflammatory pathway and M1 activation phenotype. miR-711 and miR-145 serve as predictors of mediating anti-inflammatory signaling and M2 activation phenotypes [79]. According to the above studies, miRNAs may be the most important regulators mediating microglial polarization and activation, presenting new therapeutic opportunities for microglia-associated CNS dysfunction. Thus, the role of miRNAs in CNS disorders and their mechanisms are increasingly being investigated.

*Acute CNS Injuries.* Mounting numbers of studies have indicated that miRNAs function in acute CNS injuries by regulating the activation of microglia. For example, miR-424 protects against permanent focal cerebral ischemic injury in mice involving the suppression of microglial activation [75]. In addition, knockdown of miR-377 inhibits the activation of microglia and the release of pro-inflammatory cytokines, alleviating ischemic brain injury in the rat middle cerebral

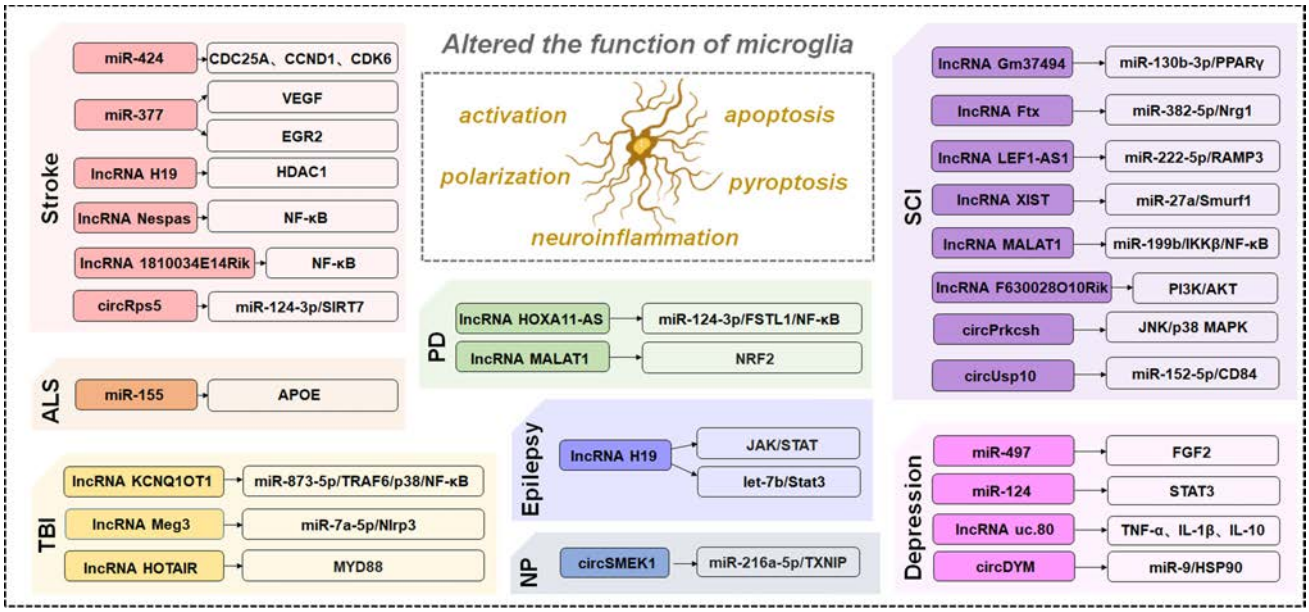


**Fig. 3** ncRNAs involved in CNS diseases by regulating microglial function. The mechanism of action of ncRNAs on microglial function and CNS diseases [stroke, spinal cord injury (SCI), amyotrophic lateral sclerosis (ALS), traumatic brain injury (TBI), Parkinson's disease (PD), epilepsy, neuropathic pain (NP), and depression].

artery occlusion model [80]. In addition, exosome-miRNAs derived from microglia also play an important role in brain injury. Zhang *et al.* demonstrated that exosomal miR-137 is upregulated in BV2-exosomes and participates in the partial neuroprotective effect of BV2-exosomes. Further analysis indicated that BV2-Exo alleviates ischemia-reperfusion brain injury through transporting exosomal miR-137, providing novel insight into microglial exosome-based therapies for the treatment of ischemic brain injury [71].

*Chronic Neurodegenerative Diseases.* Studies have shown that miR-155 is a key regulator of inflammation [81]. Targeting miR-155 in SOD1 mice restores dysfunctional microglia and ameliorates disease [72]. These findings identify miR-155 as a therapeutic target for the treatment of ALS.

*Other CNS diseases.* MiRNAs have also been associated with depression. For example, miR-497 aggravates the activation of hippocampal microglia in CUS-induced depression in rats by targeting FGF2 [82]. Furthermore, miR-124 ameliorates depressive-like behavior by targeting STAT3 to regulate microglial activation [83].

## LncRNAs and Microglia

LncRNAs are involved in various neurological disorders. In this part of review, we summarize the roles of lncRNAs in microglial function *via* different regulatory modes in different CNS diseases.

*Acute CNS Injuries.* LncRNAs are also important regulators mediating microglial polarization and activation. Some lncRNAs are involved in acute CNS injuries by shifting microglial M1/M2 polarization, such as the lncRNAs H19 [84], Gm37494 [85], and KCNQ1OT1 [86]. Moreover, many lncRNAs contribute to inflammation and the activation of microglia *via* different mechanisms including the lncRNAs Nespas [76], Ftx [87], LEF1-AS1 [78], XIST [88], Meg3 [89], 1810034E14Rik [90], HOTAIR, [91], and MALAT1 [92]. For example, Xiang *et al.* found that the lncRNA Ftx/miR-382-5p/Nrg1 axis improves the inflammatory response of microglia, which might improve SCI repair [87]. Meng *et al.* demonstrated that the lncRNA Meg3 regulates microglial inflammation by targeting the miR-7a-5p /Nlrp3 pathway [89]. Zhang *et al.* reported that lncRNA 1810034E14Rik overexpression suppresses the activation of microglial cells and inhibits the phosphorylation of p65 [90]. It is noteworthy that the lncRNA F630028O10Rik functions as a competing endogenous RNA (ceRNA) for the miR-1231-5p/Col1a1 axis and enhances microglial pyroptosis after SCI by activating the PI3K/AKT pathway [77].

*Chronic Neurodegenerative Diseases.* Altered levels of some lncRNAs in PD alleviate inflammation by regulating gene expression. For example, downregulation of the lncRNA HOXA11-AS ameliorates neuronal damage and microglial activation in a PD model [93]. Cai *et al.* also found that silencing the lncRNA MALAT1 in LPS/ATP-treated BV2 cells and PD mice model attenuates inflammasome activation [94].

*Other CNS Diseases.* Glial activation also contributes to inflammation in epilepsy. Studies have shown that the

expression of lncRNA H19 in the hippocampus of epileptic rats is significantly higher than in normal rats. In a rat model of temporal lobe epilepsy, lncRNA H19 has been shown to promote hippocampal microglial and astrocytic activation *via* JAK/STAT signaling [95]. In another rat model of temporal lobe epilepsy, Han *et al.* demonstrated that lncRNA H19 binding to let-7b promotes hippocampal glial cell activation and epileptic seizures by targeting Stat3 [96]. In addition, lncRNA Peg13, another lncRNA associated with epilepsy, binds with miR-490-3p to upregulate the activity of microglia and astrocytes in epileptic hippocampal tissue [97]. Another group found that lncRNA uc.80 is downregulated in the hippocampus of depressed rats. Overexpression of lncRNA uc.80 ameliorates depression in rats by promoting the M2 polarization of microglia [98].

### CircRNAs and Microglia

Although the role of circRNA in CNS diseases has been reported, more studies are needed to elucidate how circRNA regulates microglial function.

*Acute CNS Injuries.* Several circRNAs shift microglial M1/M2 polarization in acute CNS injuries. For example, circPrkcsh contributes to the polarization of microglia towards the M1 phenotype induced by SCI and acts *via* the JNK/p38 MAPK pathway [99]. In another study, circRps5-modified exosome was shown to improve cognitive function by decreasing neuronal damage and shifting microglia from an M1 to M2 phenotype in the hippocampus [100]. In addition to shifting polarization of microglia, another circRNA, circUsp10, was found to promote microglial activation and induce neuronal death by targeting miRNA-152-5p/CD84. This finding suggests that circUsp10 can be used as a diagnostic biomarker and potential target for SCI treatment [101].

*Other CNS Diseases.* Chronic stress dynamically affects microglial activity. CircDYM, acting as an endogenous sponge to inhibit miR-9 activity, inhibits microglial activation by regulating HSP90 ubiquitination in depression [102]. Similarly, circHivep2 regulates microglial activation in the progression of epilepsy by interfering with miR-181a-5p to promote SOCS2 expression, indicating that circHivep2 may serve as a therapeutic tool to prevent the development of epilepsy [103]. In addition, some studies have revealed the vital role of circRNAs in neuropathic pain (NP). For example, Xin *et al.* affirmed that circSMEK1 facilitates NP inflammation and microglial M1 polarization by modulating the miR-216a-5p/TXNIP axis, providing a new molecular target for the future treatment of NP [104].

## NcRNAs and Oligodendrocytes

OLs are the myelin-forming cells of the CNS and lifelong partners of neurons [105–108]. They are responsible for myelin sheath production, which is essential not only for the efficient conduction of electrical impulses along axons but also for axonal integrity [109]. NcRNAs are a key part of multifaceted transcriptional complexes participating in neurogenic commitment and the regulation of postmitotic cell function [110]. In recent years, an increasing number of studies have identified ncRNAs as promising therapeutic targets by regulating OL function in CNS diseases (Fig. 4).

### MiRNAs and Oligodendrocytes

MiRNAs regulate various biological processes in different CNS diseases. In recent years, increasing numbers of studies have reported that miRNAs are required for proper OL differentiation and myelination in CNS diseases, providing new targets for myelin repair [111–113].

*Acute CNS Injuries.* Recent studies have found that miRNAs such as miR-146a [114], miR-219 [115], miR-24 [116], miR-26b [117], miR-200, and miR-9 [118] contribute to the pathology of acute CNS injuries by regulating OL progenitor cell (OPC) differentiation and remyelination. For example, Liu *et al.* described the pivotal role of miR-146a in promoting OPC differentiation into myelinating OLs. Overexpression of miR-146a in primary OPCs increases, whereas attenuation of endogenous miR-146a suppresses the expression of myelin proteins [114]. In another study, Nazari *et al.* demonstrated that miR-219 promotes the differentiation and maturation of OPCs after transplantation and can be used in cell therapy of SCI [115].

*Multiple Sclerosis.* MS is a relapsing or progressive immune-mediated, inflammatory, demyelinating disease characterized by the loss of OLs [14, 119]. Remyelination requires the differentiation of OPCs into myelinating OLs, which is often inhibited in MS. Several researchers have noted the importance of miRNA in MS. During their maturation to myelinating cells, OPCs follow a very precise differentiation program, which is finely orchestrated by miRNAs, such as miR-146a [120], miR-19b [121], miR-27a [122], miR-219 [123], miR-125a-3p [124], and miR-145-5p [125]. For example, Zhang *et al.* demonstrated that an increase of miR-146a level in the CNS fosters OPC differentiation and remyelination by inhibiting the TLR2/IRAK1 signaling pathway. This study provides insight into the cellular and molecular bases for the therapeutic effects of miR-146a on OPC differentiation and remyelination, and suggests the potential of enhancing miR-146a as a treatment of demyelinating disorders [120]. Another miRNA, miR-19b, is significantly reduced in an EAE mouse model and its restoration probably has therapeutic effects by affecting PTEN in

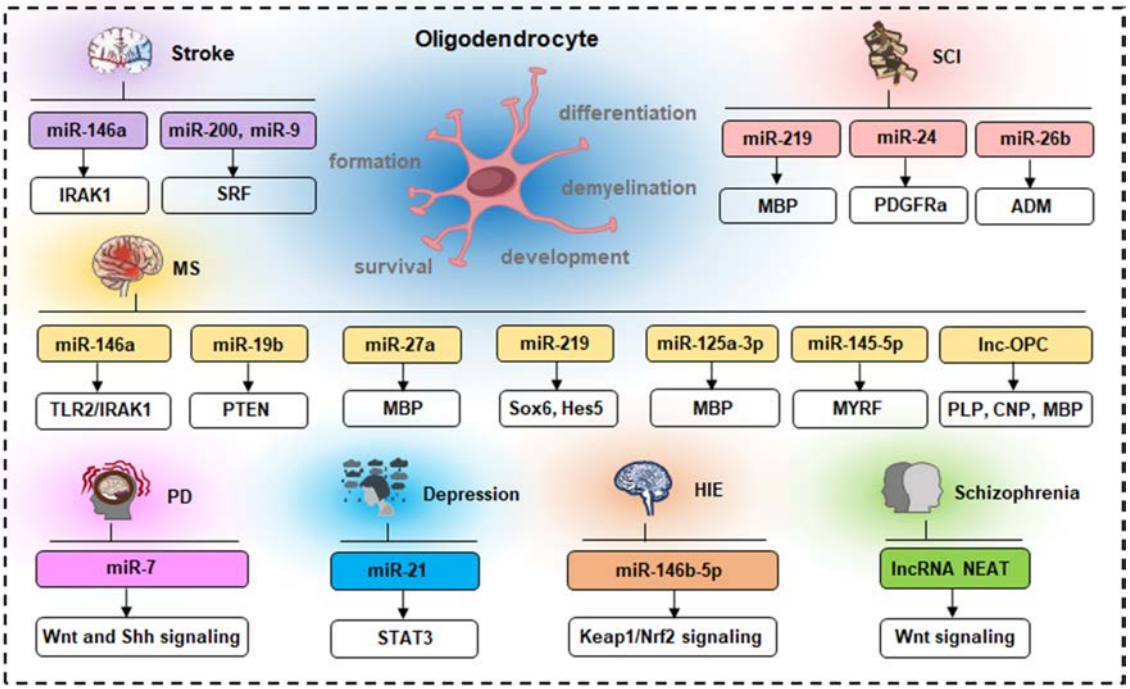


**Fig. 4** Regulation of oligodendrocyte function by ncRNAs in CNS disease. The mechanism of action of ncRNAs on OL function and CNS diseases [stroke, spinal cord injury (SCI), multiple sclerosis (MS), Parkinson's disease (PD), depression, hypoxic–ischemic encephalopathy (HIE), and schizophrenia].

the pathogenesis of EAE [121]. Taken together, mounting evidence shows that miRNAs play important roles in the development of MS, with much evidence coming from EAE animal models. Further understanding the functional roles of dysregulated miRNAs in MS is expected to help to unravel disease pathogenesis and may provide new potential remyelination therapeutic targets.

*Other CNS Diseases.* The injury of OPCs also contributes to the pathology of other CNS diseases, such as miR-7 in PD [126], miR-21 in depression [127], and miR-146b-5p in hypoxic–ischemic encephalopathy [128]. Taken together, these results suggest a crucial role for miRNAs in OL function and provides new insights into the development of an miRNA-mediated neuroprotective therapeutic approach.

### LncRNAs and Oligodendrocytes

Recent studies have found that lncRNAs regulate OL differentiation, myelination, and remyelination *via* different mechanisms in CNS disease. Researchers have identified lncRNAs that are regulated during OPC differentiation from neural stem cells (NSCs) and that are likely to be involved in oligodendrogenesis. Furthermore, the depletion of lnc-OPC significantly reduces OPC formation and affects the global expression of genes associated with oligodendrogenesis upon the differentiation of OPCs from NSCs [129]. In another study, He *et al.* investigated the lncRNA transcriptome in OL lineage cells and found that overexpression of lncOL1 promotes precocious OL differentiation in the developing brain, whereas genetic inactivation of lncOL1 causes defects in CNS myelination and remyelination following injury [130]. Katsel *et al.* demonstrated that lncRNA NEAT1 is significantly downregulated in the cerebral cortical region in schizophrenia compared with controls. $NEAT1^{-/-}$ mice revealed a significant impact on processes related to OL differentiation and RNA posttranscriptional modification with the underlying mechanisms involving Wnt signaling, cell contact interactions, and regulation of cholesterol/lipid metabolism [110].

### CircRNAs and Oligodendrocytes

Previously, studies of circRNAs have rarely addressed OL function. In recent years, Li *et al.* revealed dynamic regulation of human OL circRNA landscapes during early differentiation and suggested critical roles of the circRNA-miRNA-mRNA axis in advancing human OL development [131].

## Conclusions and Future Perspectives

There is increasing evidence that ncRNAs play important roles in glial function and CNS diseases. In this review, we have summarized recent studies demonstrating how ncRNAs

regulate glial function in CNS diseases, including acute CNS injuries, chronic neurodegenerative diseases, and other CNS diseases (Table S1). In addition, we have provided a summary of recent studies on the expression pattern of these ncRNAs in glial cells (Table S2). Here, we found that ncRNAs display positive or negative roles in glial cell-mediated CNS diseases. In this work, we collected many reports of ncRNAs affecting CNS disease in clinical samples, animal models, or *in vitro*, which were usually identified using RNA-seq, deep sequencing, and microarrays. There has been tremendous development both in clinical applications and diagnostic approaches. However, more studies are needed, and the pharmacokinetic and toxic of ncRNAs need to be tested. In conclusion, elucidating the mechanisms of these ncRNAs in biological systems in CNS diseases may create opportunities for identifying biomarkers and novel therapeutic targets of CNS diseases *via* regulating glial function.

NcRNA-based therapeutics have become promising strategies for treating CNS diseases. However, several elements need to be addressed before ncRNAs are ready for therapeutic use, including generation, stability, delivery, and safety. Many developments can be used to solve these problems. First, chemical modifications might be used to improve the stability of ncRNAs. Second, different delivery systems are also being used, including lipid-based vectors and exosome delivery systems. Taken together, the development of ncRNA-based treatments of glial cells for CNS diseases may be a novel and potentially effective therapeutic strategy, which could also further enhance our understanding of ncRNA biology. We expect that the development of research will lead to the discovery of more therapeutic strategies based on ncRNAs in glial cells, leading to the development of safe and effective strategies for use in clinical practice. Increasing clinical trials and clinically effective ncRNA drugs will certainly enhance the future of this nascent field.

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

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REVIEW

# Coordinated Regulation of Myelination by Growth Factor and Amino-acid Signaling Pathways

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**Abstract** Myelin-forming oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) are essential for structural and functional homeostasis of nervous tissue. Albeit with certain similarities, the regulation of CNS and PNS myelination is executed differently. Recent advances highlight the coordinated regulation of oligodendrocyte myelination by amino-acid sensing and growth factor signaling pathways. In this review, we discuss novel insights into the understanding of differential regulation of oligodendrocyte and Schwann cell biology in CNS and PNS myelination, with particular focus on the roles of growth factor-stimulated RHEB-mTORC1 and GATOR2-mediated amino-acid sensing/signaling pathways. We also discuss recent progress on the metabolic regulation of oligodendrocytes and Schwann cells and the impact of their dysfunction on neuronal function and disease.

**Keywords** Oligodendrocyte · Schwann cell · myelination · RHEB · mTORC1 · MIOS · GATOR2 · mitochondria · lactate

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## Myelination of Axons

### Why Myelination?

Vertebrates have evolved central and peripheral nervous systems that coordinately control organismal functions in the steady state and in response to external stimuli. The basic task the nervous systems perform uses energy to process and transmit information in the form of nerve impulses (action potentials). One of the critical logistic challenges facing the nervous systems is to transmit nerve impulses along axons at speed and with energetic efficiency. The transmission speed is directly correlated to the axon diameter. Therefore, one primitive mechanism animals evolved for speedy transmission is enlargement of the diameter of axons. Apparently, there is a limit to the extent of axon enlargement in tissue development; the enlargement of axons increases the biomass of the tissue and thus energy consumption. Also, there is the issue of spatial constraints imposed by the skull and vertebral column. As the nervous systems evolved to perform more complex functions for organismal survival, specialized neuroglia developed and the cellular composition and neural networks of the nervous systems became more complex. For better survival, organisms needed to apply a new mechanism to deal with the balance/optimization of fast transmission and energy cost. Hence, the myelination of axons that might have first evolved in fish [1]. Myelin is first found in the most ancient vertebrate, the Chondrichthyes (cartilaginous fish) but not in the Agnatha (jawless fish). Evidence further suggests that compacted myelin and genes critical to myelination such as myelin basic protein likely appeared concurrently with the emergence of jaws in vertebrates [1, 2].

Myelination of axons occurs primarily in the nervous systems of vertebrates, although a limited number of

invertebrate species appear to develop certain structural features analogous to vertebrate myelin. Vertebrate myelin is extended glial membrane (rich in cholesterol and glycosphingolipids and specialized myelin protein) that enwraps axons and forms compact, concentric insulating sheaths in segments. These segments are discontinuous; periodic gaps known as the "nodes of Ranvier" exist between them. Voltage-gated $Na^+$ channels are concentrated at the node of Ranvier. These $Na^+$ channels and the arrangement of myelin sheaths allow nerve impulses to "jump" between myelin segments (saltatory conduction); therefore, the nerve impulses are propagated along axons much faster (up to 100 times) with precision and energetic efficiency. Producing compact myelin membrane or myelinogensis is an energy-demanding process [3], but myelination is a better solution to the balance of fast conduction and conservation of space and energy. Myelin structure is not static but dynamic. It undergoes remodeling under the influence of neural activity (adaptive myelination), and this activity-dependent myelin remodeling is implicated in higher cognitive functions, such as motor-skill learning [4]. Deficits in myelin formation also underlie developmental and neurodegenerative brain disorders [5–8].

## CNS *versus* PNS Myelination

The myelination of axons in the CNS and PNS is performed by oligodendrocytes and Schwann cells (SCs), respectively. Although the myelination by oligodendrocytes and SCs serves the same function of speedy conduction of nerve impulses, the ways they myelinate axons are different. In the CNS, the bodies of the oligodendrocytes are distant from the target axons they myelinate and one oligodendrocyte can extend multiple cytoplasmic processes to enwrap multiple axons (on average 16 axons in rat optic nerves) [9]. Unlike oligodendrocytes, SCs in the PNS cling to peripheral axons "like flattened pearls on a string" and each myelinates one segment of axon, forming an internode. Hundreds of SCs are attached to a large diameter axon, in a series along its entire length. The myelin wrappings of the peripheral nerves are generally thicker relative to the axon diameter. Although certain myelin proteins, such as myelin basic protein (MBP), 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP), and proteolipid protein (PLP), are shared in CNS and PNS myelin, they are more abundant in CNS myelin, and many of the proteins in CNS and PNS myelin are different [10–12].

Myelin-forming oligodendrocytes and SCs are derived from different progenitor cell sources in development. Before becoming mature, myelin-forming cells, both oligodendrocyte and SC progenitors go through the following processes: (1) specification, proliferation, and migration of the progenitor cells to the target axons; (2) exit from the cell division cycle to become differentiated cells starting to express myelin genes (immature myelinating cells); and (3) functional and morphological maturation of immature myelinating cells to mature myelinating cells.

SCs are derived from multi-potent neural crest cells in embryonic development (Fig. 1, lower panel). Neural crest cells emerge at the most dorsal part of the neural tube, from where they delaminate and extensively migrate to various locations in the embryo. Neural crest cells are specified to form Schwan cell precursors (SCPs) in early embryonic development (E12/13 in mouse and E14/15 in rat). SCPs differentiate gradually to immature and mature myelinating SCs (E15/16 in mouse and E17/18 in rat) [13]. In this process, immature SCs sort out the axons to be myelinated (radial sorting) and establish a 1:1 ratio of the SC and the axon. Under the influence of axon-derived signaling, immature SCs become fully mature myelinating SCs wrapping axons.

The formation of oligodendrocytes (oligodendrogenesis) in the CNS starts with the specification of oligodendrocyte precursor cells (OPCs) from neural stem/progenitor cells that originate from neuroepithelium in the ventricular zone [14] (Fig. 1, upper panel). First, in the spinal cord where CNS myelination first takes place, OPCs appear in the ventral ventricular zone near the floor plate around embryonic day 12.5 (E12.5) in mice. These OPCs are generated from the pMN (progenitor of motor neuron) domain through a neuron-to-glia fate switch. Soon after, these ventrally-derived OPCs proliferate rapidly and migrate in all directions, becoming evenly distributed through the cord in both gray and white matter by ~E15 in mice [15]. At this point, a second wave of OPCs is generated from the radial glial cells of the dorsal spinal cord [16] and displace those ventrally-derived OPCs in the dorsal region and eventually account for 20% of the total spinal OPC population. Around E18.5 (shortly before birth), some OPCs in the spinal cord start to differentiate and produce oligodendrocyte lineage cells expressing MBP and PLP. The spinal myelination reaches its peak ~2–4 weeks after birth [17].

In the brain, OPCs are generated in three separate waves; the first-two waves start from the ventral ventricular zone (the medial ganglionic eminence [E12.5] and later lateral the ganglionic eminence [E15.5]). Some of these ventrally-derived OPCs migrate as far as to the cerebral cortex. Just before birth at E17.5, the dorsal (the third) wave of oligodendrogenesis, characterized by the genesis of epidermal growth factor receptor-positive/platelet-derived growth factor receptor-positive cells, begins in the forebrain through the stimulation of a type of multipotent intermediate progenitor by Sonic Hedgehog [18, 19]. These OPCs later replace the majority of the cortical OPCs derived from the ventral ventricular zone, and contribute 80% of the OPCs in the forebrain [20]. These ventrally- and dorsally-derived OPCs share

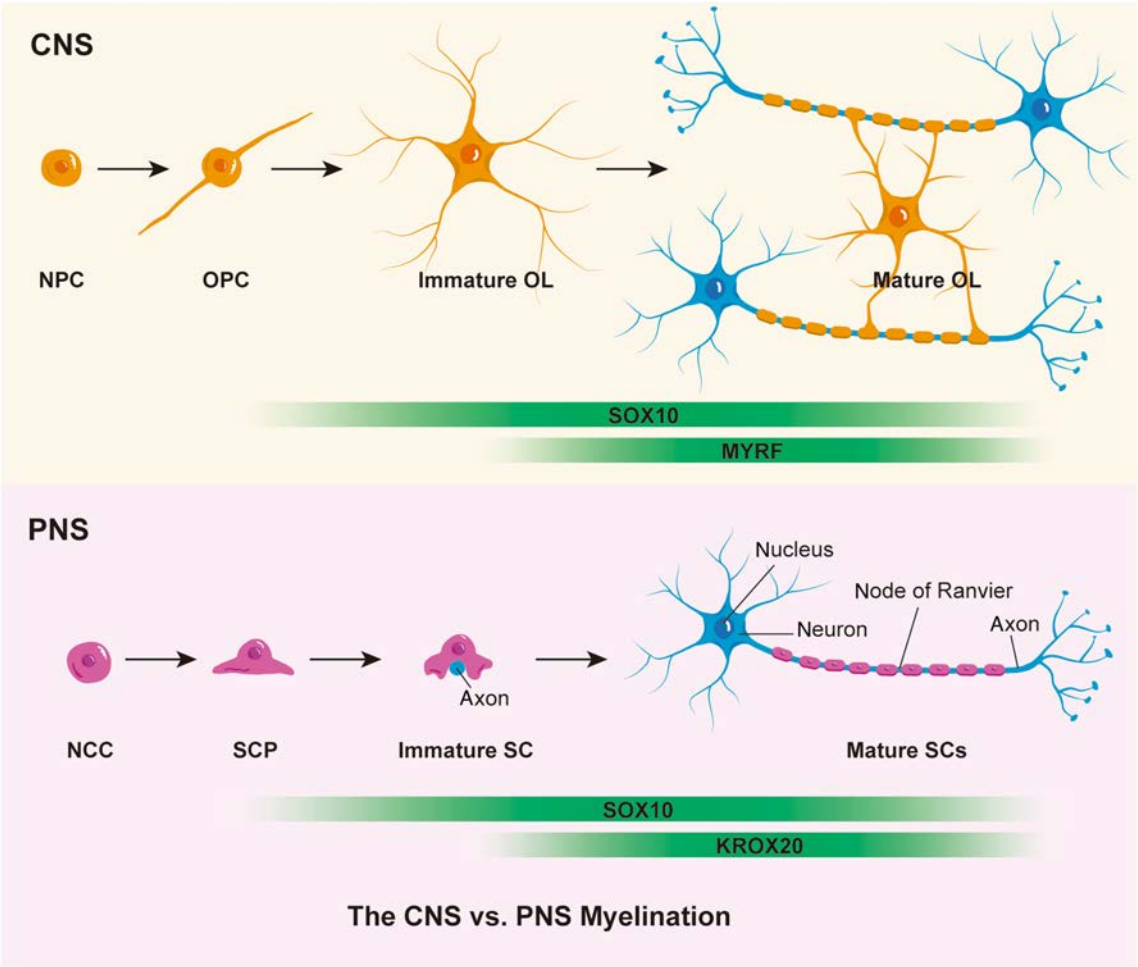


**Fig. 1** Similarities and differences between CNS and PNS myelination. NPC, neural progenitor cell; OPC, oligodendrocyte progenitor cell; OL, oligodendrocyte; NCC, neural crest cell; SCP, Schwan cell precursor; SC, Schwan cell.

similar transcriptional profiles in postnatal differentiation [21], and they are functionally interchangeable [20]; OPCs ablated from one origin can be completely replaced by those of another origin. But OPCs become heterogeneous (in terms of functional state and differentiation potential) in different brain regions and at different ages [22], and a recent study using *in vivo* clonal analysis has shown that the fate of these OPCs from different origins are inherently different: dorsally-derived OPCs can expand during adult life, but ventrally-derived OPCs cannot and are eventually eliminated after the weaning period [23]. This study further showed that OPCs undergo 2–3 rounds of symmetrical division before exiting the cell cycle and becoming committed to a program of differentiation [23]. The differentiation of OPCs to fully mature myelinating oligodendrocytes is a tightly-regulated process in which extracellular signals are transmitted through intracellular signaling pathways to regulate transcriptional and metabolic changes in oligodendrocyte lineage cells.

## Coordinated Regulation of Oligodendrocyte Myelination by Growth Factor and Amino-acid Signaling Pathways

Despite the different origins of SCs and oligodendrocytes, the myelin they produce is similar in chemical composition and structural organization. How such completely different cell types acquire the same function remains incompletely understood. It is plausible that certain regulatory networks and mechanisms governing myelinating glia are co-opted by both the central and peripheral systems early on in myelin evolution. This notion is supported by the results of several studies. For example, the well-established roles of the transcription factor SOX10 (Sry-related HMg-Box gene 10) in both central and peripheral myelination provide strong support to the notion that gene transcription programs in oligodendrocyte and SC lineages are organized along similar general principles, but differentially implemented. SOX10 regulates multiple steps in the formation of myelinating SCs,

including the terminal maturation of myelinating SCs where SOX10 induces and cooperates with the transcription factor KROX20 to express a battery of myelin genes [24]. In the oligodendrocyte lineage, SOX10 also plays a critical role in regulating the terminal maturation of oligodendrocytes that involves the induction of MYRF by SOX10 and the physical interaction of SOX10 with MYRF in inducing myelin gene expression [25]. While the gene transcription programs that differentially regulate oligodendrocyte and SC myelination are better known, less is known about the intracellular signaling pathways that link external cues to gene transcription programs in myelination.

## Growth Factor/RHEB-mTORC1 Pathway in CNS and PNS Myelination

The growth factor-activated mTORC1 kinase complex (consisting of RAPTOR, MLST8, DEPTOR, and PRAS40) is well known for its role in regulating cellular metabolism, including energy metabolism and the biosynthesis of molecules [26] (Fig. 2). In the presence of amino-acids, growth factors signal to activate the small GTPase RHEB that directly binds the mTOR protein and activates its kinase activity [27–29]. The activity of RHEB is inhibited by the GTPase-activating protein (GAP) complex TSC (tuberous sclerosis complex) [30]. Under growth factor stimulation, the GAP activity of the TSC on RHEB is inactivated by phosphorylation of the TSC protein, resulting in mTORC1 activation. Loss-of-function mutation of TSC, as seen in TSC patients, causes persistent RHEB-mTORC1 activation [31]. It is well established that RHEB-mTORC1 plays a critical role in the regulation of CNS and PNS myelination.

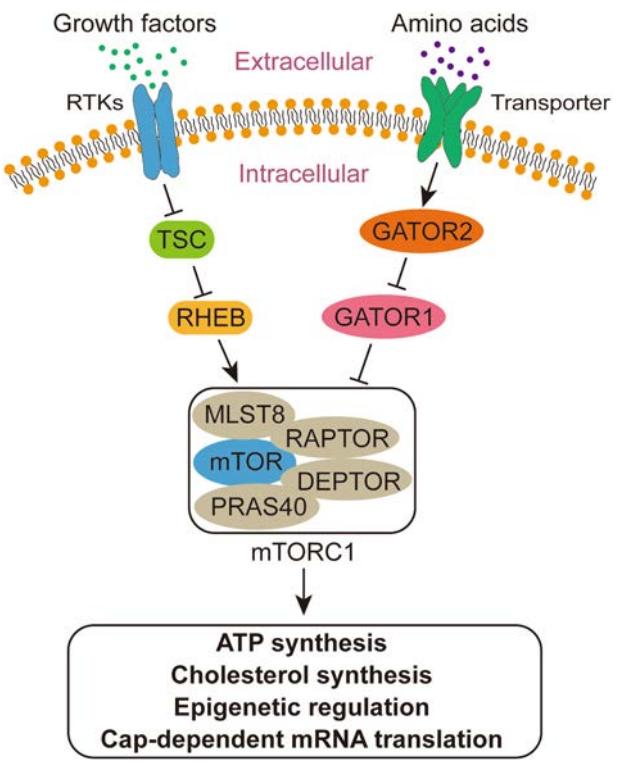


**Fig. 2** Pathways of growth factor and amino-acid signaling to mTORC1.

### *RHEB-mTORC1 and CNS Myelination*

Study of *Rheb* conditional knockout (KO) mice indicates that RHEB-mTORC1 plays a role in oligodendrocyte differentiation and myelination [32]. Genetic deletion of *Rheb* in neural stem cells (Nestin-Cre) reduces mTORC1 activity in all types of neural cells derived from neural stem cells (neurons, astrocytes, and oligodendrocytes), but selectively impairs the differentiation of OPCs and causes severe developmental hypomyelination in brain [32]: Disrupting mTORC1 activation by *Rheb*-KO in neural stem cells does not affect neurogenesis and astrogliogenesis. The loss of *Rheb* does not affect the proliferation of OPCs, but affects the differentiation of OPCs into postmitotic oligodendrocytes. Deletion of *Rheb* in OPCs (Olig1-Cre or Olig2-Cre) results in an increase in the number of OPCs and a decrease in the number of differentiated oligodendrocytes [33]. The oligodendrocyte deficit and developmental hypomyelination are reproduced by deleting mTOR using a similar approach, further suggesting that RHEB regulates oligodendrocyte differentiation and myelination through mTOR or mTORC1. The role of mTORC1 in oligodendrocyte myelination is further supported by genetic deletion of *Raptor* using CNP-Cre [34, 35], although a myelination deficit occurs primarily in the mouse spinal cord [35]. How mTORC1 regulates OPC differentiation remains unknown.

The process of OPC differentiation into mature myelinating oligodendrocytes is represented by a continuum of oligodendrocytes in different states/conditions [36]. mTORC1 appears to act on the transition of late-stage OPCs into immature oligodendrocytes starting to express myelin [37]. The timing for OPCs to exit the cell cycle and become differentiated seems to be controlled by an internal molecular clock mechanism [23, 38, 39] that may involve the activation of positive transcription factors and simultaneous suppression of the expression of negative transcription factors for OPC differentiation, such as ID2 and ID4 [40, 41] and HES5 and Master-Mind 1 [42–44]. RHEB-mTORC1 could participate in controlling the internal clock through epigenetic regulation of the expression of these inhibitory transcription factors, involving histone modification.

Previous studies have established a role of histone acetylation in OPC differentiation and myelination [45–47]. Histone deacetylases (HDACs) are primarily expressed in OPC stages and HDAC inhibitors affect OPC differentiation [48]. Histone acetylation is involved in the control of expression

of inhibitory transcription factors, including ID2 and ID4 [49]. The notion that RHEB-mTORC1 can regulate OPC differentiation through epigenetic regulation is consistent with the finding that inhibiting mTORC1 of OPCs in culture increases the expression of ID2 and ID4 [50]. Recent studies have suggested that RHEB-mTORC1 regulates the histone modification of differentiating OPCs through mitochondrial metabolism. Both histone acetylation and methylation are critically dependent on the status of cellular energy and the activity of the mitochondrial tricarboxylic acid cycle [51]. RHEB is a critical activator of acetyl-CoA and ATP production by mitochondria [52]. When *Rheb* is deleted, mitochondrial production of acetyl-CoA and ATP is significantly reduced in oligodendrocyte lineage cells. As a result, histone acetylation and methylation of the nucleosomes is reduced and such altered transcription programs in differentiating oligodendrocytes impair OPC differentiation [53, 54]. Therefore, the role for RHEB-mTORC1 plays in oligodendrocyte differentiation is to establish a permissive metabolic state for the epigenetic regulation of OPC differentiation to myelinating oligodendrocytes.

mTORC1 also regulates oligodendrocyte myelination through regulation of lipid and myelin protein synthesis. As with the maturation of myelinating oligodendrocytes, the demand for lipids surges. Cholesterol synthesis is critical for oligodendrocyte myelination [55]. Studies of zebrafish further indicate that mTORC1-regulated synthesis of cholesterol promotes myelin gene expression in myelin development [56]. In further support, a landmark study using DRG (dorsal root ganglion) neuron culture showed enhanced formation of a cholesterol-rich signaling domain between oligodendrocytes and axons and this cholesterol-rich domain is critical for the local synthesis of MBP in myelin and the induction of myelination [57]. Therefore, mTORC1-regulated cholesterol may play a role in organizing a signal ensemble or microdomain in the membrane for regulating myelin gene transcription that is essential for oligodendrocyte differentiation and maturation. On the other hand, cholesterol promotes mTORC1 activation by stimulating its lysosomal localization [58], which provides a positive-feedback mechanism to maintain mTORC1 activity that might be required for sustained myelin synthesis.

mTORC1 is well known for its role in regulating the Cap-dependent translation of mRNAs [59] and in regulating myelination through local synthesis of myelin protein. Because myelin growth is dependent on the extension from the distal end of oligodendrocyte processes, it makes sense for oligodendrocytes to use local signals in coordination with external (axonal) cues to coordinate myelin production locally [57, 60]. Not only are mRNAs encoding various myelin proteins including MBP and myelin-associated oligodendrocytic basic protein (MOBP) present in myelin [61], but RNA-binding protein [62] and regulators of protein synthesis are also present in myelin [63, 64]. A recent study of mTOR mutant zebrafish demonstrated that mTORC1-regulated Cap-dependent local translation of mRNAs in myelin plays a role in myelin expansion during development [64], highlighting a role for mTORC1 in the growth/stability of myelin sheaths.

Once the development of myelination is complete, the role of mTORC1 in the maintenance of myelin needs additional clarification. The maintenance of myelin integrity in mouse is dependent on the persistent synthesis of myelin protein. This is demonstrated by the inducible genetic deletion of the master regulator of myelin gene expression MYRF in adult oligodendrocytes [65]; loss of *Myrf* in adult oligodendrocytes causes rapid, wide-spread demyelination in the brain and spinal cord. mTORC1 does not seem to play a role in maintaining myelin protein expression and perhaps myelin structure in the adult mouse; deletion of *Rheb* in mature oligodendrocytes using Tmem10-Cre or deletion of *Mtor* using PLP-CreER does not alter myelin protein expression and causes demyelination in the brain and spinal cord months after the onset of gene deletion [33, 66].

### *RHEB-mTORC1 and PNS Myelination*

The roles of mTORC1 activity in SC myelination of the peripheral nervous system have been examined using various genetic mouse models. In the early stage of myelin development, SC precursors undergo extensive proliferation stimulated by signals from developing axons. Gradually, SC precursors slow their proliferation and initiate a differentiation program. The results of studies with genetic mouse models support the notion that a gradual decline in mTORC1 activity of the SC precursor cells is necessary for their exit from the cell cycle and further differentiation, and at later stages, mTORC1 is needed again to promote lipid synthesis for myelinogenesis [67–70]. Excessive mTORC1 activity (above a certain threshold) in SC precursors in the TSC1 and TSC2 mutants keeps SC precursors in the proliferative state and blocks their differentiation, causing a myelination deficit [68, 70]. Too much mTORC1 activity in immature SCs also suppresses the expression of the transcription factor KROX20 that initiates the transcription program of myelin genes [71]. However, when SCs mature to a certain stage, increased mTORC1 activity, as shown by the deletion of TSC1 and TSC2 in mature SCs, causes myelin overgrowth [68, 70].

In contrast to the critical role of RHEB-mTORC1 in oligodendrocyte linage formation, loss of RHEB-mTORC1 in SC precursors has no apparent effect on the formation of SC lineage [72, 73]. When *Rheb* is genetically deleted from SC precursors (using Dhh-Cre and P0-Cre), the formation of SC lineage is preserved, although mTORC1 is inactivated in the cells of SC lineage. The numbers of $SOX10^+$ and

KROX20$^+$ cells in the sciatic nerves are comparable between normal control mouse and *Rheb* KO mouse, suggesting that mTORC1 is not required for the proliferation and differentiation of SC precursors. The expression of myelin protein is basically comparable to the normal control, except MBP exhibiting a modest reduction. The number of myelinated axons in the sciatic nerves are normal, albeit with modestly reduced thickness of myelin wraps [73]. The reduced myelin thickness could be due to lower synthesis of lipids because of reduced mTORC1 activity [72]. While mTORC1 is not required for SC differentiation, mTORC1 is transiently reactivated in SCs under injury and plays a role in their dedifferentiation that facilitates injury repair [74].

In summary, balanced, dynamic RHEB-mTORC1 activity plays critical regulatory roles in the proper myelination of both CNS and PNS, but the regulation is implemented differently. In the CNS, mTORC1 does not play a critical role in the regulation of the proliferation of OPCs, but is critical in the regulation of their differentiation into mature oligodendrocytes and myelinogenesis (in the late stage of myelination). In contrast, mTORC1 in SC precursors controls their proliferation, but is not required for the differentiation of SC precursors to immature SCs. Gradual downregulation of mTORC1 activity is necessary for the full maturation of myelinating SCs. Like mTORC1 in myelinating oligodendrocytes, mTORC1 activity in myelinating SCs plays a role in enhancing myelinogenesis. The role of mTORC1 in myelin maintenance needs additional studies, although reports suggest that persistent activation of mTORC1 may be implicated in the peripheral demyelination caused by mitochondrial dysfunction [75].

## Amino-acid/GATOR-Rag Signaling Pathway in Myelination

### *Amino-acid Sensing and the GATOR-Rag Pathway*

Oligodendrocyte proliferation and differentiation, and myelinogenesis, are dependent on nutrient availability. Amino-acids account for most of the increase in cell mass of dividing cells [76]. Amino-acids are not only building blocks of protein synthesis, but also signal molecules. Amino-acid signaling intersects with growth factor signaling to regulate TSC and RHEB activity in lysosomes and affects mTORC1 activation [77–79]. While the roles of the growth factor-stimulated mTORC1 pathway in the oligodendrocyte and SC lineage are well documented, the study of nutrient-sensing/signaling pathways in these myelinating cell lineages is just beginning.

Over the past decade, enormous progress has been made towards a biochemical understanding of amino-acid sensing/signaling pathways. Cells use different mechanisms to sense the availability of different amino-acids [80–84]. Amino-acids can either be sensed directly by their binding proteins or indirectly through their metabolite (acetyl-CoA). Many organelles, such as lysosomes and mitochondria, participate in amino-acid sensing/signaling. In the case of threonine sensing/signaling, protein interactions between the lysosome and mitochondria are involved for threonine to signal to mTORC1 [80]. Studies of many organisms have revealed that the major components of amino-acid sensing/signaling pathways are conserved across species. While much is known about the machinery and pathways involved in amino-acid sensing/signaling, their biological functions at the organismal level remain obscure.

The best-studied amino signaling pathway is the lysosome-based GATOR-Rag pathway signaling to mTORC1. GATOR, a protein complex, is positioned between amino-acid sensors (Sestrin and Castor) and the Rag GTPase complex. In the presence of amino-acids, the Rag complex becomes activated on lysosomes, binds to the mTORC1 component RAPTOR and anchors the mTORC1 complex onto the surface of lysosome at the site where mTORC1 is activated by RHEB. The GATOR complex is composed of two sub-complexes—GATOR1 and GATOR2 [85]. GATOR1 (made up of DEPDC5, NPRL2, and NPRL3) inhibits the downstream Rag GTPase complex and therefore suppresses mTORC1 activation. GATOR2 (made up of MIOS, WDR24, WDR59, SEH1L, and SEC13) is upstream of GATOR1 and inhibits GATOR1 function, therefore activating mTORC1. Biochemical studies have shown that GATOR2 directly links the amino-acid sensors to GATOR1 and disruption of the GATOR2 complex by deleting a single component of GATOR2 (e.g., MIOS) inactivates GATOR2 function, as indicated by the loss of mTORC1 activity. The roles of amino-acid sensing/signaling in myelin biology has not been studied until recently [86].

### *GATOR2-Mediated Amino-acid Signaling and Myelination*

When GATOR2 function is disrupted by genetic deletion of its component Mios in neural stem cells or OPCs, oligodendrocyte lineage formation is impaired: the number of OPCs is reduced, together with the differentiated oligodendrocytes expressing the CC1 marker. Cell cycle analysis shows that *Mios*-KO OPCs exhibit a prolonged cell cycle, S-phase in particular. The altered cell cycle of OPCs reduces their proliferation and differentiation, resulting in impairment in the formation of oligodendrocyte lineage cells in the *Mios*-KO mouse. Accordingly, the *Mios*-KO mouse manifests widespread hypomyelination in the brain, as indicated by reduced myelin protein, number of myelinated axons, and thickness of myelin wrapping the optic nerves. The myelin deficit persists into adulthood. These findings indicate a role of Mios in the early stages of OPC proliferation and differentiation. The reduced thickness of myelin sheaths also suggests a role for

MIOS/GATOR2 in myelinogenesis. Surprisingly, the effect of *Mios*-KO on cell lineage formation is cell-type specific; deletion of *Mios* in neural stem cells using a Nestin-Cre driver does not affect the embryonic neurogenesis and later astrocyte formation, but oligodendrocyte lineage is affected.

Also surprising is that the requirement of MIOS/GATOR2 in amino-acid signaling to mTORC1 is cell-type-specific: genetic deletion of *Mios* in neural stem cells does not affect mTORC1 activity in neurons, but selectively impairs it in the oligodendrocyte lineage. Furthermore, loss of *Mios* renders OPCs unresponsive to changes in amino-acid availability in culture. Given the role of growth factor-stimulated mTORC1 activity in oligodendrocytes and myelin formation, it appears that all the effects of *Mios*-KO on the oligodendrocyte lineage are a result of mTORC1 deficiency in the oligodendrocyte lineage, like *Rheb*- or *Raptor*-KO in that lineage. But the primary defect in OPCs of *Mios*-KO *versus* the loss of mTORC1 (e.g., in *Rheb*- or *Raptor*-KO) is different: *Mios*-KO significantly impairs the proliferation of OPCs in the early postnatal stage and therefore affects the formation of a sizable pool of OPCs in the developing brain. The effect of *Mios* deletion on oligodendrocyte formation and myelination involves more than MIOS/GATOR2 signaling to mTORC1 activation. When mTORC1 deficiency in the oligodendrocyte lineage is rescued by expressing the constitutively active *Rheb* transgene (RhebS16H) [32], the numbers of OPCs and differentiated oligodendrocytes are not restored, and the reduced myelin protein and hypomyelination remain in the *Mios*-KO mouse [86]. These findings indicate that coordinated regulation of the formation of the oligodendrocyte lineage and the myelination of axons by growth factor and amino-acid signaling in the CNS involves more than mTORC1 activation in the oligodendrocyte lineage, and the role of MIOS/GATOR2-mediated amino-acid signaling goes beyond activating mTORC1 in oligodendrocytes.

Oligodendrocyte myelination regulated MIOS/GATOR2 involves the Rag-TFEB/TFE3 axis. TFEB and TFE3 are amino-acid sensitive transcription factors that control the transcription of lysosomal biogenesis genes [87]. They are functionally redundant. In mouse, both TFEB and TFE3 are expressed in the oligodendrocyte lineage, TFE3 being more abundant than TFEB in OPCs. The expression of TFEB is increased in newly-differentiated premyelinating oligodendrocytes [88]. GATOR2 transduces signals to the Rag GTPase complex that signals not only to mTORC1, but also to TFEB/TFE3. mTORC1 also regulates the transcriptional activity of TFEB by regulating its nuclear translocation [89]. Studies of zebrafish mutants have identified RagA as a regulator of CNS myelination, but not PNS myelination [90]. Homozygous RagA ($Rraga^{-/-}$) mutants exhibit reduced *Mbp* mRNA expression in the CNS, while *Mbp* expression in the PNS is normal. Similarly, the expression of *Plp1b* mRNA is reduced in $Rraga^{-/-}$ homozygous mutants. In this mutant, oligodendrocytes are present, but the number of myelinated axons in the CNS is significantly reduced, and no effect on the peripheral myelination has been noted. This role of RagA in CNS myelination is mediated by the nutrient-sensitive transcription factor TFEB (independent of mTORC1), because the loss of *Raga* function activates transcriptional activity of *Tfeb* and mutating the *Tfeb* gene ameliorates the myelination deficit caused by *Raga* mutation. Transgenic expression of constitutively-active *Tfeb* appears to inhibit the expression of MBP, suggesting an inhibitory role for TFEB in myelination. This inhibitory role has been confirmed by the homozygous *Tfeb* mutant that exhibits an increased number of myelinated axons in the spinal cord, and increased MBP expression in certain brain regions. All these results suggest that GATOR-mediated amino-acid signaling regulates the oligodendrocyte lineage through the RagA-TFEB axis in zebrafish. And balanced TFEB/lysosomal activity is essential for oligodendrocyte myelination. The role of TFEB in proper CNS myelination is also manifested in the mouse. Deletion of TFEB in OPCs does not affect the differentiation of OPCs into postmitotic, premyelinating oligodendrocytes (presumably because of functional redundancy with TFE3); however, TFEB regulates the apoptosis of excessively-generated premyelinating oligodendrocytes *via* the TFEB-PUMA axis. Loss of TFEB increases the number of mature oligodendrocytes in certain regions, resulting in ectopic myelination, for example in the cerebellum, indicating that TFEB exerts spatiotemporal control of brain myelination [88]. All these studies highlight that the GATOR-Rag-mediated amino-acid signaling pathway regulates oligodendrocyte myelination in an mTOR1-dependent and an mTORC1-independent manner involving the GATOR-Rag-TFEB/TFE3 axis (Fig. 3).

## Oligodendrocyte and Schwann Cell Metabolism and Tissue Homeostasis

Oligodendrocytes and SCs not only myelinate axons, but also provide metabolic support to neurons, rendering them key players in maintaining nervous tissue homeostasis. How the metabolic support of neuronal metabolism is executed is an area of active investigation.

### Schwann Cell Metabolism and Maintenance of the PNS Axons

The mitochondrial energy metabolism of SCs is critically linked to the maintenance of both myelinated and unmyelinated axons [73, 91, 92]. Disruption of SC mitochondrial metabolism by genetic ablation of mitochondrial transcription factor A (*Tfam*) demonstrates that blunting the transcription of the mitochondrial genome of developing

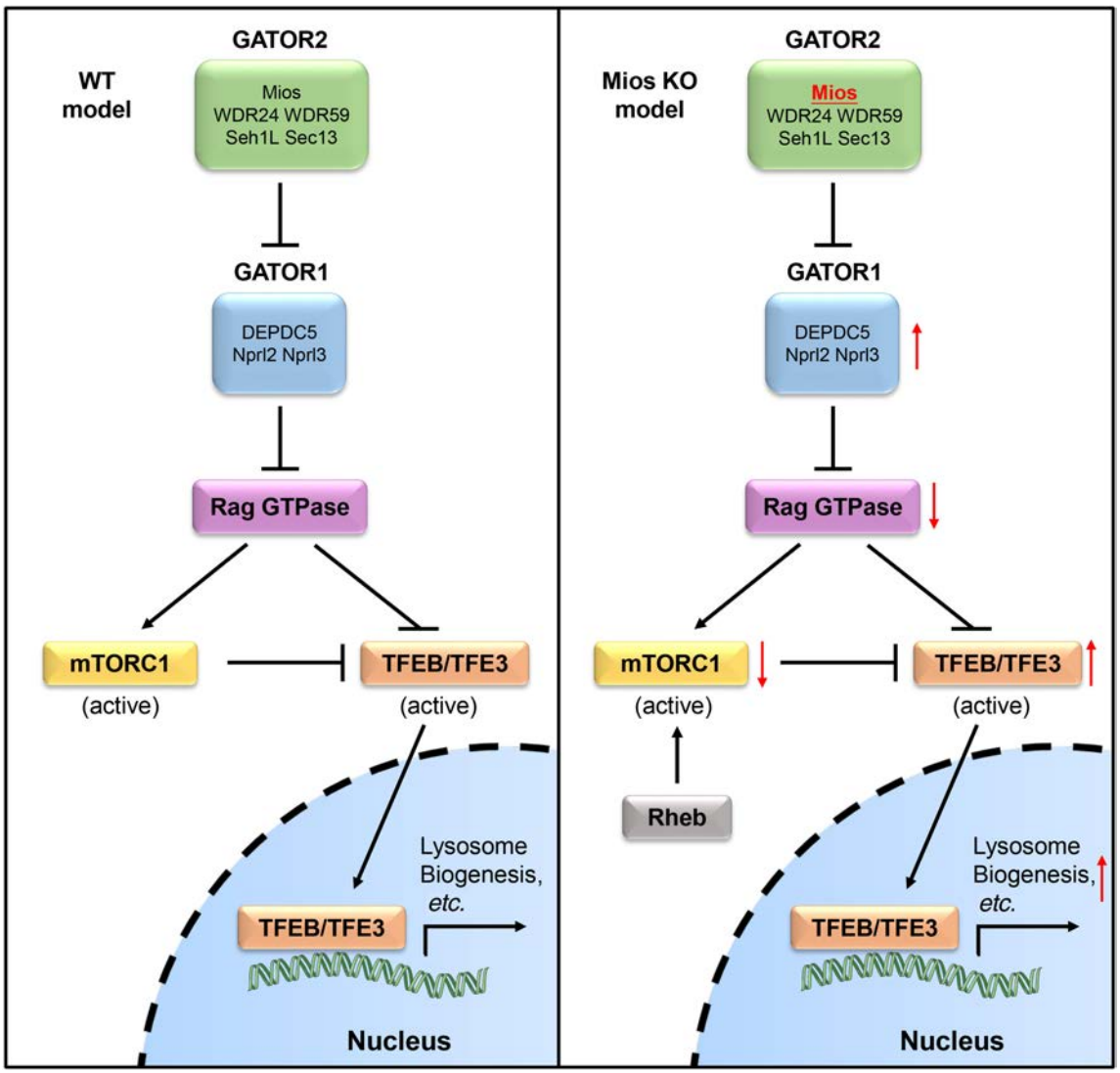


**Fig. 3** The GATOR-Rag-mediated amino-acid signaling pathway signals to mTOR1 and transcription factors TFEB and TFE3.

SCs impairs mitochondrial activity and causes peripheral axonopathy [91], although SC survival is not affected. Over time, the *Tfam*-KO mouse develops peripheral neuropathy and demyelination, with unmyelinated small-caliber axons first affected and myelinated, large-caliber axons affected later. This study suggests a link between SC mitochondrial metabolism and the structural and functional homeostasis of peripheral nerves. Another investigation based on disruption of mitochondrial respiration by genetic deletion of *Cox10* in SCs also showed that normal mitochondrial respiration is required for maintaining the integrity of peripheral axons [92].

The mechanistic understanding of the functional interactions between SCs and peripheral axons is not well understood. A follow-up study of the *Tfam* mutant mouse identified altered lipid metabolism as a culprit for axonal pathology in the *Tfam*-KO model [93]. The metabolic stress caused by *Tfam* deletion evokes a stress response, shifting lipid synthesis to oxidation and resulting in the accumulation of toxic acylcarnitines in SCs. The excessive lipid oxidation is likely to cause demyelination of peripheral axons and the release of acylcarnitines from SCs is likely to cause peripheral axonopathy.

Our study of mitochondrial pyruvate metabolism regulated by RHEB GTPase indicated that persistently increased lactate production/release by SCs causes neuronal oxidative damage to peripheral axons, resulting in axonal degeneration, a condition similar to diabetic axonopathy [73]. Lactate has long been known to support neuronal energy metabolism under certain conditions and helps preserve neuronal function under injury [94, 95]. Therefore, lactate could be a double-edged sword for neurons.

Rheb not only activates mTORC1 but activates pyruvate dehydrogenase (PDH) independently of mTORC1 [52]. When *Rheb* is deleted, the enzymatic activity PDH is decreased (the inactive, phosphorylated PDH increases); and when the *Rheb* transgene is expressed, active PDH (non-phosphorylated) is increased. When SC *Rheb* is deleted, the enzymatic activity of PDH to oxidize pyruvate to acetyl-CoA is reduced [73]. As a result, more pyruvate is reduced to lactate in *Rheb*-KO SCs, leading to increased lactate production and release. The released lactate is then imported by neuronal axons and persistently elevates the mitochondrial metabolism of neurons. Increased neuronal metabolism increases the production of reactive oxygen species and causes oxidative damage to neurons, leading to age-dependent axon degeneration (see Fig. 4). The long-term deleterious effect of SC *Rheb*-KO on axon is not likely a result of loss of mTORC1 activity, because deletion of mTOR does not cause axon pathology [96]. Preventing the overproduction and subsequent release of lactate prevents oxidative damage to neurons in the SC *Rheb*-KO mouse, which links excessive lactate uptake by neurons to neuronal damage [73].

## Oligodendrocyte Metabolism and Maintenance of CNS Axons

Oligodendrocytes are highly metabolically active, using glycolysis and mitochondrial metabolism to meet their high energy demand [97]. Therefore, oligodendrocytes are susceptible to energy deprivation [98]. In the meantime, oligodendrocytes support neuroenergetics, which adds a further demand on their metabolism. How oligodendrocytes control energy metabolism to meet the metabolic demand remains a matter of controversy. The focal point is the extent to which mitochondrial metabolism contributes to oligodendrocyte energy production. Many of the *in vitro* studies based on OPC/oligodendrocyte cultures under various conditions show that developing oligodendrocytes have highly active mitochondrial energy metabolism [99, 100], and the relative contribution of glycolysis and mitochondrial oxidative phosphorylation may depend on the state/stage in differentiation and the age of animals from which the oligodendrocyte cultures are derived. When developmental myelination is complete, mature oligodendrocytes rely on glycolysis to maintain myelin structure, because dysregulation of mitochondrial metabolism does not affect myelin integrity [92, 101]. Oligodendrocyte metabolism is closely linked to neuronal function. Oligodendrocytes increases glucose uptake and metabolism in response to neuronal activity and export more lactate to support neuronal metabolism [102, 103]. In the mouse corpus callosum, oligodendrocytes support neuronal energy metabolism directly by providing glucose to neurons [104]. Oligodendrocytes produce exosomes to transport regulatory proteins such as SIRT2 and perhaps metabolites to neurons [105, 106].

However, it has long been appreciated that oligodendrocytes metabolically support neurons by providing lactate [92, 107], which is exported by oligodendrocytes through the MCT1 transporter, and is imported by neurons through the MCT2 transporter. Neurons in the brain metabolize lactate

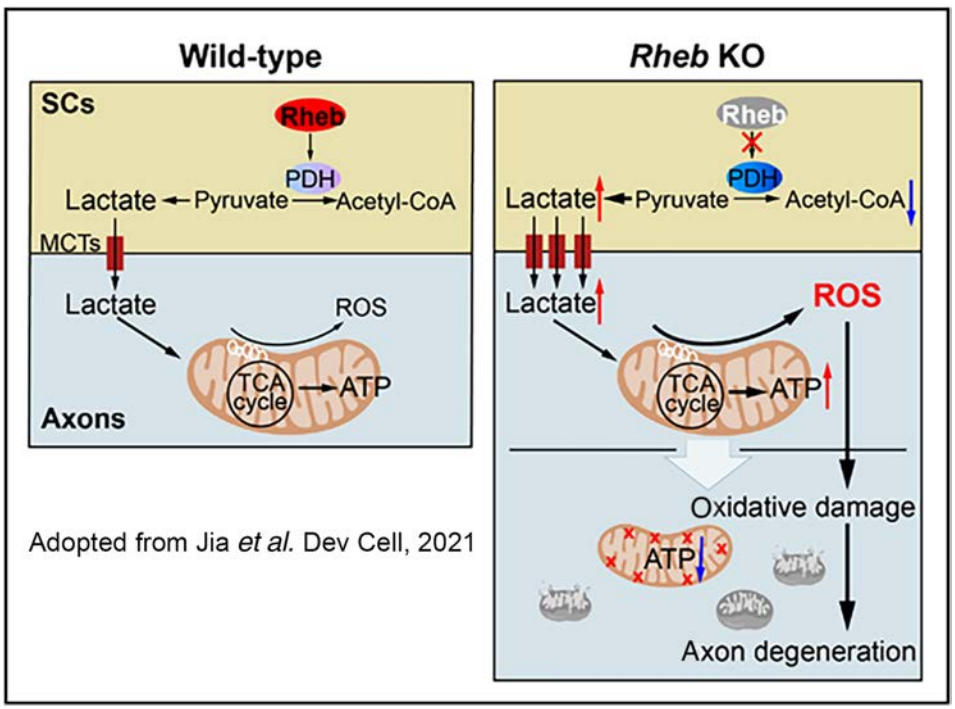


**Fig. 4** RHEB in Schwann cells regulates the energy metabolism of neuronal mitochondria. *Rheb*-KO in Schwann cells increases the production and release of lactic acid, resulting in neuronal oxidative stress and axon degeneration.

rapidly [92]. In neurons, lactate is converted to pyruvate that is oxidized to acetyl-CoA for the mitochondrial tricarboxylic acid cycle. Neuronal energy metabolism is sustained by lactate/pyruvate metabolism when neuronal activity increases [108]. Disruption of the lactate exchange between oligodendrocytes and neurons by genetic ablation of MCT1 causes age-dependent neurodegeneration and is associated with the pathogenesis of amyotrophic lateral sclerosis [107]. Oligodendrocyte lactate can also regulate neuronal function as a signaling molecule that activates signaling pathways (AKT/Stat3) and induces the expression of plasticity-related genes [109], with implications for whole-body metabolism [110].

How mitochondrial dysfunction in mature oligodendrocytes affects oligodendrocyte-neuron metabolic exchange and neuronal function remains to be clarified. Oligodendrocyte mitochondrial dysfunction often increases lactate production, as noted in patients with multiple sclerosis [111]. How persistently increased lactate input to brain and spinal neurons affects their function remains unknown. High lactate content in brain is seen in normal aging, in prematurely-aging mice [112], in Alzheimer's patients and *Drosophila* model [113, 114], suggesting that persistently increased lactate production by brain cells can have deleterious effects on neurons. This notion is consistent with the finding that increased lactate production by neurons or glia in *Drosophila* models accelerates brain aging and degeneration [115]. Future study is needed to clarify the effects of excessive lactate production by oligodendrocytes and other glia on neuronal function and brain homeostasis.

## Concluding Remarks and Future Perspectives

Oligodendrocytes and SCs are integral to neuronal function and nervous tissue homeostasis. A better understanding of the formation and function of oligodendrocytes and SCs would provide critical insights into brain function and disease. Growth factor and amino-acid signaling pathways are coordinated to regulate oligodendrocyte and SC function, but the regulation is implemented differently in these cells. The coordinated regulation involves more than activation of the growth factor-activated RHEB-mTORC1 pathway. The roles of other components of the GATOR-mediated amino-acid sensing/signaling pathway in oligodendrocyte differentiation and myelination remain to be defined. Future studies are also needed to clarify the RHEB-mTORC1- and GATOR-mediated metabolic regulation of oligodendrocyte and SC formation and their support for neuronal metabolism and function.

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REVIEW

# Glia Connect Inflammation and Neurodegeneration in Multiple Sclerosis

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**Abstract** Multiple sclerosis (MS) is regarded as a chronic inflammatory disease that leads to demyelination and eventually to neurodegeneration. Activation of innate immune cells and other inflammatory cells in the brain and spinal cord of people with MS has been well described. However, with the innovation of technology in glial cell research, we have a deep understanding of the mechanisms of glial cells connecting inflammation and neurodegeneration in MS. In this review, we focus on the role of glial cells, including microglia, astrocytes, and oligodendrocytes, in the pathogenesis of MS. We mainly focus on the connection between glial cells and immune cells in the process of axonal damage and demyelinating neuron loss.

**Keywords** Multiple sclerosis · Inflammation · Neurodegeneration · Glial cells

## Introduction

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating disease of the central nervous system (CNS) with progressive neuroaxonal degeneration [1]. The diagnosis and monitoring of MS have benefited from the advances in magnetic resonance imaging (MRI) and positron emission computed tomography (PET) methods that enable the visualization of features related to chronic inflammation in MS [2].

Chronic inflammation in MS arises from the activation of innate and adaptive immune responses in the CNS. The immune system and particular immune pathways play a central role in the pathogenesis of MS. In the context of MS neuroinflammation, the cerebral cortex, cerebrospinal fluid (CSF), the meninges, and choroid plexus are filled with innate and adaptive immune cells. With the development of single-cell sequencing [3], mass cytometry, and fate-mapping techniques, researchers have discovered various immune cell populations in the CNS, largely residing in the interfaces between the brain and the periphery [4].

The blood-brain barrier (BBB) is a diffusion barrier, which impedes the influx of most compounds from blood to the brain. Three cellular elements of the brain microvasculature compose the BBB—endothelial cells, astrocyte end-feet, and pericytes (PCs) [5]. Endothelial cells at the BBB are tightly packed and have low pinocytotic activity, enabling them to block inflammatory cells and molecules in the peripheral blood from entering the CNS [6].

During the development of MS, the integrity of the BBB is chronically impaired [7], peripheral immune cells are activated and then cross to the CNS *via* the damaged BBB, ultimately leading to immune-mediated tissue injury. Finally, fibrinogen appears perivascularly at the edges of MS lesions and is histopathologically associated with developing demyelination and inflammation, suggesting that blood proteins leak into the CNS and maintain chronic inflammation [8].

Furthermore, at sites of CNS injury and disease, the recruitment of peripheral immune cells modulates tissue-resident glial cells. Peripheral immune cells crosstalk with glial cells by secreting diverse cytokines. They influence each other bidirectionally. On the other hand, neuroinflammation including peripheral immune cells and glia affects

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synaptic transmission at different levels in MS. Glia forms a bridge connecting inflammation and neurodegeneration.

## Immunopathogenesis in MS

### Adaptive Immunity

The role of adaptive immune responses in the pathogenesis of MS has been extensively studied. Peripheral immune cells are first activated outside the brain; T cells are activated by molecular mimicry between foreign peptides and self-peptides presented by dendritic cells (DCs), and then cross to the CNS *via* the damaged BBB, ultimately leading to immune-mediated tissue injury. This is the most accepted hypothesis for the development of MS. However, a study reported that the pathogenesis of some cases of MS begins within the CNS - oligodendroglial and axonal injury are independent of peripheral immune cell infiltration - then continues with peripheral immune activation [9, 10].

In the neuroinflammation context, the main way in which T cells enter the CNS involves direct interactions between the T cells and the endothelium of post-capillary blood vessels. The central lymphatic system associated with the dural venous sinuses allows DCs and T cells to migrate into the deep cervical lymph nodes for antigen presentation. In the same way, B cells shuttle between cervical lymph nodes and the CNS parenchyma as a major functional consequence of chronic inflammation in the CNS [11, 12]. Reflux in the paravascular lymphatic system facilitates the transport of brain antigen proteins into the blood [13, 14].

B cells that can differentiate into plasmablasts and plasma cells are also found in the brain parenchyma of MS patients [15]. B cells and plasma cells found in the brain parenchyma, meninges, and CSF of MS patients produce anti-myelin antibodies that are detectable by the presence of oligoclonal bands [16, 17].

Recent studies have confirmed that the complement system is activated in MS. Functional immunoglobulins secreted by B cells and plasma cells lead to myelin destruction by activating the classical complement system [18]. Recent anti-CD20 antibody treatment has revealed the importance of B cells in the immunopathogenesis of MS [19]. The antibody-independent function of B cells is to attract and activate T cells and myeloid cells for recruitment to the CNS, thereby enhancing cellular immune responses.

### Innate Immunity

The innate immune system in the CNS contains innate immune cells and soluble inflammatory mediators. These innate immune cells mainly include macrophages, microglia, and astrocytes. All these cells are involved in MS immunopathogenesis. The innate immune response begins with pattern recognition receptors on myeloid cells recognizing potential external threats such as pathogen-associated molecular patterns (PAMPs) or internal threats such as damage-associated molecular patterns [9].

Traditional clinical MRI imaging technology has low sensitivity for the detection of this chronic inflammation. Over the past 20 years, clinical quantitative dynamic MRI scanning with contrast agents has increased the sensitivity to inflammatory signatures, including a reduction in BBB integrity associated with inflammation. Quantitative lesion volume measures and magnetic resonance susceptibility imaging are sensitive to the activity of macrophages in the rims of white matter lesions. PET and magnetic resonance spectroscopy methods can also be used to detect activated innate immune systems in the brain and spinal cord. These high-level imaging methods for the visualization of chronic inflammation make a great contribution to clinical applications [2].

## The Correlation Between Inflammation and Neurodegeneration in MS

MS is a chronic inflammatory disease of the CNS, characterized by axon demyelination and neuroaxonal degeneration which becomes more dominant as the disease progresses [1]. Patients are gradually deprived of their memory, motor abilities, and ability to handle everyday tasks [20]. In most MS patients, MS begins with a relapsing-remitting course (RRMS) process, and after 15-25 years, RRMS transforms into progressive neurodegeneration, called secondary progressive MS (SPMS) [21]. At the same time, 10–15% of MS patients directly enter the neurodegenerative stage at the onset of the disease, which is called primary progressive MS (PPMS) [1, 22]. In RRMS and the progressive forms of MS, chronic central inflammation plays a major role in causing neurodegenerative processes [23, 24]. The pathogenesis of RRMS is the transient infiltration into the CNS by peripherally adaptive or innate immune cells, whereas the mechanism of progressive MS is mainly driven by immune cells residing in the CNS or its rim.

The first lesions in the brain of MS patients are usually focal areas of demyelination in the white matter, caused by the invasion of peripheral immune cells through the BBB. Further sustained activation of innate immune cells such as bone marrow-derived macrophages and infiltration of peripheral immune cells, including B cells and $CD8^+$ T cells, eventually lead to myelin degeneration, oligodendrocyte loss, and axonal damage [1, 20, 25–27].

In the healthy CNS, lipid-rich myelin sheaths encase axons and aid in the conduction of electrical impulses. CNS myelin has two functions: it provides metabolic support to

the axon and allows rapid transmission of action potentials along the axon [28–30]. In the healthy CNS, oligodendrocytes are mature, terminally differentiated oligodendrocyte progenitor cells that form myelin sheaths around axons. Myelin sheath formation from mature myelin oligodendrocytes is divided into 3 steps. First, the mature myelin oligodendrocytes express myelin proteins such as myelin basic protein (MBP). Then, myelin proteins build up an elaboration of the myelin membrane to initially wrap the axon. Finally, the continued elaboration of the membrane further wraps the axon to form the multilayered and compacted sheath [31].

In MS, neurodegeneration is dependent on inflammation, which is highly correlated with axonal damage [32]. The autoimmune attack (including the pathological activation of T cells/B cells and macrophages) drives inflammatory demyelination, the myelin sheath is lost, but the underlying axon remains intact [31]. Existing oligodendrocytes whose sheaths have been damaged do not contribute to the regenerative process and are cleared away by activated microglia and astrocytes [33]. The remyelination response generates new sheaths from newly formed oligodendrocytes. When remyelination is hindered, energy-efficient conduction cannot be restored due to the absence of remyelination; this leads to the accumulation of mitochondria at the node and ultimately axonal degeneration [34]. This degeneration triggers a secondary inflammatory response, characterized by the presence of activated macrophages around the degenerating axon.

In MS, the activity of lesions reflects the degree of degeneration and the disease process. Based on the number of inflammatory cells in the autopsy brains of patients with different MS pathogenesis, it has been found that inflammation in the brain not only exists in RRMS but also in progressive MS; peripheral plasma cell infiltration is the most evident in MS. Meningeal B cells form ectopic lymphoid-like aggregates and play a major role in inflammatory demyelination and neurodegeneration of the cerebral cortex [35]. A marker of meningeal B cell aggregation is CXCL13 in serum and CSF [36], which is elevated in patients with PPMS or SPMS [37]. The presence of ectopic leptomeningeal lymphoid tissue in the brains of SPMS patients is associated with extensive cortical demyelination and neurodegeneration [38]. In addition to the complete demyelination of the affected area, the loss of neurons and synapses in adjacent areas can also be substantial [39].

Neuropathologically, white matter lesions in MS are classified according to the characteristics and extent of microglia-macrophage-mediated inflammation and demyelination [26]. Lesions with persistent demyelination or the presence of microglia and macrophages throughout the volume are called active lesions. Lesions with central demyelination or thin myelin sheaths associated with remyelination, and with microglia-macrophage lesions at their borders are classified as mixed active–inactive lesions, and these lesions become dominant as MS progresses [26, 40, 41]. During the pathogenesis of MS, chronic inflammation persists in white matter lesions. Although patients with progressive MS have fewer active lesions in the white matter region than RRMS, mixed active–inactive is seen in the postmortem brains of almost all people with MS [42]. Mixed active–inactive plaques are most abundant in the brains of people whose disease duration is >10 years and who are >50 years of age at death [43].

Inflammation of the CNS can lead to a series of molecular changes in immune cells. In both active lesions and mixed active-inactive lesions, bone marrow-derived macrophages change from a state in which they mainly express the non-inflammatory molecule CD206 [44] to a pro-inflammatory phenotype, with the expression of markers such as CD68, CYBA, major histocompatibility complex class II antigens, inducible nitric oxide synthase, and ferritin [32, 45]. Also, disease-associated microglia reduce the expression of the homeostatic markers and up-regulate the expression of pro-inflammatory markers [46]. During active demyelinating lesions, the myelin breakdown product iron accumulates in macrophages [47].

In addition, several growth factors and immune signaling pathways establish a link between inflammation and neurodegeneration in MS, such as the growth factor TGF-β, the complement cascade, and the extracellular receptor TREM2 [48]. Immune cells secrete neurotoxic products, including reactive oxygen species (ROS), glutamate, cytokines, and chemokines. They further elicit an immune response, altering the cellular metabolism of neurons and their axons. In the short term, these immune cell products are essential for tissue defense and stress responses, but in the long term, they cause intrinsic stress in the CNS, disrupt homeostasis, and ultimately lead to neurodegenerative diseases [49].

Besides the detrimental aspect of neuroinflammation in MS, glial cells play a positive role in remyelination after demyelination [50]. In the area of mixed active-inactive lesions, activated microglia and astrocytes mainly exhibit an anti-inflammatory phenotype and promote remyelination [51]. Activated glial cells drive remyelination by facilitating OPC recruitment and differentiation into oligodendrocytes in three ways. First, they clear the damaged myelin debris *via* CX3CR1, triggering receptor expressed on myeloid cells 2 (TREM2) [52] and colony-stimulating factor 1 receptor (CSF1R) signaling [53], and they clear cholesterol *via* apolipoprotein E, LXRα, ABCA1, and ABCG1 [54]. Second, they secrete pro-regenerative factors, such as activin A [55], insulin-like growth factor 1 (IGF1) [56], galectin 3 [57], tumor necrosis factor (TNF) [58], and IL-1β [59] to recruit OPCs to demyelination zones and support oligodendrocyte lineage cell responses. Third, they facilitate OPC responses to the extracellular matrix during remyelination

*via* the secretion of matrix metalloproteinases (MMPs) and TGM2 [60].

## CNS Glial Cells Connect Inflammation and Neurodegeneration in MS

### Glial Cells at Sites of MS Pathology

Microglia and astrocytes are widely distributed in normal cerebral white matter, and microglia in non-lesion white matter areas of MS patients mainly exert the function of removing damaged neurons and cell debris. Activation of microglia in the white matter of the brain becomes more representative of MS progress [41]. The number and features of microglia in MS lesions can represent the progression of MS disease. Active lesions are characterized by macrophages and microglia residing throughout the area of the lesions, while mixed active–inactive lesions have a hypocellular lesion center, and a small number of macrophages and microglia are limited to the edge of the lesion. In inactive lesions, macrophages and microglia display an almost non-inflammatory phenotype [44]. Astrogliosis is rare in normal white matter and is evident in areas surrounding MS lesions. Microglial activation and astrogliosis in normal white matter can predict the onset of MS lesions [61].

In the acute lesions of SPMS, iron accumulation is evident in activated microglia and macrophages [62]. Intracellular iron accumulation occurs after activated microglia and macrophages engulf myelin debris, dead oligodendrocytes, and leaking red blood cells [63]. The use of high-field MRI has demonstrated that iron rim lesions appear in MS foci with iron accumulation in microglia-enriched areas [64]. These iron rim lesions are more destructive than non-iron rim lesions [65], and iron rim lesions are almost absent around the remyelinated shadow plaques [66], indicating that iron accumulation in glia has negative effects on the recovery of MS. In progressive MS, abnormal iron accumulation in microglia promotes the establishment of a proinflammatory phenotype in microglia [67]. For example, exhaustive transcriptome analysis of microglia in human tissues found that iron accumulation in microglia drives glycolysis in cells, making them more efficient in producing ATP, promoting morphological changes, and secreting cytokines, thereby a pro-inflammatory cell phenotype is established [68].

### Microglia Connect Inflammation and Neurodegeneration in MS

Microglia are ubiquitous resident phagocytes among the innate immune cells in the CNS, where they can self-replenish. Unlike macrophages, microglia originate from the yolk sac [69]. In neural homeostasis, microglia have important functions such as pruning synapses, helping remyelination, removing debris, producing cytokines, tissue surveillance, and supporting other brain cells [70–72].

Microglia are the first responders to neuroinflammation or neural injury, and they rapidly adjust their phenotype in response to the brain environment [73]. Microglia are not just quiescent sentinels ready for neuroinflammation, they are also highly reactive to brain dynamics, actively producing signaling molecules to maintain homeostasis or trigger diseases. Microglia play an important role in all MS lesions and during advanced neurodegeneration. Activated microglia are found in lesions and white matter areas in MS patients. Microglia with a pro-inflammatory phenotype are usually scattered in small clusters in white matter areas. These disease-associated microglia reduce the expression of the homeostatic marker molecule P2ry12 and up-regulate the expression of the pro-inflammatory markers TMEM119 and CD68, antigen presentation MHCII antigen, and ROS markers [46]. The microglia with a pro-inflammatory phenotype also produce ROS, nitrogen [74], pro-inflammatory cytokines [75], and crosstalk with adaptive immune cells [76], activating the complement system and affecting neurotransmitter transmission [77], ultimately causing axonal degeneration [78], oligodendrocyte and myelin damage, and contributing to MS progression (Figs 1 and 2) [46].

Microglia have multiple cellular phenotypes and different transcriptional phenotypes depending on the stage of MS (relapsing/remitting or progressive) and lesion type (active or chronic). In the late stage of MS, microglia swing between homeostatic and pro-inflammatory phenotypes and exert beneficial effects including phagocytosis, brain-derived neurotrophic factor secretion, and remyelination support. Microglia contribute to remyelination by secreting IGF-1 and fibroblast growth factor and inducing the proliferation of OPCs.

Traditional studies have identified different states of microglia according to their morphological phenotypes. The classification of quiescent and reactive microglia is usually distinguished by the number of cell branches. However, the cell morphology of microglia is highly dynamic; further studies of gene transcripts to distinguish different states of microglia through new technologies have the potential to allow a better description of dynamic microglial states [79].

### Astrocytes Connect Inflammation and Neurodegeneration in MS

Astrocytes account for almost 50% of all glial cells in the CNS, with many stellate protrusions per cell. This special cellular structure helps astrocytes to make close contact with synapses, blood vessels, and other glial cells. Astrocytes are central resident cells necessary for synapse formation and function. They directly affect the function of local synapses

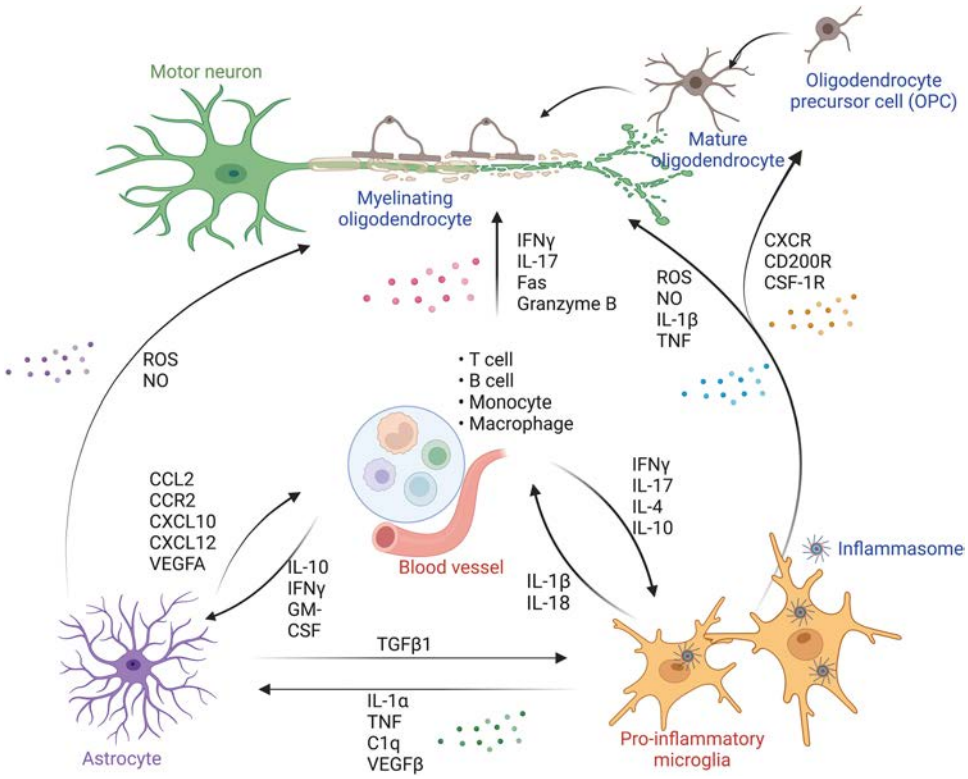


**Fig. 1** Pathophysiological roles of glial cells and peripheral non-neuronal cells in MS. In MS, peripheral non-neuronal cells, including T cells, B cells, and cells of the monocyte-macrophage lineage, are recruited to the CNS in response to neuronal damage. Crosstalk between different cell types by several immune cytokines and chemokines exacerbates this neurotoxicity. Peripheral immune cells promote the activation of resident glia. Glial cells are thought to acquire neurotoxic properties, comprising both gain-of-function toxicity and loss of supportive functions. Glial cells in turn regulate peripheral non-neuronal cells, followed by synaptic loss and demyelination. Chemotactic factors, such as C–C motif chemokine 2 (CCL2). NO, nitric oxide; ROS, reactive oxygen species; TGFβ1, transforming growth factor β1; TNF, tumor necrosis factor.

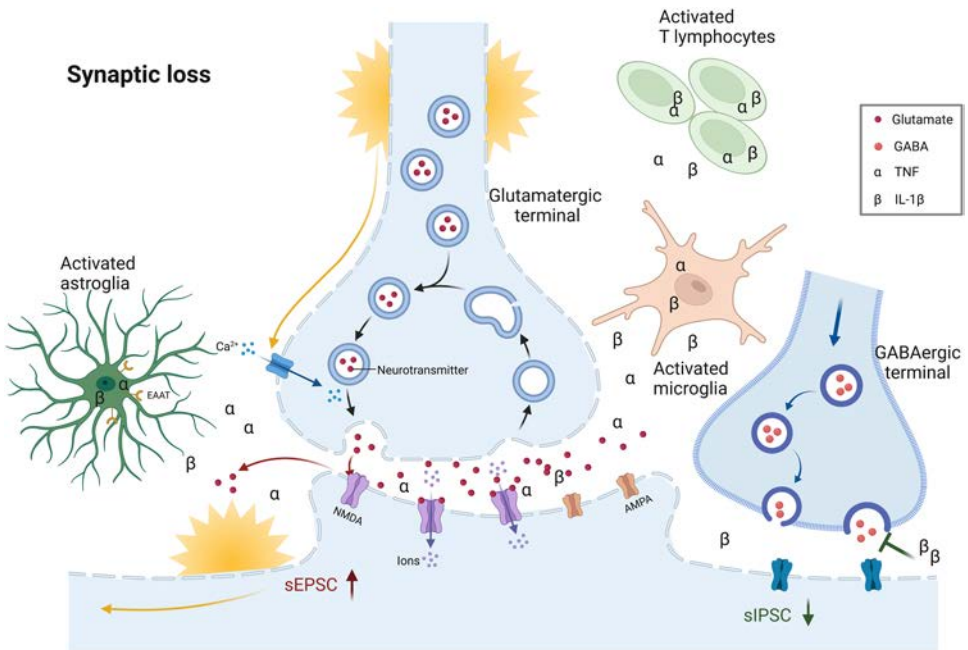


**Fig. 2** Inflammatory synaptopathy in MS and EAE. Proinflammatory cytokines (including TNF and IL-1β) released by activated lymphocytes, microglia, and astrocyte induce glutamate accumulation and GABA reduction in the synaptic cleft in MS and EAE. Furthermore, inflammation can induce structural alterations, comprising synaptic loss characterized by degeneration of the presynaptic and/or postsynaptic site.

through rapid neurotransmitter uptake and extracellular potassium balance (Fig. 2). Astrocytes also support neurons through sophisticated regulation of metabolism, neuronal excitability, and plasticity. Astrocytes are also neurovascular units, an important component of the BBB, maintaining cerebral blood flow. During CNS injury or disease, the BBB, including astrocytes, restricts the entry of peripheral immune cells into the CNS parenchyma. In the context of neuroinflammation, crosstalk between astrocytes and microglia promotes the occurrence of inflammation.

In the nineteenth century, Jean-Marie Charcot identified astrocytes as key components of MS lesions; this was the first evidence of the importance of astrocytes in MS pathology. The subsequent experimental

autoimmune encephalomyelitis (EAE) mouse model further elucidated the role of astrocytes in neuroinflammation. In the acute EAE B6 mouse model, depletion of reactive astrocytes increases the disease severity and CNS inflammation [80]; while in the EAE non-obese diabetic mouse model, depletion of reactive astrocytes ameliorates the pathogenesis of disease and decreases the recruitment of active microglia [81], suggesting the complex and diverse functions of astrocytes. We found that astrocytes with different phenotypes are enriched in MS, such as a subset of neurotoxic reactive astrocytes abundant in MS and characterized by complement component 3 expression. In addition, a subset of astrocytes driven by transcription factor (MAFG) and GM-CSF signaling promotes inflammation and neurodegeneration in EAE and MS. There are also some anti-inflammatory phenotypes of astrocytes, such as a subset of anti-inflammatory $LAMP1^{+}TRAIL^{+}$ astrocytes, which limit EAE pathogenesis by inducing T cell apoptosis [82].

CNS inflammation in MS is typically triggered by BBB dysfunction and peripheral immune cell invasion, followed by glial including astrocyte activation, which respond to pro-inflammatory cytokines and PAMPs. In MS patients, astrocytes in active lesion areas are hypertrophic and strongly express GFAP, secreting pro-inflammatory cytokines, chemokines, and demyelination signaling molecules [83]. Among these, the chemokines CCL2, CXCL10, and CXCL12 produced by reactive astrocytes attract leukocytes including T cells, B cells, and plasma cells to the perivascular space and CNS parenchyma, further activate the complement system, and cause axonal damage and demyelination. Conversely, astrocyte responses are regulated by T cells, and IL-10 produced by regulatory T cells limits astrocyte activation and reduces neuroinflammation [84]. GM-CSF signaling also limits astrocyte anti-inflammatory functions by downregulating TRAIL expression [85] (Fig. 1). In MS lesions, astrocytes can also phagocytose myelin debris, followed by intracellular iron accumulation, but their phagocytic capacity is not as good as that of microglia and macrophages [86, 87]. In the chronic inflammation of progressive MS, iron accumulation disrupts astrocyte antioxidant pathways, resulting in its damage [88].

In the healthy CNS, astrocytes contribute to the maintenance of neuronal function through a variety of mechanisms involving ion channels, neurotransmitters, and metabolites [89]. Astrocytes trap glutamate and prevent it from accumulating in the synaptic cleft, thereby protecting the synapse from glutamate toxicity. Furthermore, astrocytes act as modulators of synaptic inhibition. In MS, astrocytes influence the glutamine neurotransmission across the synaptic cleft by increasing the release and/or decreasing the reuptake of glutamate into astrocytes, causing excessive stimulation of glutamate receptors (GluRs) and, consequently, excitotoxic damage of neurons and oligodendrocytes [90].

Crosstalk between CNS glia is a regulatory mechanism in MS pathogenesis. Recently described microglia–astrocyte interactions mediated by the semaphorin4D–plexinB1/2 and ephrinB3–EPHB3 axis, promote CNS inflammation [82]. Neurotoxic astrocytes similar to those induced by microglia in the EAE mouse model have been observed in the postmortem brain tissue of MS patients [91]. In EAE mice, microglia-derived TGFα reduces disease severity by directly regulating astrocytes, whereas microglia-derived VEGFβ aggravates disease by regulating astrocytes [92].

## Oligodendrocytes Connect Inflammation and Neurodegeneration in MS

Myelinating oligodendrocytes are derived from OPCs in the early stages of neural development. During embryogenesis, neural stem cells generate highly proliferative OPCs. The differentiation of OPCs into mature and myelinating cells is a multistep process [93], tightly controlled by spatiotemporal activation and repression of specific growth and transcription factors [94, 95]. Mature oligodendrocytes spread throughout the gray and white matter of the CNS, wrap around neuronal axons, and are responsible for the production of myelin, maximizing axonal conduction velocity [96]. Myelin is an extension of the oligodendrocyte cell membrane with a highly complex structure.

An undifferentiated pool of OPCs is maintained in the adult CNS and contributes to the production of myelinating oligodendrocytes throughout adulthood. Following nerve injury or inflammation, axonal damage requires newly generated and differentiated mature oligodendrocytes to accelerate recovery.

Components of the myelin sheath [MBP-, myelin oligodendrocyte glycoprotein, or proteolipid protein] are the main targets of autoimmune attack, mainly including the antigen-specific $CD4^{+}$ T cells in the CNS [97].

Also, inflammation plays an important role in OPC/oligodendrocyte survival, proliferation, differentiation, and remyelination. The blockade of OPC differentiation contributes to failed remyelination in MS owing to the inflammatory microenvironment and the presence of an array of inhibitory molecules like Lingo-1 and PSA-NCAM [98]. The inflammatory microenvironment is associated with the presence of ROS, which may in turn affect the fate of OPCs [99]. The inflammatory microenvironment is also associated with the lack of certain factors such as IGF1, TGF-β1, GGF2, or integrins, contributing to limited remyelination [58, 59, 100]. Moreover, in experimental inflammatory demyelination, OPC differentiation is inhibited by effector T cells and IFNγ. This study found that induction of immunoproteasomes in OPCs occurs in human demyelinated MS brain lesions [101]. The crucial immunoproteasome subunit PSMB8 (also known as LMP7) increases MHC class

I expression on OPCs, rendering OPCs a more prominent target for the cytotoxic $CD8^+$ T cells that are abundant in MS lesions [102].

In addition, recent evidence suggests that oligodendrocytes also have immune-related functions. Falcão *et al.* reported that disease-specific oligodendroglia are also present in human MS brains. OPCs express MHC-II in response to IFNγ, exhibit phagocytic capacity, and regulate T cell survival and proliferation, activating memory and effector CD4-positive T cells. They claimed that oligodendrocytes and OPCs are not only passive targets but instead active immunomodulators in MS [103]. Niu *et al.* reported that interaction between oligodendroglia and the vasculature in MS disrupts the BBB, triggering CNS inflammation [104]. Also, OLCs regulate neuronal network activity through direct connections to the glial syncytium [105].

## Crosstalk Between Peripheral Immune Cells and Glia

There is little peripheral lymphocyte infiltration in the normal brain, but lymphocytes are recruited to the CNS as part of a neuroinflammatory response. The migration of B cells into the CNS is regulated by $T_{FH}$ cells, based on studies in EAE and evidence in the CSF of patients with MS [106]. B cells with a neurotoxic phenotype have been observed in patients with MS and shown to secrete soluble factors that directly kill oligodendrocytes and neurons *in vitro* [97, 107].

The progression of MS is mainly the result of interactions between peripheral immune cells and glial cells in the CNS, especially microglia. At sites of CNS injury and disease, the recruitment of peripheral immune cells modulates tissue-resident glial cells [108]. IFNγ and IL-4 are known T cell-derived cytokines driving the pro-inflammatory and anti-inflammatory phenotypes of microglia in culture, respectively [109], suggesting T cells directly modulate resident microglia during injury and disease. Microglia can also present antigens to lymphocytes by expressing MHC class II molecules; their role as antigen-presenting cells is seemingly dispensable for the induction of EAE [110].

T cells, similar to microglia, produce multiple molecules, eventually altering the activation state of astrocytes. Activated astrocytes in turn recruit peripheral immune cells into the injured BBB by secreting CCR2, activating inflammasomes, and further causing axonal damage. In addition, activated astrocytes produce fewer neurotrophic factors, thereby failing to further support neuronal integrity, eventually accumulating neurodegeneration [111].

After CNS injury, microglia transform into an inflammatory state and further recruit mononuclear macrophages into the CNS; the macrophages in turn regulate microglial function and state by promoting microglia to express a range of inflammatory mediators *in vitro* [112]. Peripheral monocyte-derived macrophages stimulated by interferon-γ facilitate microglia to induce the expression of TNF and inducible nitric oxide synthase. Microglia and macrophages interact to enhance each other's phagocytic capability, which is detrimental to neuronal survival, eventually causing CNS damage and disease [113]. Monocyte-macrophages can regulate not only the activity and function of microglia but also the function of astrocytes. Soluble factors secreted by inflammatory macrophages enhance the release of CCL2 and MMP9 by astrocytes, which further recruit peripheral mononuclear macrophages into the CNS. The crosstalk between astrocytes and macrophages further accelerates CNS inflammation and damage [114].

Neutrophil clearance is one of the key events in the resolution of neuroinflammation [115]. The clearance of neutrophils is mediated by specific factors derived from macrophages and glia, which ultimately resolve inflammation and promote recovery after CNS injury [116]. Activated microglia and peripheral infiltrating neutrophils are in contact with each other during CNS inflammation, as shown by microscopy *in vivo*. Microglia accelerate the clearance of neutrophils by secreting specific inflammatory factors. The clearance of neutrophils by macrophages and microglia in turn affects the function and phenotype of phagocytes. Also, complement components released by neutrophils promote the differentiation, maturation, and migration of astrocytes from neural stem cells *in vitro* [117]. In addition, neutrophils produce a series of cytokines and proteolytic enzymes affecting microglia, astrocytes, and oligodendrocytes [118].

The inflammasome, for example, as a mediator connecting glial and immune cells, is an intracellular multiprotein complex that mediates innate immune responses against PAMPs. NLRP3, mainly localized to microglia, was the first inflammasome to be studied in the brain. Activation of the NLRP3 inflammasome has also been found in other CNS cells, such as astrocytes, neurons, endothelial cells, and pericytes. Also, immune cells (T cells and myeloid cells) express components of the NLRP3 inflammasome complex in MS. One of the hallmarks of NLRP3 inflammasome activation is the production and secretion of the proinflammatory cytokines IL-1β and IL-18 [119]. Activation of the NLRP3 inflammasome in glial cells amplifies neuroinflammation and promotes EAE progression. Similar to the EAE mouse model, the IL-1β mRNA level is increased in the peripheral blood mononuclear cells of MS patients. The mRNA and protein levels of NLRP3 inflammasome components and pro-inflammatory cytokines are upregulated in MS patients. IFN therapy effectively inhibits the level of IL-1β in serum and further improves the clinical manifestations of MS by reducing the expression of inflammasome and the secretion of IL-1β [120].

## Glia Connect Inflammation and Neurotransmitters

Brain-infiltrating autoreactive T cells and glial cells in the CNS are significantly activated during MS, and these activated immune cells broadly modulate synaptic transmission. Infiltrating T cells and glial cells are important sensors of the local microenvironment of the CNS and secrete many cytokines, growth factors, or neurotransmitters. Activated microglia affect synaptic structure and function by shedding microvesicles [121] or by stripping synapses after inflammatory injury [122]. Therefore, these glial cells connect inflammation and neurotransmitters (Fig. 2).

Recent studies have found that incubation of $CD3^+$ T cells isolated from the spleen of EAE mice with brain slices from normal mice alters normal brain glutamatergic and GABAergic synaptic transmission [123, 124], indicating that immune cells can change the conduction of neurotransmitters and affect the function of synapses. Similarly, co-incubation of activated microglia from EAE mouse brains with brain slices from normal mice results in synaptic defects similar to those in EAE mice [123]. Interestingly, the effects of $CD3^+$ T cells and activated microglia on synaptic function are completely reversed by specific cytokine antagonists, suggesting that cytokines secreted by immune cells contribute to synaptic defects [123].

Pro-inflammatory and anti-inflammatory cytokines secreted by glial cells play an important role in regulating bidirectional communication between glial and neurons, as well as in regulating synaptic transmission [125]. Therefore, in the pathological state of MS, synaptic transmission and neuronal survival depend on intrasynaptic cytokine concentrations, the balance between pro-inflammatory and anti-inflammatory cytokines, and the expression of neuronal surface receptors [126].

Neuroinflammation affects synaptic transmission at different levels. On the one hand, inflammation alters the frequency of presynaptic neurotransmitter release, and the amplitude and duration of the postsynaptic current [124]. On the other hand, neuroinflammation affects spontaneous excitatory and inhibitory postsynaptic currents (sEPSCs and sIPSCs) of neurons, ultimately altering synaptic excitability and disrupting neuronal function [125]. In MS, the effects of neuroinflammation on synaptic function are commonly studied using two approaches: (1) correlation of cortical excitability and plasticity—as measured by transcranial magnetic stimulation—with levels of cytokines in the CSF [127]; and (2) isolation of CSF from MS patients and co-incubation with healthy mouse brain slices to explore the effects of specific cytokines in the CSF of MS patients on synaptic transmission [128].

## Dysregulation of Neurotransmitters in MS

The concentrations of glutamate, GABA, and other neurotransmitters at the synaptic cleft depend on the regulation of their synthesis, release, degradation, and reuptake. In MS, these regulatory processes are disturbed, and the receptors involved in neurotransmission are abnormally expressed, ultimately leading to disturbances in neurotransmitter transmission [90].

### The Glutamatergic System in MS

There is increasing evidence that free glutamate in the synaptic cleft is increased in MS, and abnormally high levels of glutamate have been detected in the CSF, white matter, and gray matter of MS patients [129]. Increased glutamate concentration is involved in many processes depending on the interaction between immune cells and nervous systems. Neurons, T cells, macrophages, astroglia, and activated microglia are all able to synthesize and release glutamate [130], guaranteeing a continuous local supply of glutamate neurotransmitters. The abnormal accumulation of glutamate in the synaptic cleft is due to increased glutamate release in MS. At the same time, in oligodendrocytes from MS patients, the expression of the metabolizing enzyme glutamate dehydrogenase is down-regulated, while the expression of the glutaminase enzyme is increased [131], the uptake of glutamate by astrocytes is insufficient, and blockade of the uptake of glutamate in the synaptic cleft also leads to disruption of glutamate homeostasis. Abnormal accumulation of glutamate in the synaptic cleft leads to overstimulation of GluRs, which eventually leads to excitotoxic damage to neurons and oligodendrocytes. In MS, glutamate excitotoxicity is an important bridge linking neuroinflammation and neurodegeneration [132, 133].

Glial cells in the nervous system play an important role in the disturbance of the glutamate neurotransmitter system in MS. In MS, activated microglia inhibit the expression of neuronal cell surface glutamate transporters (GluTs: EAAT1, EAAT2, and EAAT3) involved in glutamate uptake [131, 132, 134]. Microglia also secrete chemokines to recruit peripheral immune cells to infiltrate and enhance glutamatergic transmission, including monocytes, macrophages, and lymphocytes. Activated microglia promote TNF-α release and further trigger the excitation of post-synaptic currents [135].

In an *ex vivo* MS-CSF incubation study on striatal slices, TNF (expressed at higher levels in the CSF of patients with progressive MS than in patients with RRMS) promoted glutamatergic transmission by increasing the duration of sEPSCs, leading to neuronal swelling [136]. This suggests that TNF acts as a major neurotoxic molecule during MS progression (Fig. 2). In MS, increased pro-inflammatory

cytokines and free glutamate in the synaptic cleft upregulate neuronal GluR expression and exacerbate synaptic dysfunction, thereby enhancing local glutamate excitotoxicity [137]. This is the main mechanism of neurodegeneration caused by glutamate excitotoxicity in MS, so drugs that modulate GluR expression and function may have a protective effect on neuronal excitotoxicity. GluR antagonists have been shown to ameliorate motor deficits and neuropathology in the EAE model [138]. In addition, drugs that inhibit the release of glutamate from nerve cells and immune cells have been reported to have beneficial effects in EAE [139], and inhibition of glutamate release from immune cells has also shown promising therapeutic prospects in MS patients [129]. Therefore, modulating extracellular glutamate levels appears to be one of the promising therapeutic strategies for preventing neurodegeneration in MS.

### The GABAergic System in MS

In contrast to the abnormal accumulation of glutamate neurotransmitters in the synaptic cleft in MS patients, lower levels of GABA have been detected in their CSF and blood [140]. CSF from MS patients with acute brain injury impairs GABAergic transmission in normal mouse striatal slices and the growth factor IL-1β may be responsible for this effect. Exogenous IL-1β mimics the effect of MS CSF on synapses and the phenotype is blocked by incubation with IL-1R antibody. More recent transcriptomic and proteomics analyses have shown that molecular proteins regulating GABA concentrations in the synaptic cleft are abnormally expressed in MS patients [141, 142]. The GABA concentration in the synaptic cleft is known to be regulated by both NMDA and $GABA_A$ receptors. *Ex vivo* experiments have demonstrated that the reduced $GABA_A$-mediated neuronal inhibition depends on IL-1β (Fig. 2).

In addition to its neurotransmitter function, GABA is an important immunomodulator that affects a variety of immune cell functions, including cytokine release, cell proliferation, and the phagocytic activity of immune cells [143, 144]. The role of GABA in regulating immune system function enriches our understanding of its role as a neuroprotective and anti-excitotoxic molecule.

## Conclusions and Perspectives

During the pathogenesis of MS, the roles of adaptive and innate immune cells depend on the disease process and the spatial region of the brain. We need to better understand the immune cell populations and pathways that regulate glial interactions with neurons, oligodendrocytes, and myelin by using advanced technologies, such as massive single-cell sequencing datasets of glia in health and disease, cerebral organoids, clustered regularly interspaced short palindromic repeats (CRISPR) screening, transgenic knockout of specific cells in the mouse model, high content imaging, and constantly improving sequencing technologies. The diverse and dynamic properties of the microglia, astrocytes and other glial cells present in disease pathology are potential therapeutic targets. The ability to treat MS in the future could rely on a strategy that combines immune intervention with early neuroprotective, remyelinating drugs.

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REVIEW

# Association of Glial Activation and α-Synuclein Pathology in Parkinson's Disease

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**Abstract** The accumulation of pathological α-synuclein (α-syn) in the central nervous system and the progressive loss of dopaminergic neurons in the substantia nigra pars compacta are the neuropathological features of Parkinson's disease (PD). Recently, the findings of prion-like transmission of α-syn pathology have expanded our understanding of the region-specific distribution of α-syn in PD patients. Accumulating evidence suggests that α-syn aggregates are released from neurons and endocytosed by glial cells, which contributes to the clearance of α-syn. However, the activation of glial cells by α-syn species produces pro-inflammatory factors that decrease the uptake of α-syn aggregates by glial cells and promote the transmission of α-syn between neurons, which promotes the spread of α-syn pathology. In this article, we provide an overview of current knowledge on the role of glia and α-syn pathology in PD pathogenesis, highlighting the relationships between glial responses and the spread of α-syn pathology.

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**Keywords** Parkinson's disease · α-synuclein pathology · Microglial activation · Astrocyte activation · Neuroinflammation

## Introduction

Parkinson's disease (PD) is the second most common age-related neurodegenerative disease following Alzheimer's disease (AD), characterized by motor symptoms of bradykinesia, rigidity, resting tremor, and postural instability as well as non-motor symptoms of constipation, depression, sleep disorders, and cognitive decline [1]. The onset of disease generally occurs in individuals >60 years old, and it is estimated that ~10 million people worldwide have PD [2–4]. Currently, there are no effective therapies to block the progression of PD [5]. Although 5%–10% of PD cases are familial, the majority of cases are sporadic [3, 6]. The hallmarks of pathological changes in PD are the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the deposition of α-synuclein (α-syn) in Lewy bodies (LBs) and neurites (LNs) that are widely distributed in the brains of PD cases [6–9]. The LBs are composed of phosphorylated α-syn (S129), ubiquitinated proteins, and other damaged organelle components [10–12]. Pathological α-syn can recruit and convert unfolded α-syn to form pathological amyloid fibrils in neurons. The fibrils undergo fragmentation to form small fragments and oligomers that are secreted from the neuron. The oligomers then enter the next neuron through receptor-mediated endocytosis, leading to the spread of pathological α-syn throughout the brain in a prion-like manner [13–15]. In diseased PD brains, glial activation and the accumulation of α-syn in neurons are often accompanied by LB and LN pathology and neurodegeneration [16–19]. Although it is accepted that glial activation is

a response to neuronal damage by misfolded α-syn that is toxic to neurons, recent studies suggest that the reaction of glia may be more than just a passive response; it may contribute to the spread of α-syn pathology and the development of PD-related pathology [19, 20].

In this review, we discuss the association of glial activation and α-syn pathology in PD. We summarize the studies on the activation of glial cells by α-syn species to discuss the response of glial cells to α-syn aggregates. We also described the roles of glial cells in the clearance and transmission of α-syn aggregates that contribute to the spread of α-syn pathology.

## α-Syn Pathology

### α-Syn Aggregation and Toxicity

The α-syn protein is encoded by the *SNCA* gene, and has an average molecular weight of ~14 kDa [21]. α-Syn protein is mainly located at the presynaptic terminal in the central nervous system (CNS) and is involved in the release of synaptic vesicles by promoting soluble NSF attachment protein receptor (SNARE)-complex assembly [22, 23]. α-Syn natively exists as soluble compact monomers or α-helically folded tetramers to avoid aggregation [13, 14]. However, under some pathological conditions, α-syn monomers may be misfolded to form soluble pathological oligomers, or insoluble β-sheet-rich fibrils [24–26].

α-Syn oligomers are more toxic than other assemblies (monomers, fibrils, or aggregates) [27]. The point mutation of α-syn with E35K, E57K, or E46K is prone to oligomerization and leads to neuronal damage [27–29]. In lentivirus-infected brains, both wild-type (WT) α-syn and the E57K mutant form SDS-insoluble α-syn oligomers that are detected in the membrane fractions, suggesting that α-syn oligomers interact with lipid membranes. However, more oligomers are formed by α-syn E57K than WT α-syn. α-Syn E57K prefers to form oligomers, but WT α-syn rapidly forms fibrils from oligomers. Moreover, more DA neuronal loss has been reported in mice infected with E57K mutant α-syn than with WT α-syn, further suggesting toxic effects of α-syn oligomers on DA neurons [27]. In induced pluripotent stem cell (iPSC)-derived neurons, overexpression of α-syn with the familial mutation E46K or an artificially-induced E57K mutation results in more α-syn oligomers than those expressing WT α-syn. Moreover, α-syn E46K or E57K mutation leads to abnormalities in axonal and synaptic integrity due to the abnormal distribution of motor factors for the anterograde axonal transport of mitochondria [29]. In addition, transgenic mice that harbor the E57K mutation show evident synaptic abnormalities and neuronal loss, with learning and memory defects [28], suggesting that oligomeric α-syn plays crucial roles in α-syn-induced PD pathology.

The cell membranes and mitochondria are the most common targets of exogenous α-syn oligomers in various types of neuron [30–35]. α-Syn oligomers have a high affinity for the lipid bilayer of the cell and organelle membranes, resulting in a disruption of membrane integrity [32–34]. The high affinity of the oligomers for cell membranes also determines their ability to enter neurons, which may be superior to other forms of α-syn [34]. After entering neurons, α-syn oligomers target and accumulate in the outer or inner membrane of mitochondria [33, 36, 37]. It has been reported that α-syn oligomers interact with the translocase of the outer mitochondrial membrane 20 (TOM20), resulting in an inhibition of mitochondrial protein import [36, 38]. Moreover, α-syn oligomers impair the respiratory chain complexes to decrease ATP production and increase oxidative stress when they are translocated to the mitochondrial inner membrane [37]. Interestingly, neutralizing α-syn oligomers with the A11 antibody, which is a polyclonal antibody against oligomers, blocks α-syn oligomers from entering cells and attenuates oligomer toxicity [34], further suggesting a role for α-syn oligomers in neuronal toxicity. Thus, the conformational changes of α-syn under pathological conditions induce α-syn misfolding and aggregation, leading to the formation of α-syn oligomers and fibrils [34]. Blocking oligomer entry into cells might be a promising strategy to inhibit the toxicity of α-syn aggregation (Fig. 1).

### Pathological α-syn Transmission

The hypothesis of α-syn transmission was first proposed by Braak and colleagues [39]. Their study showed that the distribution of α-syn pathology in the brain is correlated with the severity of PD symptoms. At the early stage of PD, α-syn pathology often appears in the olfactory nucleus and olfactory bulb, the lower brain stem, and the dorsal IX/X motor nuclei [39, 40]. With the progress of disease, α-syn pathology is observed in the midbrain at the developing stage and in the neocortex at the last stage. Based on these findings, it has been speculated that α-syn pathology is initiated in the lower brain stem, spreads through neural interconnections, and eventually reaches the neocortex [39].

More convincing evidence that suggests α-syn transmission between cells came from clinical findings in 2008 [41, 42]. α-Syn- and ubiquitin-positive LBs and LNs have been found in transplanted DA neurons in the grafts from PD patients who died within 11–16 years after bilateral transplantation with human fetal mesencephalic tissue, suggesting that the α-syn pathology in grafted DA neurons comes from the propagation of pathological α-syn from surrounding host neurons [41, 42].

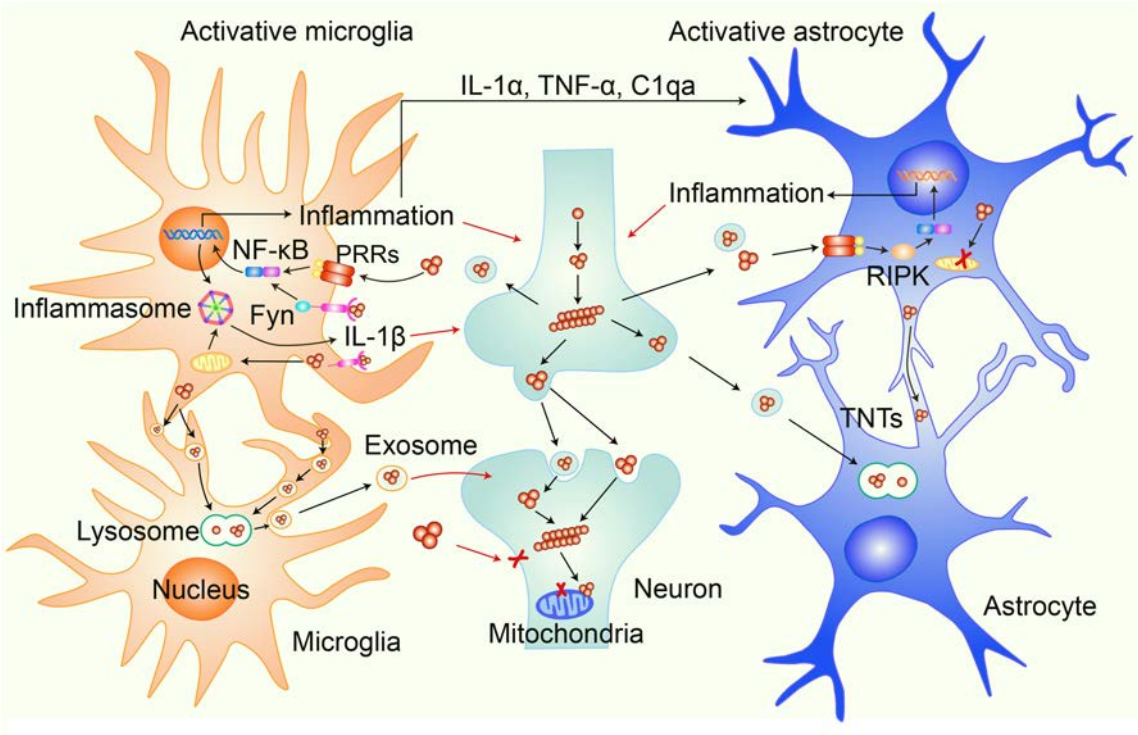


**Fig. 1** Schematic of the impact of α-syn aggregates on the activation of microglia and astrocytes and their contributions to α-syn pathology. α-Syn aggregates propagate between neurons and are released from them. Extracellular α-syn aggregates activate microglia by initiating the NF-κB-dependent inflammatory response and inflammasome activation *via* pattern recognition receptors (PPRs) or Fyn. Extracellular α-syn aggregates induce the NF-κB-dependent inflammatory response in astrocytes, and this is associated with the activation of RIPK signaling. Meanwhile, microglia phagocytose extracellular α-syn aggregates and transport them to lysosomes for degradation. Overloaded α-syn in microglia or astrocytes can be transmitted between microglia through cellular networks or between astrocytes through tunneling nanotubes (TNTs). In addition, microglia and astrocytes activated by α-syn aggregates produce pro-inflammatory factors, which damage neurons and promote the transmission of α-syn pathology between neurons.

In recent years, various *in vitro* and *in vivo* models of α-syn transmission have been established to confirm the α-syn-spreading hypothesis based on findings from PD patients [43–47]. It is well accepted that the transmission of α-syn is due to the prion-like properties of its misfolded form, similar to other pathogenic proteins in neurodegenerative diseases [25, 26]. Prions are composed of misfolded prion proteins (PrPs) that can infect the host and force the native normal structure of PrPs to form an anomalous misfolded structure and aggregate in host cells. Once the aggregates reach a certain extent, they undergo fragmentation to form new templates (seeds), thus initiating a chain-reaction of PrP misfolding and aggregation [25]. Without misfolded α-syn, the transfer of WT α-syn between cells does not induce α-syn pathology. However, like prions, misfolded α-syn is prone to aggregation, which induces the structural change of normal α-syn monomers that are recruited to α-syn aggregates and form pathological forms, ranging from small oligomers to large β-sheet-rich fibrils [25, 48]. Under certain conditions, these fibrils undergo fragmentation to generate new small aggregates called seeds to propagate the pathogenic form of α-syn, which results in the spread of α-syn pathology from cell-to-cell and brain region-to-brain regions when α-syn seeds are released from one cell and taken up by another [25, 26].

α-Syn monomers can be induced to form misfolded α-syn and then β-sheet-rich fibrils *in vitro* [49]. Smaller pre-formed fibrils (PFFs) are obtained by ultrasonic fragmentation of fibrils [49]. The introduction of PFFs into human cell lines by liposomes induces overexpressed WT α-syn to form amyloid-like fibrils that are highly ubiquitinated and phosphorylated, sharing properties with those in PD patients [43, 50]. In mouse primary neurons, PFFs induce endogenous α-syn to form LB/LN-like structures in a time-dependent manner, resulting in synaptic dysfunction and neuronal death. In addition, the severity of the defects in neuronal network activity matches the development of α-syn pathology, similar to the progression of α-syn pathology in PD patients [44]. In animals, a single brain site injection with recombinant PFFs or brain homogenates containing pathological α-syn assemblies into A53T transgenic mice that overexpress the human *SNCA* gene with the familial A53T mutation induces

the development of α-syn pathology in extensive areas of the brain. This confirmed the α-syn transmission hypothesis for the first time *in vivo* [46]. In addition, intrastriatal inoculation with recombinant PFFs in WT mice also induces phospho-α-syn-positive LB/LN aggregates from endogenous α-syn, showing the distribution of α-syn pathology along with interneuronal connectivity in the brain. These mice also show time-dependent α-syn pathology, neuronal loss, and motor defects similar to sporadic PD-like pathology [51]. Moreover, fewer motor defects and α-syn pathology have been reported in *Snca*+/– heterozygous mice, further suggesting the involvement of WT α-syn in α-syn pathology after PFF treatment [51]. Therefore, the PFF mouse model has been widely used in PD studies. Most interestingly, in addition to the transmission of α-syn pathology in the CNS, peripheral PFF inoculation also induces α-syn pathology in the CNS [52, 53]. Inoculation of PFFs into the gastroduodenal tract in WT mice induces α-syn pathology in multiple brain regions, including the dorsal motor nucleus, brainstem, midbrain, and cortex [53]. The mice present olfactory dysfunction, motor deficits, and cognitive decline, in which the phenotypes are similar to the clinical symptoms of PD patients, indicating that the abnormal aggregation of α-syn in the gastroduodenal tract can initiate the transmission of α-syn pathology from the gut to the CNS and affect neuronal functions in multiple brain regions [53]. In addition, α-syn pathology is present in multiple brain regions, starting from the dorsal motor nucleus of the vagus in the medulla and locus coeruleus in the pons 1 month after the injection of PFFs into the muscle layers of the pylorus and duodenum, and then spreads to the amygdala, SNpc, striatum, hippocampus, and cortex [53]. Loss of DA neurons and defects in motor behavior have also been reported in mice that receive a PFF injection into the pylorus and duodenum. Importantly, truncal vagotomy or knockout of the *Snca* gene blocks the transmission of α-syn pathology from the gut to the CNS and prevents DA neuronal loss and motor deficits, showing that the vagus nerve is necessary for the transmission of α-syn pathology from the peripheral nervous system (PNS) to the CNS and that WT α-syn progresses into pathogenic α-syn, contributing to the spread of α-syn pathology [53] (Fig. 1).

Both *in vitro* and *in vivo* data support the Braak hypothesis that α-syn is transmitted from cell to cell in a prion-like manner. The presence of LB/LN pathology in multiple brain regions in PD patients may be a result of α-syn transmission in a time- and region-dependent manner. The toxicity of pathological α-syn, with a wide distribution and spread of toxic α-syn species to multiple regions in the CNS and PNS, may cause dysfunction of neurons, which is responsible for the motor and non-motor symptoms in PD patients.

## Microglia and α-Syn Pathology

Microglia are resident myeloid macrophages in the CNS that play important roles in maintaining neuronal homeostasis by the pruning of synapses, elimination of protein aggregates and dead cells, maintenance of synaptic plasticity, and immune monitoring in CNS development and diseases [54, 55]. Microglia express a large number of pattern-recognition receptors, such as toll-like receptors (TLRs), to recognize damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), which play roles in the immune response [55]. Activated microglia can internalize pathogenic species and degrade them through various endocytic pathways. Activated microglia also increase the expression of relevant inflammatory modules, including chemokines and interferons, which are major components in neuroinflammation [55, 56]. The increased levels of inflammatory factors in PD patients and animal models indicate that microglial activation plays a key role in PD pathogenesis [57–59]. Inhibition of microglial activation by MCC950, a small molecule that inhibits inflammasomes by blocking the activation of NLR family pyrin domain containing 3 (NLRP3), or by NLY01, a glucagon-like peptide-1 receptor agonist that inhibits microglial activation, decreases PFF-induced α-syn pathology and DA neuronal loss as well as motor behavior deficits in a PFF mouse model in which a single striatal injection of synthetic α-syn fibrils drives the transmission of pathological misfolded α-syn in WT mice [60, 61], which suggests a link between microglial activation and α-syn pathology in PD.

### Induction of Microglia-associated Neuroinflammation by Different α-Syn Species

A growing body of evidence indicates that various receptors on the surface of microglia are involved in binding to different α-syn species to mediate neuroinflammation, causing augmentation of neurotoxicity by the release of different inflammatory cytokines that affect neurons in different cell or animal models [62–66] (Table 1). Kim and colleagues first reported that α-syn oligomers secreted by neuronal cells are endogenous TLR2 receptor agonists for microglial activation [62]. After treatment with conditioned medium from differentiated SH-SY5Y cells in which human WT α-syn is overexpressed, primary microglia can be activated, as evidenced by morphological change to an amoeboid shapes, the production of pro-inflammatory cytokines, and an increase in proliferation [62]. Transcriptome analyses suggest the involvement of the TLR and Jak-STAT signaling pathways in α-syn oligomer-induced inflammatory activation. Blocking TLR2 signaling by knocking out the *Tlr2* gene in microglia or applying anti-TLR2 antibodies inhibits inflammatory activation. The conditioned medium from SH-SY5Y cells

**Table 1** Different receptors respond to diverse α-syn forms to initiate microglial activation

| Receptor | Signaling | Syn forms | References |
|---|---|---|---|
| TLR1/2 | TLR1/2-MyD88-NF-κB & inflammasome priming | Monomers, oligomers & fibrils | [62, 64, 65, 95, 109] |
| TLR-4 | TLR1/2-MyD88-NF-κB | Monomers, oligomers & fibrils | [63, 110] |
| TLR5 | Inflammasome activation | Monomers & oligomers | [64] |
| CD36 | CD36-Fyn-PKCδ-NF-κB & inflammasome activation | Monomers & fibrils | [65, 111] |
| FcγR | FcγR-NF-κB | Fibrils | [112] |

that overexpress α-syn mainly contains β-sheet-rich oligomers of α-syn but also monomers. Both α-syn monomers and oligomers derived from SH-SY5Y cells induce microglial activation, but the effects of monomers are weak. Injection of these oligomers into the cerebral cortex induces inflammatory activation in WT mice but not in *Tlr2*-knockout mice [62]. Thus, the data suggest that TLR2 is an effective agonist responsible for α-syn-induced microglial activation.

In addition to TLR2, TLR4 can also recognize oligomeric α-syn and initiate the TLR4-MyD88 signaling pathway, thereby activating transcription and releasing inflammatory factors. Moreover, in comparison to TLR2, TLR4 has better selectivity for the α-syn oligomer and mediates a stronger inflammatory response. The use of the TLR4 inhibitors RSLA and TAK242 or the molecular chaperone clusterin that binds to oligomeric α-syn blocks the microglial activation induced by α-syn oligomers in BV2 microglial cells [63]. In addition to TLR2, α-syn monomers and oligomers also bind to TLR5 to promote inflammasome assembly and activate the inflammatory response [64]. The different α-syn species bind to various TLRs and have differential activities in NLRP3 inflammasome activation in lipopolysaccharide (LPS)-primed primary microglia [64].

In addition to the classical TLR pathway, Fyn, a non-receptor Src family tyrosine kinase, mediates aggregated α-syn PFF uptake and NLRP3 inflammasome activation [65]. The aggregated α-syn PFF produced by *in vitro* incubation of human recombinant α-syn activates CD36, which recruits and activates Fyn, further inducing a PKCδ-dependent NF-κB signaling pathway. Infection with AAV-α-syn induces microglial activation in the SNpc in WT mice but not in *Fyn*-knockout mice [65]. The activation of microglia by aggregated α-syn also increases the toxic effects of α-syn on DA neurons [67]. In a mouse model, overexpression of human α-syn by the injection of AAV-α-syn into the SN induces the activation of NF-κB two weeks after injection in WT mice but not in mice in which the family of Fc gamma receptors (FcγRs) is deficient [68]. In addition, the activation of microglia and the degeneration of DA neurons induced by overexpression of α-syn are attenuated in FcγR–/– mice. As FcγR is expressed on the surface of microglia but not neurons in the CNS, the attenuation of DA neuronal loss suggests that the activation of microglia contributes to α-syn-induced neurodegeneration [68].

Pattern recognition receptors (PPRs) on microglial membranes, including TLR2, TLR4, and TLR5, as well as non-receptor-dependent kinases play a crucial role in α-syn-induced inflammatory activation. PPRs, along with inflammasomes, sense PAMPs and DAMPs upon neuronal damage. In PD brains, PPRs are strongly expressed and closely associated with microglial activation [62–66]. Recently, inflammasomes have received great attention as contributors to α-syn-induced neuroinflammation [61, 64, 65, 69]. The inflammasome in immune cells is a multiprotein complex that regulates inflammatory responses by sensing PAMPs or cellular stress [70]. Dysfunction of inflammasomes is associated with autoimmune diseases, neurodegenerative diseases, and cancers [70]. The most important type of inflammation is the NLRP3 inflammasome, which was first shown to be involved in cryopyrin-associated periodic α-syndrome [71]. The NLRP3 inflammasome is assembled by the sensor element NLRP3, the adaptor element apoptosis-associated speck-like protein (ASC), and the effector element caspase. Upon stimulation, NLRP3 oligomerizes and recruits ACS through the amino-terminal pyrin domain, which in turn recruits caspase-1 through the carboxy-terminal caspase recruitment domain. Caspase-1 is a protease that is able to cleave the precursor forms of interleukin-1β (IL-1β) and IL-18, producing mature IL-1β and IL-18 that are secreted from cells and initiate inflammatory responses. Activation of the NLRP3 inflammasome is usually considered to be a two-step process: priming and activation. Priming involves sensing PAMPS or DAMPs by PPRs to initiate NF-κB signaling, leading to an increase in the transcription of inflammatory factors and inflammasome components. In the activation stage, NLRP3 senses various stimuli, including bacteria, viruses, ATP, or cell stresses such as mitochondrial oxidative stress, followed by the assembly of inflammasomes, leading to the processing of precursor inflammatory cytokines and the release of inflammatory cytokines from cells [71]. Neuroinflammation mediated by the NLRP3 inflammasome has been widely reported in AD [71–73]. Pathological Aβ aggregates activate microglia to induce the assembly of inflammasomes and the release of IL-1, subsequently damaging

neurons [72]. Moreover, IL-1, by binding to IL-1R on neurons, increases the phosphorylation and aggregation of Tau through the activation of tau-associated kinase and phosphatase signaling pathways, contributing to the Tau pathology in AD [73].

α-Syn aggregates not only activate the transcription of NLRP3 inflammasome-related factors through the PPR-mediated NF-κB signaling pathway but also promote the assembly of the NLRP3 inflammasome due to mitochondrial dysfunction induced by α-syn PFFs [65, 69]. Blocking inflammasome activation with the inflammasome inhibitor MCC950 inhibits α-syn aggregate-induced secretion of IL-1β and improves motor behavior in a PFF mouse model [61]. In addition to membrane receptors, the membrane-associated intracellular tyrosine kinase Fyn also regulates α-syn PFF uptake into microglia, which results in oxidative stress due to mitochondrial damage by α-syn, contributing to inflammasome activation [65]. Compared to the activation of inflammasomes by a combination of LPS with ATP treatment [65], α-syn PFFs can induce both the priming and the activation of inflammasomes, which is closer to the activation of microglia under pathological conditions, further indicating that the microglial inflammation caused by α-syn aggregates involves multi-step processes (Fig. 1).

## Failure of Microglial Phagocytosis and Degradation Contributes to α-Syn Spreading

Microglia, as immune cells in the CNS, play a crucial role in the recognition and degradation of extracellular materials in the brain by phagocytosis [55]. In the CNS, phagocytosis is involved in the clearance of myelin debris, dead cells, and protein aggregates, as well as the pruning of synapses. Dysfunction of microglial phagocytosis disrupts brain homeostasis and leads to neurological disorders [55]. Phagocytosis is involved in the recognition of targets by the appropriate receptors on the cell membrane, the formation of phagosomes though membrane extension mediated by actin polymerization, and the transport of phagosomes to lysosomes for degradation [74, 75]. Some phagocytic components involved in the formation and transport of phagosomes to lysosomes overlap with components involved in autophagy [76–78]. For example, the formation of autophagic vesicles acquires microtubule-associated protein 1A/1B-light chain 3, which is also responsible for the maturation of phagocytic vesicles [78]. The impairment of microglial phagocytic function is closely related to aging [79, 80]. During aging, microglia increase the production of inflammatory factors and decrease phagocytosis, which contribute to aging-related diseases, such as AD and PD [79, 80]. The involvement of microglial phagocytosis in the processing of extracellular Aβ aggregates has been well documented in AD [79, 81, 82]. Using single-cell sequencing analyses, a presumably protective phagocytic microglial population in an AD mouse model has been identified, called disease-associated microglia (DAM) [82]. DAM activation requires the downregulation of homeostatic checkpoints and the initiation of a TREM2-dependent signaling pathway that enhances the cellular phagocytosis and degradation [82]. Loss of TREM2 leads to an exacerbation of Aβ pathology in an AD mouse model [82, 83], demonstrating the important roles of microglial phagocytosis in neurodegenerative diseases.

The involvement of microglial phagocytosis in α-syn pathology is supported by a recent study showing that microglia phagocytose and degrade α-syn aggregates by the redistribution of fibrillar α-syn through intercellular connections [84]. Under physiological conditions, microglia clear α-syn fibrils by the phagocytosis of extracellular α-syn aggregates into cells and the transport of α-syn aggregates into lysosomes for degradation, which prevents the spread of α-syn. The transfer of α-syn fibrils from activated microglia to the surrounding naïve microglia promotes the degradation of α-syn aggregates and decreases the inflammatory activity in α-syn-overloaded microglia [84]. IL-4 secreted from mesenchymal stem cells can modulate M2 microglial polarization, which promotes the phagocytosis and degradation of α-syn *in vitro* and *in vivo* and has anti-inflammatory effects [85, 86]. In a mouse model, α-syn released by neurons is degraded by selective autophagy after the endocytosis of α-syn by microglia, a process that is TLR4-dependent [87]. Moreover, α-syn accumulates to form high molecular weight species at 6 weeks after AAV-α-syn injection into mice in which microglial autophagy is deficient but not in WT mice [87]. Thus, the data support the hypothesis that microglia play a beneficial role in restricting α-syn accumulation and spread by the phagocytosis and degradation of α-syn aggregates released from neurons under α-syn transmission.

Although microglia can block α-syn spreading by the phagocytosis and degradation of extracellular α-syn aggregates under physiological conditions, the capacity for α-syn clearance by microglia declines under pathological conditions or aging [79]. With aging, microglial phagocytosis is impaired, resulting in a decrease in α-syn phagocytosis and an increase in α-syn accumulation in the brain [79, 88–90]. Microglia isolated from adult mice show decreased phagocytosis of α-syn oligomers compared with those isolated from young mice, and these microglia release more inflammatory cytokines [91]. Consistent with this conclusion, upregulation of CD22 expression is closely associated with age-related decrease in microglial phagocytosis. Anti-CD22 treatment increases the phagocytosis of pathological α-syn fibrils in microglia [79]. Moreover, α-syn aggregates interfere with the phagocytosis of microglia through the activation of SHP-1 [88], a negative regulator of phagocytosis [92]. Furthermore, autophagy proteins are decreased during aging, which directly affects the autophagic clearance

of α-syn [87, 89]. Although microglial phagocytosis is generally enhanced when microglia are activated, continuous inflammation may impair this process [64, 80]. Continuous activation of microglia by α-syn aggravates inflammasome activation and IL-1β release, while inhibition of inflammasomal activation increases the phagocytosis and degradation of α-syn oligomers [64], indicating that inflammasome activation impairs the microglial phagocytosis and degradation of α-syn. In line with this finding, the transmission of α-syn pathology from the striatum to the SNpc region is significantly reduced in mice that receive a single injection of PFFs into the striatum if the mice are treated with the NLRP3 inflammasome inhibitor MCC950 [61]. Therefore, the impairment of microglial phagocytosis and degradation caused by pathological factors and aging accelerates the transmission of α-syn pathology (Fig. 1).

### Acceleration of α-Syn Spreading by Neuroinflammation

The evidence that neuroinflammation is linked to α-syn pathology in the human PD brain comes from a study by Olanow and colleagues. They showed that there are many DA neurons at 18 months after transplantation of fetal mesencephalic tissue into the striatum of PD patients, and only diffuse monomeric but not aggregated α-syn is present in the grafts until 14–16 years after transplantation. However, activated microglia are present in all grafts between 18 months and 16 years, much earlier than α-syn aggregation, suggesting that the activation of microglia plays roles in α-syn pathology [93]. This finding has been supported by a study in which the effects of microglia on α-syn transmission were evaluated in a mouse model [86]. In this model, the animals first received an injection of AAV virus that expressed human α-syn into the SN, followed by an intrastriatal injection of LPS two weeks later, and the animals then received an intrastriatal injection of healthy mouse embryonic DA neurons one week after LPS treatment. Human α-syn in TH-positive grafted DA neurons was significantly increased in animals treated with LPS, suggesting that inflammation promotes the cell-to-cell transmission of α-syn [86]. The cell-to-cell transmission of α-syn also increases when using a colony stimulating factor 1 receptor inhibitor to remove microglia in the brain [86]. This study demonstrates that microglia are involved in the clearance of α-syn; however, the activation of microglia under pathological conditions promotes the spread of α-syn pathology.

Activated microglia can secrete a wide range of inflammatory factors or exosomes into the extracellular environment and act on other cells, playing a key role in the communication between microglia and other cells [94]. The pro-inflammatory cytokines IL-1β and tumor necrosis factor α (TNF-α) that are released by microglia or other immune cells induce NF-κB activation in neurons, which is able to upregulate α-syn gene transcription through the recruitment of both the NF-κB subunits p65 and p50 and the cofactor p300 to the α-syn gene promoter, suggesting that α-syn-mediated neuroinflammation in turn accelerates α-syn transmission through an increase in α-syn expression in neurons [95]. Aging microglia have been shown to have increase inflammatory responses, releasing various inflammatory factors that may also affect α-syn expression in neurons. It has been reported that microglia treated with iron show certain characteristics of senescence. Treatment of neurons with conditioned media from these aging microglia also increases α-syn expression and aggregation [96]. Primary microglia treated with PFFs phagocytose α-syn aggregates and secrete α-syn oligomers *via* exosomes. Activation of microglia by LPS significantly increases exosome release after PFF treatment [97]. Treatment of primary neurons with microglia-derived exosomes induces α-syn aggregation in neurons that is more severe in combination with cytokines. Injection of exosomes from PFF-treated microglia or from the cerebrospinal fluid of PD patients into mouse brains induces α-syn aggregate formation in neurons, further suggesting that exosomes from microglia contribute to the transmission of α-syn pathology [97]. Thus, microglia promote α-syn transmission in multiple ways, either by the reduction of α-syn endocytosis and increase of the release of α-syn in microglia or by the induction of α-syn transmission between neurons, which contributes to the spread of α-syn pathology in the CNS (Fig. 1).

## Astrocytes and α-Syn Pathology

As the most abundant cell population in the CNS, astrocytes perform a range of actions to maintain brain function and homeostasis, including blood–brain barrier formation and maintenance, neurotransmitter transmission, regulation of synaptic plasticity and brain metabolism, and neuroimmunity [98–100]. Impairment of these actions lead to various neurological disorders and neurodegeneration [98, 99]. Postmortem and clinical studies have shown that astrocytes play a crucial role in the α-syn pathology in PD [101]. α-Syn aggregates released from neurons during α-syn transmission not only induce microglial activation but also activate astrocytes to exacerbate inflammation [102]. In postmortem PD brains, α-syn aggregates are present not only in DA neurons but also in astrocytes [101, 103]. Under physiological conditions, α-syn is rarely expressed or expressed at lower levels in astrocytes [101]. Therefore, the α-syn aggregates in astrocytes are believed to originate from neurons.

## Astrocyte-associated Neuroinflammation Caused by α-Syn Species

The first experimental evidence demonstrating that α-syn oligomers are transferred from neurons to astrocytes and subsequently activate astrocytes came from the study by He-Jin *et al.* [102]. In a co-culture system in which differentiated SH-SY5Y cells that overexpress α-syn are co-cultured with primary astrocytes, neuron-derived α-syn oligomers can be transferred to astrocytes [102]. In transgenic mice that overexpress human α-syn under the control of neuronal promoters, α-syn aggregates form in astrocytes, suggesting the transmission of α-syn from neurons to astrocytes [102]. The endocytosis of neuron-derived α-syn oligomers by astrocytes results in the accumulation of α-syn in astrocytes, leading to an inflammatory response of astrocytes, which enhances inflammatory cytokine production by astrocytes. Moreover, α-syn is co-localized with LAMP2, a lysosomal protein in astrocytes. Furthermore, the inhibition of lysosomes by bafilomycin A1, a lysosomal inhibitor, increases the accumulation of detergent-insoluble α-syn in astrocytes, suggesting that endocytic α-syn oligomers undergo lysosomal degradation. Importantly, the secretion of pro-inflammatory factors by astrocytes is dramatically increased in bafilomycin A1-treated astrocytes, demonstrating a correlation between the increased inflammatory response and the accumulation of α-syn in astrocytes [102].

Many molecules are involved in the α-syn aggregate-mediated activation of astrocytes [63, 104]. TLR4 is involved in α-syn oligomer-mediated astrocyte activation [63]. In human primary neurons, blockade of TLR4 does not affect α-syn oligomer-induced neuronal death. However, in a co-culture of human primary neurons and astrocytes, TLR4 receptor antagonists decrease the TNF-α levels and neuronal death induced by α-syn oligomers, suggesting that TLR4-mediated astrocyte activation by α-syn promotes neurodegeneration [63]. Moreover, NF-κB signaling is involved in the PFF-induced activation of astrocytes [104]. The expression and nuclear translocation of NF-κB are increased in human primary astrocytes after overnight treatment with PFFs [104]. Inhibition of NF-κB signaling with BAY, which inhibits the NF-κB upstream kinase inhibitor of IκB kinase, blocks the PFF-induced activation of NF-κB signaling and the production of inflammatory chemokines. Meanwhile, BAY also downregulates the gene expression profiles of A1 astrocytes, a neurotoxic state, and upregulates the gene expression of A2 astrocytes, a neurotrophic state, in PFF-treated astrocytes [104]. In addition, inhibition of receptor interacting protein kinase (RIPK) signaling with either RIPK3 or RIPK1 blocks NF-κB-associated gene expression and decreases chemokine CXCL10 levels in PFF-treated astrocytes, suggesting an involvement of RIPK signaling in PFF-induced astrocyte activation. In addition to activation of the pro-inflammatory response, PFFs impair the phagocytic activity of astrocytes, as evidenced by decreases in the expression of the phagocytosis-associated genes *GAS6* and *MEGF10* and in the uptake of fluorescently-labeled zymosan, an indicator of endocytosis [104].

In addition to direct activation of astrocytes by α-syn aggregates, microglia–astrocyte communications are also important in PFF-induced astrocyte activation [60]. Pro-inflammatory factors that are released by activated microglia upon PFF stimulation can convert the remaining astrocytes to the A1 type, which is neurotoxic. Three key inflammatory mediators, TNF-α, IL-1α, and complement component 1q, which are produced by activated microglia, contribute to inflammatory communication between microglia and astrocytes. Furthermore, the glucagon-like peptide-1 receptor agonist NLY01 can inhibit the activated microglia-induced conversion of astrocytes to the neurotoxic A1 type, prevent DA neuronal loss, and improve behavioral deficits in the PFF mouse model [60]. Thus, data suggest that α-syn aggregates induce inflammation in the brain through direct or indirect pathways that involve both microglia and astrocytes, and this contributes to the neurodegeneration in PD (Fig. 1).

### Astrocytes as Modulators of α-Syn Spreading

Lines of evidence suggest that astrocytes can endocytose neuron-derived α-syn aggregates and transport them to lysosomes for degradation, similar to the process in microglia [102, 105, 106]. Astrocytes degrade α-syn aggregates more effectively than neurons, which may be attributed to the higher abundance of lysosomes in astrocytes [105]. By co-culturing astrocytes with iPSC-derived DA neurons carrying *SNCA* triplications, astrocytes reduce α-syn aggregation in neurons and α-syn transmission between neurons, suggesting that astrocytes have protective effects to limit α-syn transmission. However, astrocytes with an *ATP13A2* mutation lose the capacity for endocytosis and degradation of α-syn aggregates that are released from neurons, resulting in an increase in α-syn transmission, suggesting that functional impairment of astrocytes might accelerate α-syn transmission [105]. Moreover, an increase in mitochondrial fragmentation and a decrease in ATP production has been reported in astrocytes treated with α-syn oligomers, suggesting that an overload of α-syn aggregates in astrocytes affects the normal function of astrocytes, contributing to the loss of a protective role in the inhibition of α-syn transmission [107, 108]. In addition, α-syn transmission between astrocytes has also been reported [108]. Similar to the manner in which microglia distribute α-syn PFFs through an intercellular network, astrocytes can transfer α-syn oligomers to nearby astrocytes *via* the formation of tunneling nanotubes (TNTs) [108]. The accumulation of α-syn in astrocytes induces the formation of TNTs to transfer α-syn to nearby astrocytes. Moreover,

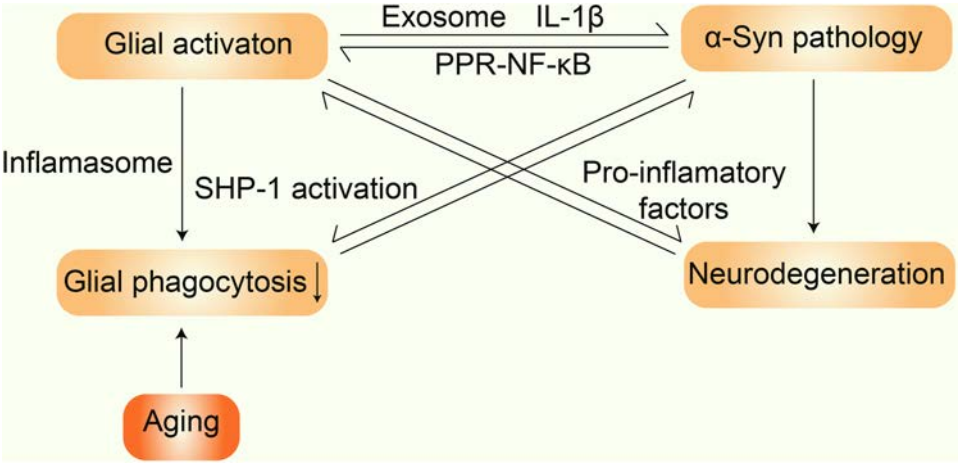


**Fig. 2** Schematic of the interactions among glial activation, α-syn pathology, and neurodegeneration in PD. α-Syn pathology leads to the activation of glia, which contributes to α-syn pathology. Neurodegeneration occurs due to either the direct neurotoxicity of α-syn aggregates or the release of pro-inflammatory factors by the activated glia. In addition, glial activation and α-syn aggregation as well as aging lead to a decrease in phagocytosis by glial cells, resulting in an accelerated spread of α-syn pathology.

α-syn oligomers lead to morphological alterations in the endoplasmic reticulum and mitochondria. Furthermore, the accumulation of α-syn impairs autophagic flux, as evidenced by an increase in the formation of autophagosomes that are not degraded by lysosomes [108].

## Conclusions and Perspectives

There is a link between glial activation and α-syn pathology in PD pathogenesis. On the one hand, the glial activation response to α-syn oligomers, at least at the early stage, promotes the phagocytosis and clearance of α-syn by glia, which inhibits the transmission of α-syn between neurons and the development of α-syn pathology. On the other hand, sustained activation of glial cells by α-syn aggregates leads to chronic inflammation, which impairs the phagocytic activity of glia and increases inflammatory cytokine levels, leading to the accumulation of α-syn and increases in the cell-to-cell transmission of α-syn, contributing to the spread of α-syn pathology. In addition, genetic and environmental factors as well as aging influence both glia and neurons, and are involved in the initiation and progression of α-syn pathology (Fig. 2). Drugs that modulate the microglial and astrocyte activation associated with α-syn alleviate the loss of DA neurons and the defects in behaviors in PFF mouse models, further demonstrating the pivotal roles of α-syn-mediated glial activation in PD pathogenesis. However, some questions remain to be elucidated. Do temporal and spatial glial activation contribute to the spread and distribution of α-syn pathology in PD and other α-syn-related neurodegenerative diseases? Why do different neurons have differential responses and susceptibilities to α-syn oligomers, although the transmission of α-syn pathology still occurs within them? In future, the identification of factors with analyses using spatial transcriptomics and single-cell sequencing in PD animal models may reveal a mechanistic connection between microglial activation and α-syn pathology. Furthermore, more microglial and astrocyte subtypes, in addition to M1, M2, A1, and A2, can be identified in PD models using single-cell transcriptomics, which may detail the roles of the subtypes of glial cells in the clearance and release of α-syn. This would also be helpful for exploring the roles of neurons in response to α-syn species and in the spread of α-syn pathology.

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REVIEW

# Novel Microglia-based Therapeutic Approaches to Neurodegenerative Disorders

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**Abstract** As prominent immune cells in the central nervous system, microglia constantly monitor the environment and provide neuronal protection, which are important functions for maintaining brain homeostasis. In the diseased brain, microglia are crucial mediators of neuroinflammation that regulates a broad spectrum of cellular responses. In this review, we summarize current knowledge on the multifunctional contributions of microglia to homeostasis and their involvement in neurodegeneration. We further provide a comprehensive overview of therapeutic interventions targeting microglia in neurodegenerative diseases. Notably, we propose microglial depletion and subsequent repopulation as promising replacement therapy. Although microglial replacement therapy is still in its infancy, it will likely be a trend in the development of treatments for neurodegenerative diseases due to its versatility and selectivity.

**Keywords** Microglia · Neuroinflammation · Multifunction · Microglial replacement · Neurodegeneration

## Introduction

Microglia were first described by the Spanish neuroscientist Pío del Río-Hortega in 1919 [1] and were gradually found to be maverick immune cells in the central nervous system (CNS) that are both specialized and diverse. In mice, microglia originate from a pool of primitive macrophages in the yolk sac on embryonic (E) day 8.5 [2]. During development, microglia survive through colony-stimulating factor 1 receptor (CSF1R) signaling [3], and further differentiation depends on the activity of the transcription factors *PU.1* and interferon regulatory factor 8 [4]. In the adult brain, microglia have a ramified morphology and express *TMEM119, CD11b,* and *P2ry12/P2ry13*, which participate in the immune response [5]. In the normal brain, microglia are highly dynamic and perpetually scan the CNS [6], influencing fundamental processes such as protein aggregation, soluble factor secretion, phagocytosis, and neural circuit refinement [7]. In the context of CNS injury, microglia undergo complex, multistage activation and respond through signaling molecules such as cytokines and chemokines for subsequent tissue repair [8].

The recent introduction of single-cell RNA sequencing and single-nucleus RNA sequencing analyses has greatly advanced our knowledge of microglial responses in neurodegenerative diseases [9–11], leading to the identification of special microglial subsets associated with neurodegeneration in both mouse models and human specimens. For example, using transcriptional single-cell sorting, Keren-Shaul *et al*. identified disease-associated microglia (DAMs) in proximity to amyloid-β (Aβ) plaques involved in the immune response in AD mouse brains [12]. Krasemann *et al.* reported that the microglial neurodegenerative phenotype is characterized by genetic upregulation of ApoE signaling and suppression of TGFβ signaling, and this phenotype partially overlaps with that of the DAMs found in AD mouse models [13]. Another study reported that activated response microglia are composed of specialized subgroups overexpressing MHC (major histocompatibility complex) type II genes and show strongly

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upregulated expression of AD risk genes in the AD mouse model [14]. By using single-cell RNA sequencing, Mathys *et al.* identified multiple disease stage-specific cell states, including IFN-I and IFN-II co-regulated DAMs in a mouse model of severe neurodegeneration with AD-like phenotypes [15]. Microglial features determined from snRNA-seq data from brain specimens of humans with neurodegenerative diseases show considerable heterogeneity and appear to be different from those in mouse models. For example, Friedman *et al*. analyzed RNA profiles in the brains of AD patients and showed that they were not replicated in animal models or individuals with early-onset and late-onset AD [16]. They also identified multiple dimensions of CNS myeloid cell activation that occur differentially in response to specific disease conditions. Beyond a detailed description of different microglial subsets, it will be essential to investigate additional biological characteristics of microglia.

It has long been assumed that microglia are long-lived cells that persist throughout the entire lifespan of mice or humans under physiological conditions. Lawson *et al.* [17] used [3H] thymidine incorporation and autoradiography and reported that 0.05% of the microglia proliferated within 1 hour. Askew *et al.* [18] analyzed the proliferation of resident microglia by using BrdU incorporation and found that 0.69% of the total microglial cells proliferated after a single pulse of BrdU (the estimated value in humans is ~2%). Therefore, at the individual cell level, microglia replicate frequently. Regarding pathological conditions, Tay *et al.* [19] established a new multicolor fluorescence fate mapping system and found that CNS pathology shifts from random self-renewal of the microglial network toward a rapid expansion of selected microglial clones. Füger *et al*. [20] imaged individually-labeled microglia in APP/PS1 mice and found increased microglial turnover in the presence of amyloid lesions.

The maintenance and local expansion of microglia are solely dependent on the self-turnover of CNS-resident cells under normal conditions. When microglia are depleted by inhibiting CSF1R signaling, newborn microglia rapidly replenish the population in the whole brain in a short time after drug withdrawal [21]. Importantly, the repopulated microglia are solely derived from residual microglia [22]. In the context of disease, numerous cells of the myeloid lineage appear in the nervous parenchyma in pathological settings, such as in AD models and experimental autoimmune encephalitis (EAE) models. Myeloid cells enter the nervous parenchyma, and some of them appear to transform into cells resembling microglia [23].

In this review, we highlight new evidence demonstrating the unique and diverse properties of microglia in the healthy and diseased brain, including in the context of Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS). In addition, we elaborate on the theoretical basis of microglial replacement and other microglia-based therapies for translational clinical applications.

## The Multifaceted Functions of Microglia in the CNS

The biological functions of microglia in the CNS are multifaceted. They exert both neuroprotective and neurotoxic effects (Fig. 1). In a healthy brain, microglia are responsible for mediating inflammation, regulating a broad spectrum of cellular responses, and eliminating microbes, dead cells, and protein aggregates [24]. Microglia were originally known for their immune functions. They are the first responders to neuroinflammation and rapidly adapt their phenotypes and functions to fit the brain milieu [1]. Microglia secrete cytokines and chemokines that contribute to various aspects of immune responses in the CNS. During infection, chronic activation of microglia leads to sustained production of proinflammatory cytokines that cause damage to the surrounding neurons. This notion has been confirmed in various brain disorders, such as AD, PD, and MS. For example, in AD patients, the neurotoxic factors produced by activated microglia cause nitration of tyrosine 10 of the Aβ peptide, which promotes the aggregation of senile plaques [25]. In PD patients, inflammatory mediators such as IL-1β and IL-6, and epidermal growth factor released by activated microglia, can lead to the death of dopaminergic neurons [26]. PET studies have indicated that motor disability is correlated with the abundance of activated microglia in MS patients [27].

In addition to immune function, as resident macrophages in the brain, microglia vigilantly perform surveillance to ensure parenchymal homeostasis. Once they migrate into the brain parenchyma, microglia actively communicate with other cells. Early after birth, microglia release neurotrophic factors such as IGF-1 to promote neuronal survival and axon fiber formation [28]. Brain-derived neurotrophic factors released by activated microglia promote neurogenesis in the CNS [29]. At the same time, microglia are also well poised to induce programmed cell death, which has been demonstrated to be CD11b-dependent [30]. Afterward, microglia clean up the resulting cellular debris by phagocytosis. Microglial phagocytosis is mediated by signaling *via* triggering receptors expressed on myeloid cell-2 (TREM2) [31]. In addition, microglia participate in maintaining synaptic homeostasis *via* neuronal pruning and synaptogenesis [32, 33]. Oligodendrocytes, another important group of cells that are pivotal for myelin production around axons, are deeply involved in microglial maturation [34, 35]. In addition, microglia establish a delicate balance with astrocytes and are responsible for the maintenance of neuronal functions and brain homeostasis [36, 37].

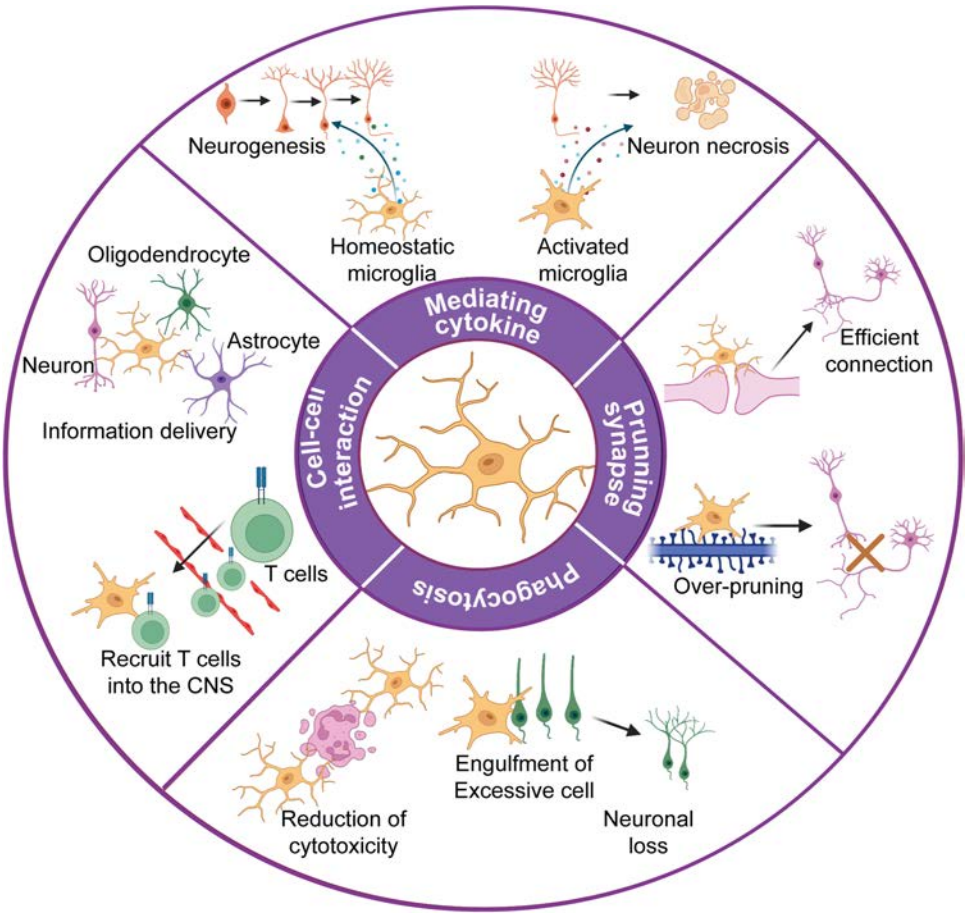


**Fig. 1** Microglia play multifaceted roles in the CNS. Upper: homeostatic microglia promote neurogenesis by releasing neurotrophic factors, whereas inflammation-associated microglia lead to neuronal cell death. Right: microglia are crucial for synaptic pruning and circuit refinement. Synaptic over-pruning induces synaptic dysfunction, which is found in neurodegenerative diseases. Lower: phagocytosis is essential for CNS homeostasis. Microglia can remove apoptotic cells and prevent the release of toxic intracellular contents. However, hyperactive microglia engulf newborn neurons, leading to a decrease in the number of mature neurons. Left: microglia–neuron/oligodendrocyte/astrocyte crosstalk promotes neurogenesis and axon formation. Microglial hyperactivation disrupts the blood-brain barrier and recruits peripheral cells into the brain during infection.

Microglia are active sensors and versatile effector cells not only in healthy brains but also in diseased brains. In the following section, we emphasize the role of microglia in neurodegenerative diseases of the CNS, including AD, PD, and MS (Fig. 2).

## Microglia are a "Double-edged Sword" in the Progression of AD

AD is the major cause of dementia and is mainly characterized by progressive neuronal loss followed by cognitive impairment. The current understanding is that AD is closely associated with genetic, aging-related, and environmental factors [38–40]. The main pathological features of AD include the deposition of insoluble Aβ peptides, as well as the aggregation of hyperphosphorylated tau protein, which lead to the formation of neurofibrillary tangles in the brain [38–40].

Reactive gliosis and the inflammatory response are hallmarks of AD. Microglia-mediated inflammation is a 'double-edged sword', performing both detrimental and beneficial functions. For example, activated microglia in AD mice show upregulation of inflammatory markers such as CD36, CD14, CD11c, MHC-II, and iNOS, which might disturb neuronal functions and lead to cognitive decline [41]. Clinical imaging studies have reported negative correlations between microglial activation measured by [11C]PK11195 PET and the structural integrity and functional activity of the brain in AD patients [42], whereas some inducers of inflammation, such as lipopolysaccharide (LPS), activate microglia to promote the degradation of Aβ [43]. Microglia interact with Aβ and amyloid-beta precursor protein (APP) through specific pattern-recognition receptors, including CD14, CD36, and Toll-like receptors, which are strongly expressed on the microglial surface [44]. This interaction increases microglial phagocytosis, resulting in the clearance of Aβ from the brain [45, 46]. As an example, the phagocytic index and total Aβ load are higher in IL-1α (+) and IL-1Ra (+) microglia and microglia producing TNF-α and IL-1β are associated with a lower Aβ load and phagocytic index in AD mice [47]. Therefore, strategies need to be developed to modulate the activation of microglia by inhibiting the release of inflammatory factors. Preclinical trials have shown that genetic and pharmacological blockade of TNF effectively alleviates amyloid pathology and tau phosphorylation [48]. Intraperitoneal administration of an antibody blocking IL-1 receptors has also been shown to decrease the

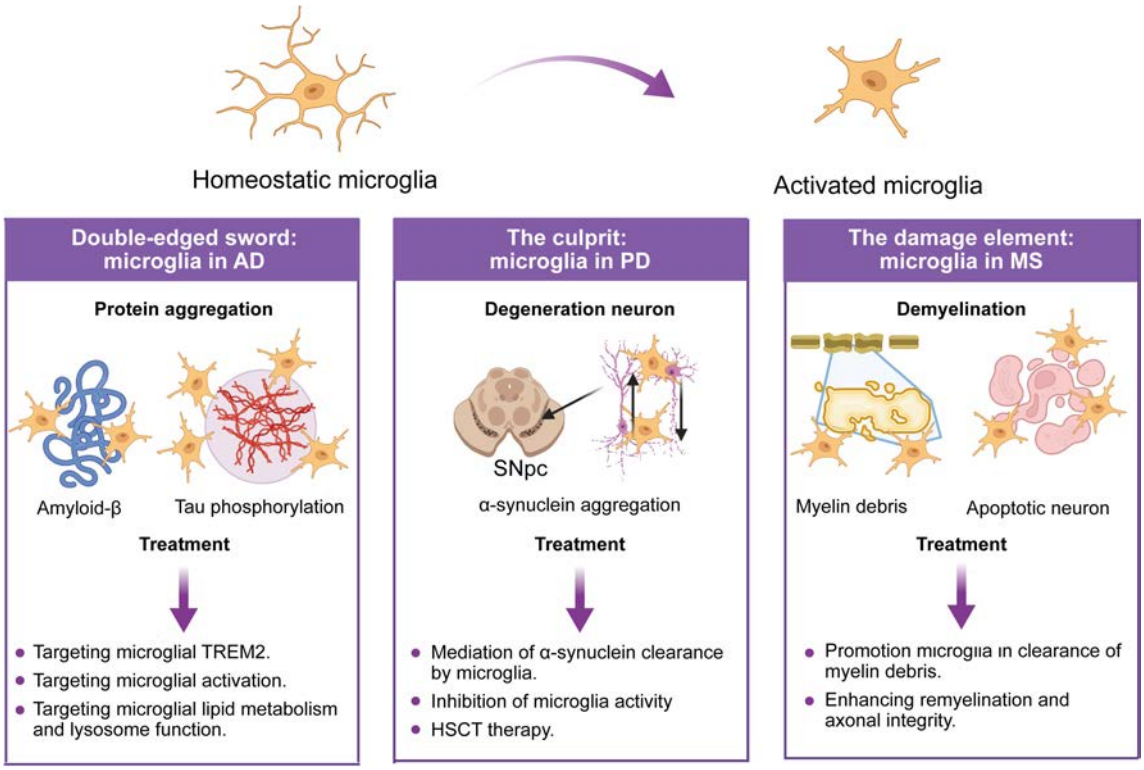


**Fig. 2** Microglia are active sensors and versatile effector cells in the brain under pathological conditions. Microglia constantly screen the microenvironment in the CNS and transform into a reactive state under certain pathological conditions. However, the tilt toward harmful or beneficial outcomes of microglial activation varies between neurological diseases. (1) Microglia in AD. AD is typified by the accumulation of extracellular Aβ peptides and intracellular deposits of hyperphosphorylated tau. Microglia are committed to an inflammatory response and can engulf Aβ deposits and impair cells. Microglial treatments for AD include targeting the microglial inflammatory response, TREM2 activation, lipid metabolism, and lysosomal function. (2) Microglia in PD. PD is primarily characterized by the death of dopaminergic neurons that project from the SNpc. Reactive microglia are involved in dopaminergic cell death as proinflammatory mediators. Reactive microglia also block the delivery of α-syn to the nigrostriatal tract. Microglia-based treatments for PD include the promotion of α-synuclein transfer and clearance, anti-inflammatory treatments, and HSCT therapy. (3) Microglia in MS. MS is a chronic inflammatory disease that leads to focal plaques of primary demyelination and diffuse neurodegeneration. Active demyelination is usually associated with microglial activation. Microglial treatments for MS include the regulation of microglial inflammatory signaling, the depletion of microglia to reduce demyelination, and the targeting of microglial phagocytosis. AD, Alzheimer's disease; PD, Parkinson's disease; MS, multiple sclerosis; SNpc, substantia nigra pars compacta; α-syn, alpha-synuclein; HSCT, hematopoietic stem cell transplantation.

activity of several tau kinases in the brains of 3×Tg-AD mice [49]. However, further clinical trials are needed to assess the safety and efficacy of this antibody in humans.

Differences exist between microglia in humans and mouse AD models. At the site of neurodegeneration, plaques are surrounded by activated microglia named DAMs [12]. Genome-wide studies have shown that some genes, such as *APOE4*, *TREM2,* and *CD33*, have unique expression patterns in DAMs [13, 50, 51]. However, no DAM signature has been found in human AD *via* single-nucleus RNA sequencing (snRNA-seq), and only a few microglial genes, including APOE and SPP1, have been identified [52]. In another study of patients, the differentially expressed microglial genes included *EEF1A1, GLULL, KIAA1217, LDLRAD3*, and *SPP1*, which differ from the characteristic genes of DAMs [53]. DAMs have been reported in other conditions, including aging, ALS, and frontotemporal dementia. The scRNA-seq profiles of dissected human brain specimens from MS patients have revealed various microglial populations expressing DAM genes [54]. Given the heterogeneity of brain pathology, certain DAM genes may be involved in different diseases. Research into the treatment of AD should consider the feasibility of implementing this approach in the clinic.

Overall, microglia, as a "double-edged sword" in the progression of AD pathology, have both beneficial and detrimental effects. A better understanding of the role of microglia in the progression of AD pathology is needed, as microglia could be a target and a tool for AD treatment.

## Microglia: The Culprit in PD

PD is the second most common neurodegenerative disorder and is characterized by motor and non-motor symptoms. The widely recognized pathology of PD involves the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the presence of cytoplasmic protein aggregates known as Lewy bodies in the remaining dopaminergic neurons [55]. In the early stages of PD, α-synuclein undergoes aggregation and fibrillization, which can lead to neurotoxicity [56, 57]. In late-onset PD, a large genome-wide association study identified a single nucleotide polymorphism in the human leukocyte antigen (HLA-DRA) gene. HLA-DR is a genetic risk factor for PD patients and is expressed by antigen-presenting cells such as microglia [58]. Another study showed that HLA-DR-positive microglia ("reactive" microglia) are also found in the substantia nigra of PD patients [59].

Although molecular profiling of microglia in animal models of PD has yet to be attempted, activated microglia are known to be prevalent in mouse models and PD patients. There is emerging evidence that microglial dysfunction contributes to PD pathogenesis and progression [60], as exhibited by a weakened inflammatory response, reduced phagocytosis, and decreased interactions with neurons. In a PD mouse model, neuroinflammation and associated "reactive" microglia precede the onset of astrogliosis and dopaminergic cell death [61]. Many studies have described reactive microglia in postmortem brain samples from PD patients. Jyothi *et al*. reported that the microglial count showed a biphasic increase in the vicinity of the few remaining nigral dopamine neurons and that microglia displayed a morphology characteristic of activated cells in PD patients [62]. A PET study using [11C] (R)-PK11195 in early PD, an *in vivo* marker of microglial activation, reported that reactive microglia are noted in brain regions such as the pons and basal ganglia [63]. These activated microglia then produce a wide range of inflammatory mediators that lead to the continuous death of dopamine neurons. Consistent with this, in PD model mice established by 1-methyl-4-phenyl-1,2,3,6tetrahydropyridine (MPTP), brain microglial activation *via* NLRP3 inflammasomes plays a role in neurotoxicity [64].

Cell-to-cell propagation of α-synuclein aggregates is thought to contribute to the pathogenesis of PD. IL-4-activated microglia effectively reduce the extracellular α-syn and decrease α-syn transfer from neuron to neuron, whereas LPS-treated microglia are less prone to carry out phagocytosis [65]. Pro-inflammatory cytokines can both be neurotoxic and attenuate microglial phagocytosis. LPS, a pro-inflammatory stimulus, attenuates microglial phagocytosis and leads to the increased presence of α-syn within microglia and grafted dopaminergic neurons in a PD mouse model [65]. Croisier *et al*. found that CD68 immunoreactivity is negatively correlated with disease duration, suggesting that elevated phagocytic activity is associated with sustained tissue destruction in PD patients [66]. Therefore, regulating the activation of microglia into a neuroprotective function is a potential therapeutic target.

PD is a chronic disease, making it likely that prevention and treatment will require long-term therapy. Thus, mediating microglia to play a neuroprotection role is a promising field for research.

## Microglia: The Damaging Element in MS

MS is a demyelinating disease of the CNS, including the brain and spinal cord. In MS, demyelination occurs when the immune system inappropriately attacks and destroys myelin, which breaks down communication between neurons, ultimately leading to a variety of sensory, motor, and cognitive problems [67]. MS is a complex and heterogeneous disease with different lesion patterns and mechanisms of tissue injury [68]. MS is also an inflammatory disease of the CNS in which microglia play an important role.

The role of microglia in MS is complex and controversial. Microglia are considered to be damaging elements in MS. Focal lesions of active demyelination and neurodegeneration are characterized by inflammation and microgliosis. A study in an EAE mouse model indicated that microglia are the first cells to take up myelin antigens and subsequently recruit leukocytes to the CNS through MHC class II molecules, thus initiating immune infiltration and the demyelination cascade in the early stage [69]. Microglia also release proinflammatory cytokines such as IL-18 and IL-6 and chemokines to aggravate MS [70]. In MS patients, TMEM119-positive microglia are less abundant in active MS, with restoration associated with disease inactivity [71]. In chronic slowly expanding MS lesions, a ring of activated microglia is also present at the site of lesion expansion [72]. CSF1R signaling (specifically in microglia) is activated in MS and might drive deleterious neuroinflammation, particularly during disease progression [73, 74]. Disruption of remyelination is another pathogenic factor in MS. TREM2 activation on microglia promotes myelin debris clearance and remyelination [75], and *TREM2* genetic deficiency might be a risk factor for MS. Thus, microglia are recognized as key players in MS pathology.

## Perspectives and Reflections on Microglial Replacement Therapy

As shown above, we demonstrated the role of microglia in the pathology of neurodegenerative disease and the potential therapeutic role of microglia in disease treatment. Some

researchers have reported that microglial repopulation can largely resolve the proinflammatory response and promote functional recovery [76]; however, the functional restoration of repopulated microglia depends on exogenous environmental and endogenous genetic changes. Here, we propose that microglia replacement by allogenic cells is an effective method to treat microglia-associated neurodegenerative disorders (Fig. 3).

## The Origin of Microglia

Microglial cells originate from cells produced at ~E7.5 in the yolk sac, at the time of the first wave of hematopoiesis [77, 78]. The second wave starts at E10.5; hematopoietic stem cells (HSCs) are produced in the aorta–gonad–mesonephros region and settle in the fetal liver and other hematopoietic organs of the embryo, where they mature and differentiate into definitive erythrocytes and all myeloid cells, including monocytes. HSCs from the fetal liver finally colonize the bone marrow, the only hematopoietic organ in adults [77]. Due to the specificity of microglial origin, non-autologous cell replacement of microglia may be an effective treatment for microglia-associated neurodegenerative diseases.

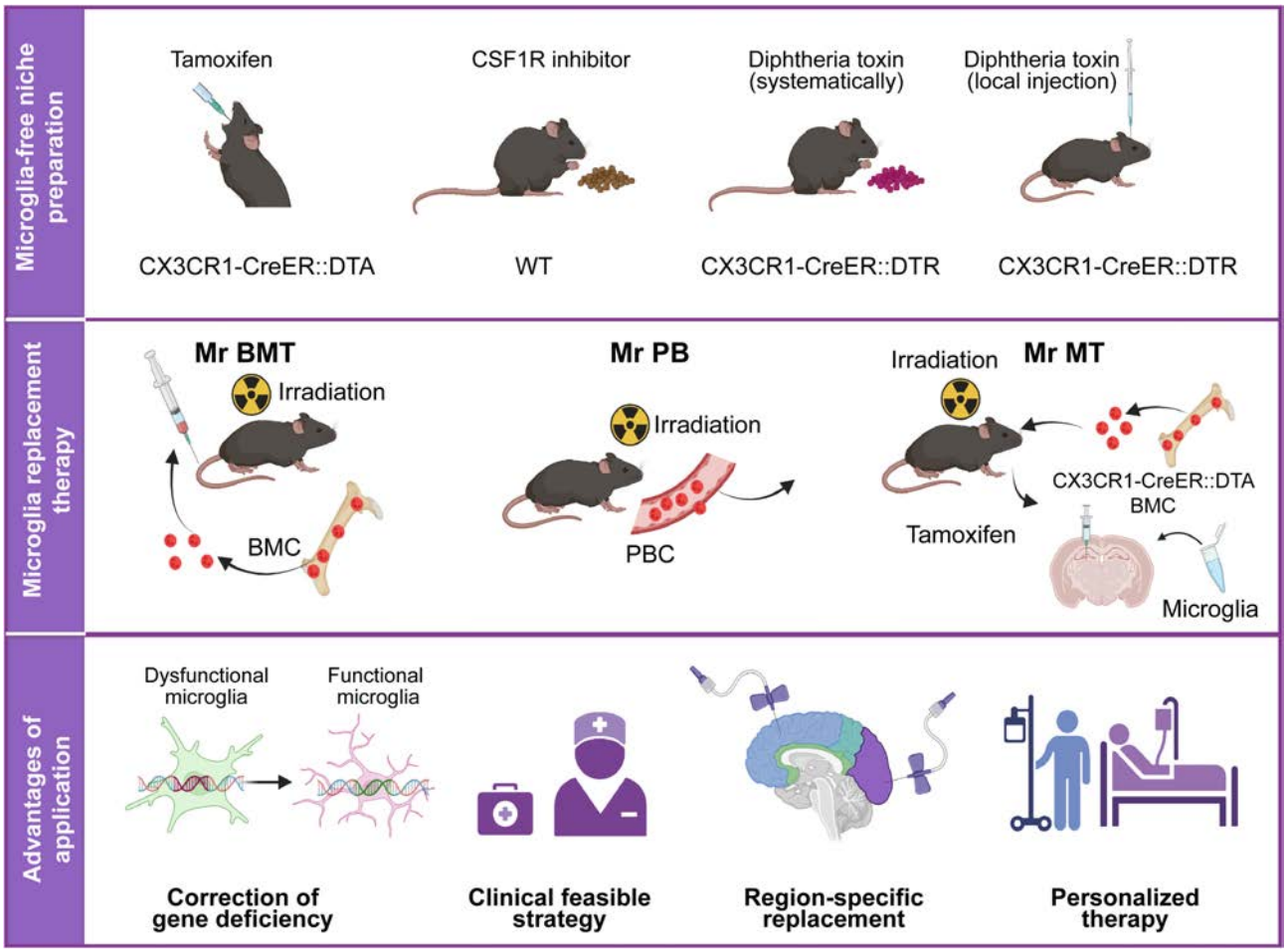


**Fig. 3** Microglial replacement therapy is a promising strategy for treating neurological disorders. A microglia-free niche is a necessary precondition for microglial replacement. The depletion of microglia is an effective model to create the niche, such as in CX3CR1-CreER::DTA mice. Pharmacological microglial depletion includes the administration of a CSF1R inhibitor or DT in the brain in CX3CR1-CreER::iDTR mice. There are three highly efficient and clinically feasible strategies for microglial replacement in the whole CNS or brain regions of interest: microglial replacement by bone marrow transplantation (Mr BMT), microglial replacement by peripheral blood (Mr PB), and microglial replacement by microglial transplantation (Mr MT). Mr PB clinically boosts donor cell availability. Mr BMT can correct gene deficiency, thus better treating gene-related diseases. Mr MT can achieve region-specific microglial replacement. These three approaches provide options for personalized treatment. DT, diphtheria toxin; DTR, diphtheria toxin receptor; DTA, diphtheria toxin activator. The figure was created with BioRender.com.

## A Microglia-free Niche is a Necessary Precondition for Microglial Re-population

The macrophage niche theory is a concept that first postulates that each macrophage cell occupies its territory [79]. When the niche is available, monocytes can efficiently differentiate into macrophages, and the same process occurs for microglia. The depletion or death of microglia may provide a space that triggers the proliferation of neighboring macrophages to repopulate the space. Microglial niches become temporarily available *via* irradiation-mediated damage or depletion [79]. The irradiation method opens the blood-brain barrier, and this helps a drug to enter the brain through blood circulation and avoids further damage to the CNS. Early approaches depleted microglia by generating mice transgenic for CD11b–thymidine kinase of the herpes simplex virus (HSVTK). In the presence of ganciclovir, HSVTK is activated and induces apoptosis in $CD11b^+$ mitotic cells [74]. While $CD11b^+$ cells are necessary for normal hematopoiesis and red blood cell production [80], completely depleting them is not a perfect way to deplete microglia. An alternative genetic approach used CX3CR1-CreER::DTA and CX3CR1-CreER::DTR mouse lines to induce diphtheria toxin-dependent microglial cell death [81]. However, DTR models kill only short-lived microglia [81]. Pharmacological microglial depletion can be achieved by targeting the *Csf1r* gene. The CSF1R inhibitors PLX5622, PLX3397, GW5622, and PLX647 are able to cross the brain-blood barrier and induce microglial depletion. Administration of PLX5622 in rodent chow depletes microglia by up to 99% after 2 weeks in adult mice [22]. However, there are still some potential toxic or adverse effects of microglial depletion and replacement. In the report by Rubino *et al*., acute and synchronous microglial depletion in adult mice triggered gray matter gliosis and progressive ataxia-like neurological behavior [82]. Bruttger *et al*. reported that microglial ablation in $DTR^{MG}$ mice leads to a cytokine storm and astrogliosis [81]. Nevertheless, Peng *et al*. reported normal motor performance after diphtheria toxin (DT) treatment in DTR mice [83]. Several papers have reported long-term observations in the PLX depletion model and did not observe motor deficits [21]. The reasons for these different behaviors may involve the dose, concentration, and duration of drug treatment. Therefore, when choosing microglial replacement therapies, we should take into account the potential risks of the method and try to minimize them.

## Current Microglial Replacement Strategies

Microglial replacement therapy has undergone several attempts and failures. Traditional bone marrow transplantation enables the partial replacement of endogenous microglia with donor cells [84]. However, the replacement efficiency is usually <5%–20%, due to the lack of a cell niche [85]. Recent studies established an experimental strategy for transplanting microglia into $RAG2^{-/-}$ $IL2Rg^{-/-}$ recipient mice with clear success. However, the treatment is limited in terms of time and clinical application. In light of these challenges, Xu *et al.* recently developed three effective strategies for microglial replacement either throughout the CNS or focusing only on brain regions of interest [86]: microglial replacement by bone marrow transplantation (Mr BMT) [87], microglial replacement by peripheral blood (Mr PB) [88], and microglial replacement by microglial transplantation (Mr MT) [89]. By choosing an appropriate strategy, the rate of microglial replacement can be effectively boosted. In this protocol, PLX5622 is used to clear microglia, and 9 Gy of whole body irradiation is administered to open the blood-brain barrier. Mr PB is able to induce peripheral blood cells to differentiate into microglia-like cells (MLCs) and can replace 80.74% of resident microglia in the brain. Mr BMT is capable of inducing allografted bone marrow cells (BMCs) to differentiate into microglia-like cells in the entire CNS, replacing 92.66% of resident microglia in the brain. The engrafted microglia after Mr MT exhibit the natural characteristics of naive microglia.

## Similar but not the Same

Microglial replacement combining genetic engineering with cell transplantation represents a cutting-edge approach for the precise treatment of microglia-associated neurodegenerative diseases. Whether transplanted microglia can act as normal microglia is the key issue. Microglial cells in the normal brain are called “true” microglia or *bona fide* microglia, while transplanted microglial cells are often called “microglia-like cells” [90]. This is because of not only their different origins but also their slightly different characteristics. Bone marrow-derived MLCs, when engrafted into the CNS, share ~90% of the transcriptome with host microglia, including the expression of some key microglial genes, such as Tgfb receptor 1 (*Tgfbr1*). In addition, MLCs exhibit their characteristics, as they show reduced gene expression of *Tmem119* and *P2ry12* [91]. Similarly, the MLC population is also maintained by local proliferation [18]. Reports suggest that microglia and MLCs are very similar, and their phenotypes are constantly shaped by the microenvironment in a time- and context-dependent manner. For instance, under pathological conditions, during the acute phase of EAE, microglia shift to a pro-inflammatory macrophage phenotype, while during the chronic phase, macrophages turn down their pro-inflammatory signature to acquire a resting microglia phenotype [92]. However, to our knowledge, the functional differences between microglia and MLCs are not well established; for example, whether they have similar functions in parenchymal surveillance and neuronal

circuit refinement remains to be elucidated. We summarize the main benefits and limitations as well as the caveats of each proposed strategy in Table 1. Therefore, these methods offer important rationales for further clinical applications, and each tactic has its own merits and limitations, which provide more choices. Importantly, these methods allow for the genetic modification of replacement microglia and the compensation of functional defects.

## Microglial Replacement Therapy in CNS Disease

Microglial replacement therapy has been effectively used to treat other diseases. For instance, transplantation of wild-type bone marrow into *mecp2*-deficient hosts leads to the implantation of bone marrow-derived cells with a microglial phenotype in the brain parenchyma, which arrests disease progression [84]. Another in-depth study indicated that whole bone marrow is heterogeneous and ill-defined, and hematopoietic stem cells play a very important role after Mr BMT transplantation [93]. BM-derived immature monocytic cells can commit to a microglia-like phenotype, and they harbor several features and functions of native microglia [94]. Since resident microglia appear to degenerate in AD, Mr BMT compensates for the deficient functions of senescent resident microglia in AD [95]. ALS is a progressive, fatal neurodegenerative disease. BMT of mSOD1-transgenic mice with BMCs altered the functional properties of microglia and improved the neural cell microenvironment [96].

Moreover, transplantation of genetically modified BM cells can reduce symptoms of CNS diseases. Mr BMT methods are also being continuously improved. Recently, a study developed an approach for rapid and near-complete replacement of microglia in mice with circulation-derived myeloid cells, eliminating the substantial variability that occurs after conventional BMT, which slows neurodegeneration and ameliorates motor dysfunction in prosaposin-mutant mice [93]. However, Mr BMT has its limitations: for example, Mr BMT is a method for whole-brain microglial replacement, not for specific brain regions. Mr MT enables microglial replacement *via* microglial transplantation into the brain area of interest. The method of targeted removal of microglia in specific regions has been studied [97]. Although the application of Mr MT in diseases is relatively rare, microglial recolonization in a brain region of interest will certainly have better application prospects. We speculate that therapeutic genes can be transduced into the stem or progenitor cells with lentiviral vectors, leading to stable integration of genes such as *MeCP2* in Rett syndrome or *TREM2* in AD mice. It is conceivable that by genetically modifying the extracted normal microglia, we can treat more intractable genetic defects and avoid immune rejection at the same time.

**Table 1** Three methods of microglial replacement and their application.

| Strategy | Mr BMT | Mr PB | Mr MT |
|---|---|---|---|
| Source | Bone marrow cells | Peripheral blood | Isolated microglia |
| Efficiency | 92.66% in the brain | 80% in the brain | 50% in a specified brain region [86] |
| | 99.46% in the retina | 74.01% in the retina [86] | |
| | 92.61% in the spinal cord [86] | | |
| Benefits | Replaced microglia are highly dynamic and phagocytic, preserve the immune response. | Replaced microglia are highly dynamic and phagocytic, preserve the immune response, in a fashion similar to microglia [101]. | Replaced microglia are highly dynamic and phagocytic, preserve the immune response. |
| | Ameliorates behavior or cognitive dysfunction and extends survival. | Easily accessible. | Replaced microglia resemble the characteristics of naive microglia. |
| | Allows for gene editing of donor cells. | CNS-wide replacement. | Allow for local replacement. |
| | CNS-wide replacement. | | |
| Limitations | Replaced microglia transcriptionally distinct from resident microglia [91, 99]. | Replaced microglia transcriptionally distinct from resident microglia [102]. | The source is rare. |
| | Replaced microglia may function differently from microglia [99]. | The replacement efficiency of Mr PB is lower than Mr BMT [88]. | The procedure for Mr MT is invasive [89]. |
| | Clinically hard to match due to transplant rejection. | | Transplanted microglia can migrate to untargeted brain regions. |
| | Possible immunosuppression and chemotherapy-associated toxicity. | | The replacement efficiency is minimal among 3 methods [86]. |
| Application | Disease progression of amyotrophic lateral sclerosis [96]; Alzheimer's disease [103]; lysosomal and peroxisomal storage diseases [104] | Relatively few studies | Relatively few studies |

## Targeting Microglial Therapeutics: Challenges and Opportunities

In this review, we summarized the critical pathological role of microglia in several neurodegenerative diseases, mostly focused on AD. We envision microglial replacement as a potential medical intervention for many neurological diseases such as PD and MS, but further research is needed.

What factors affect the replacement of microglia? Xu *et al*. reported that Mr BMT cells are mainly derived from CCR2-positive BMCs [86]. CCR2-positive cells are migratory cells that respond to neural insults [98]. Therefore, the function of CCR2 may be related to the mechanism of microglial replacement in the brain. In addition, a study has indicated that engrafted BM-derived myeloid cells display significantly increased levels of CD68, a lysosomal marker associated with a heightened activation or phagocytic state [99]; microglial engraftment affects astrocyte activation and neuronal communication [99]. Thus, molecules that affect microglial activation or interactions with other cells, such as CX3CR1/CX3CL1 and CD200/CD200R [100], might also affect microglial replacement. The removal rate of resident microglia directly affects the replacement efficiency of external them. Our recent research found that microglial debris is primarily removed by astrocytes in the brain *via* the opsonization of C4b [105]. Microglia engraftment likely affects astrocyte reactivation. Additional research into the mechanisms of microglial replacement would be beneficial for developing clinical therapies.

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REVIEW

# Updated Understanding of the Glial-Vascular Unit in Central Nervous System Disorders

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**Abstract** The concept of the glial-vascular unit (GVU) was raised recently to emphasize the close associations between brain cells and cerebral vessels, and their coordinated reactions to diverse neurological insults from a "glio-centric" view. GVU is a multicellular structure composed of glial cells, perivascular cells, and perivascular space. Each component is closely linked, collectively forming the GVU. The central roles of glial and perivascular cells and their multi-level interconnections in the GVU under normal conditions and in central nervous system (CNS) disorders have not been elucidated in detail. Here, we comprehensively review the intensive interactions between glial cells and perivascular cells in the niche of perivascular space, which take part in the modulation of cerebral blood flow and angiogenesis, formation of the blood-brain barrier, and clearance of neurotoxic wastes. Next, we discuss dysfunctions of the GVU in various neurological diseases, including ischemic stroke, spinal cord injury, Alzheimer's disease, and major depression disorder. In addition, we highlight the possible therapies targeting the GVU, which may have potential clinical applications.

**Keywords** Glial-vascular unit · Perivascular space · Blood-brain barrier · Glymphatic system · Ischemic stroke · Spinal cord injury · Alzheimer's disease · Major depression disorder

## Introduction

The concept of the neurovascular unit (NVU) was first raised in the 2001 Stroke Progress Review Group meeting of the National Institute of Neurological Disorders and Stroke, stimulating worldwide interest in stroke research (https://www.ninds.nih.gov/About-NINDS/Strategic-Plans-Evaluations/Strategic-Plans/Stroke-Progress-Review-Group). The NVU was widely recognized and applied in studies to delineate its complex and delicate crosstalk in multi-cellular structure. These cells work in concert to maintain central nervous system (CNS) homeostasis and fulfill diverse physiological functions [1–3]. Since the proposal of the NVU, brain cells and cerebral blood vessels are no more regarded as distinct entities, and neurodegenerative diseases, as well as cerebrovascular diseases, are assumed to share interdependent structural and functional mechanisms, greatly broadening our understanding of these diseases.

In recent years, the "glial-centric" network in the NVU, namely the glial-vascular unit (GVU) which focuses on the interactions of glial cells with other brain cell populations and cerebral vessels, has attracted increasing attention [2]. Given the fact that glial cells in the GVU are in close spatial contact with the vasculature and neighboring perivascular cells, they contribute to various critical functions that are associated with the relay of information in the CNS such as modulating cerebral blood flow (CBF), regulating angiogenesis, maintaining the integrity of the blood-brain barrier (BBB), and taking part in the clearance of brain

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waste-products [4]. Previous studies have also implied that glial-vascular coupling is affected in various neuroglial disorders [2, 3, 5]. Nevertheless, the comprehensive functional changes of the GVU have not yet been systematically illustrated.

In this review, we first give a short description of the components of the GVU and its physiological functions. Next, we underline the multiple roles of the GVU under pathological conditions. Finally, we highlight the possible treatments targeting the GVU in various CNS disorders. Since this review focuses mainly on the "glial-centric" network in the frame of the NVU, we aim to provide detailed and updated evidence for the indispensable roles of glial cells in supporting glial-neuro-vascular interactions.

## Components of the GVU

### Glial Cells as Components of the GVU

Astrocytes, the largest class of glial cells in the GVU, participate in nearly all vital functional activities in the CNS, correlating with their heterogeneity [6–8]. These cells are mainly subdivided into just two classes, namely protoplasmic and fibrous astrocytes. [4]. Of note, protoplasmic astrocytes in the gray matter make extensive contact with both neurons and local blood vessels. Their endfeet make contact with neurons as well as vasculature. Their fine peri-synaptic processes envelop the synapse to form a tripartite synapse and their large-diameter vascular processes encircle the cerebral microvessels and capillaries [9]. Furthermore, astrocytes are extensively coupled through gap junction channels [10]. Therefore, astrocytes actively participate in the formation of neurovascular coupling.

Making up 5%–15% of brain cells, microglia are the innate immune cells resident in the mammalian CNS [11]. They normally perform surveillance and scavenging functions and serve as the first line of defense against CNS diseases [12, 13]. In previous studies, microglia have been subdivided into the pro-inflammatory M1-type and the anti-inflammatory M2 type, which determines their dual functions in CNS disease [13]. However, this M1/M2 polarization based on the immune response is supposed to be discarded due to its failure to yield a meaningful account of the transcriptional profile of the cell and its non-macrophage-like functions [14, 15]. Instead of simply classifying microglia into M1 and M2 phenotypes, recent findings focus on both the transcriptional and the functional status of microglia [16–18]. For example, proliferation-associated microglia have been identified in development, and disease-associated microglia have been described in neurodegeneration. Considering the association between microglia and vasculature, vessel-associated microglia and capillary-associated microglia (CAMs) have also been confirmed [19]. Thus, microglia, as an indispensable part of the GVU, also play a key role in stabilizing neurovascular coupling.

Oligodendrocytes, serving as myelin-forming cells, develop from glial progenitor cells which originate in the ventricular zones in the CNS. The primary function of oligodendrocytes is wrapping axons and thus forming myelin sheaths, which facilitate the rapid transduction of action potentials in axons. Besides, oligodendrocytes also provide necessary metabolic support to other neural cells. Dysfunction of oligodendrocytes leads to demyelination of the CNS, eventually resulting in neuronal injury and cognitive impairment. Previous studies have reported an "oligovascular niche" in the CNS. Oligodendrocytes and their precursor cells crosstalk with other types of cells in the niche. Cerebral endothelial cells (ECs) secrete trophic factors that support the survival and proliferation of rat oligodendrocyte precursor cells (OPCs) [20]. Chavali et al demonstrated that OPCs also contribute to angiogenesis and vascular repair, especially in white matter, which may be an important target for vascular dementia [21].

### Perivascular Cells as Components of the GVU

Here, perivascular cells are defined as the cells located spatially around the cerebral vessels, including ECs, pericytes, and perivascular macrophages (PVMs) [6, 22, 23]. They have spatial and functional interactions with glial cells, together forming glial-vascular coupling.

Brain ECs are tightly sealed by expressed tight junctions, serving as a barrier between the blood and extracellular matrix (ECM). In fact, ECs and ECM, together with tight junctions and pericytes, form the BBB [24]. Besides, the physiological functions of ECs also include regulating vasoconstriction, secreting neurotrophic and signaling factors, and supplying the brain with nutrients by endothelial transporters, such as glucose through glucose transporter-1 [6].

Pericytes are also one of the cellular components of the perivascular space (PVS) and participate in the formation of the BBB [25]. The origin of pericytes can be traced back to 1873, and later Zimmermann coined the term 'pericytes' in 1923 [26]. Cerebral pericytes are perivascular cells located on capillaries that play an important role in CNS homeostasis. Pericytes participate in diverse neurological processes which are discussed below [25, 27].

PVMs are a group of resident macrophages located in the PVS [28], present mainly in the relatively large vessels; they are mostly found in the penetrating artery and ascending vein where smooth muscle cells (SMCs) occur [29]. Unlike microglia or meningeal macrophages developing from prenatal cellular sources in the yolk sac, PVMs originate from perinatal meningeal macrophages in an integrin-dependent

manner [30]. As innate immune cells in CNS, PVMs undertake the role of phagocytosis and mediate neutrophil recruitment [23]. Besides, PVMs also take part in the regulation of CBF, angiogenesis, and vessel permeability [31–33].

### PVS is the Functional Niche in the GVU

PVS was first described in detail by the German pathologist Rudolf Virchow and the French biologist Charles Philippe Robin, so it is also named Virchow-Robin space [34]. According to their descriptions, PVS is a gap space located under the outer membrane of cerebral vessels [34]. Until past decades, it had been acknowledged that the PVS is an extension of the subarachnoid space where vessels penetrate from the surface into the parenchyma. However, this conclusion has recently been challenged and modified [35]. It is now confirmed that the outer layer of the PVS, the glia limitans, is continuous with the pia mater rather than with the arachnoid [36]. Therefore, PVS is a virtual structure composed of the glia limitans and the vascular basement membrane, communicating directly with subpial rather than subarachnoid space [37]. At the level of capillaries, the glia limitans and basement membrane fuse to form a blind end (Fig. 1) [37].

An enlarged PVS has been observed in neuroimaging and this is associated with neurological disorders such as cerebral small vessel disease and cognitive deficits [38, 39]. However, its concrete role in CNS diseases has yet to be fully elucidated. In the past decade, it is demonstrated that the PVS participates in the clearance of brain waste-products together with other GVU components such as astrocytic endfeet [40, 41]. In short, structurally, the PVS occupies the central position in the GVU. Functionally, the PVS is a vital niche for fulfilling GVU functions.

## Multiple Physiological Functions of the GVU

Given the fact that glial cells and perivascular cells both have spatial and functional links, the synergistic contributions of both cell types may achieve various physiological functions of the GVU (Fig. 2).

### GVU Modulates CBF

One of the key functions of the GVU is regulating CBF in response to stimuli. Astrocytes cover nearly 99% of the capillary surface, suggesting they are involved in CBF regulation. A previous study first discovered the mechanism underlying functional hyperemia—the blood vessel responses related to neuronal activation. Neuronal activity increases the amount of intracellular $Ca^{2+}$ in the astrocytic endfeet, leading to the dilatation of local cerebral arterioles [42]. Later, a study showed that cortical astrocytes are responsible for rapid vasodilation. Photolysis of caged $Ca^{2+}$ in astrocytic endfeet enwrapping blood vessels results in a ~37% increase in CBF at the short latency of 1–2 s [43].

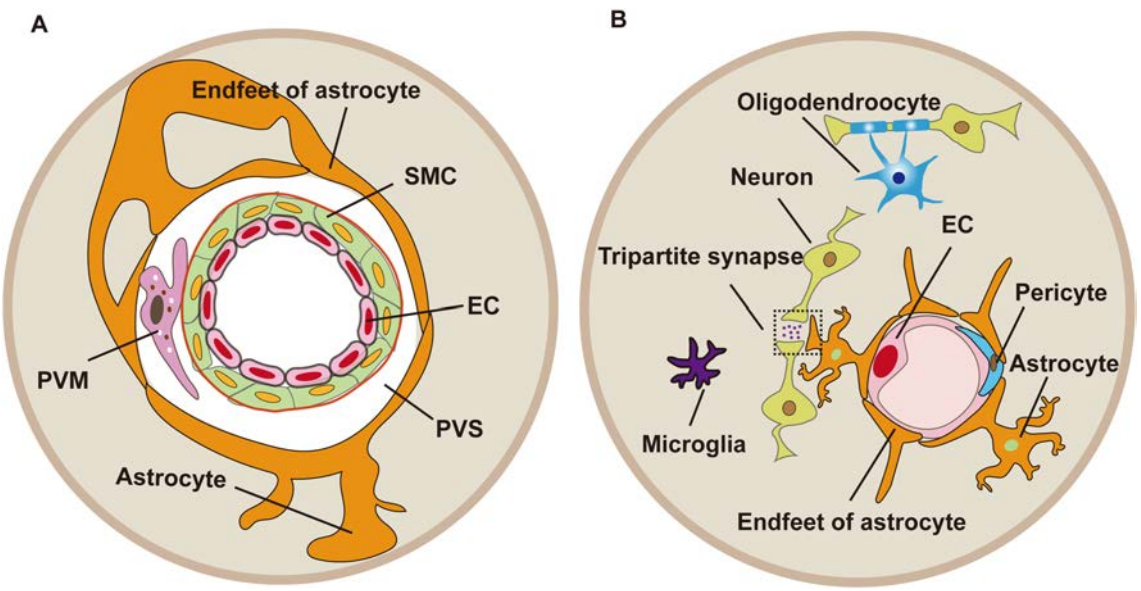


**Fig. 1** Schematic of the PVS in the GVU at the level of a penetrating artery and capillary. **A** The PVS around a penetrating artery is surrounded by astrocytic endfeet, and the inner side of the PVS is composed of vascular ECs and vascular basement membrane including SMCs. PVMs locate in the PVS, and CSF flows in the PVS from where it is transported into the parenchyma by AQP4 expressed on astrocytic endfeet, forming the glymphatic system. **B** At the level of capillaries, pericytes rather than PVMs locate in the PVS. Basement membrane and astrocytic endfeet gradually fuse, so the PVS finally diminishes and forms a blind end.

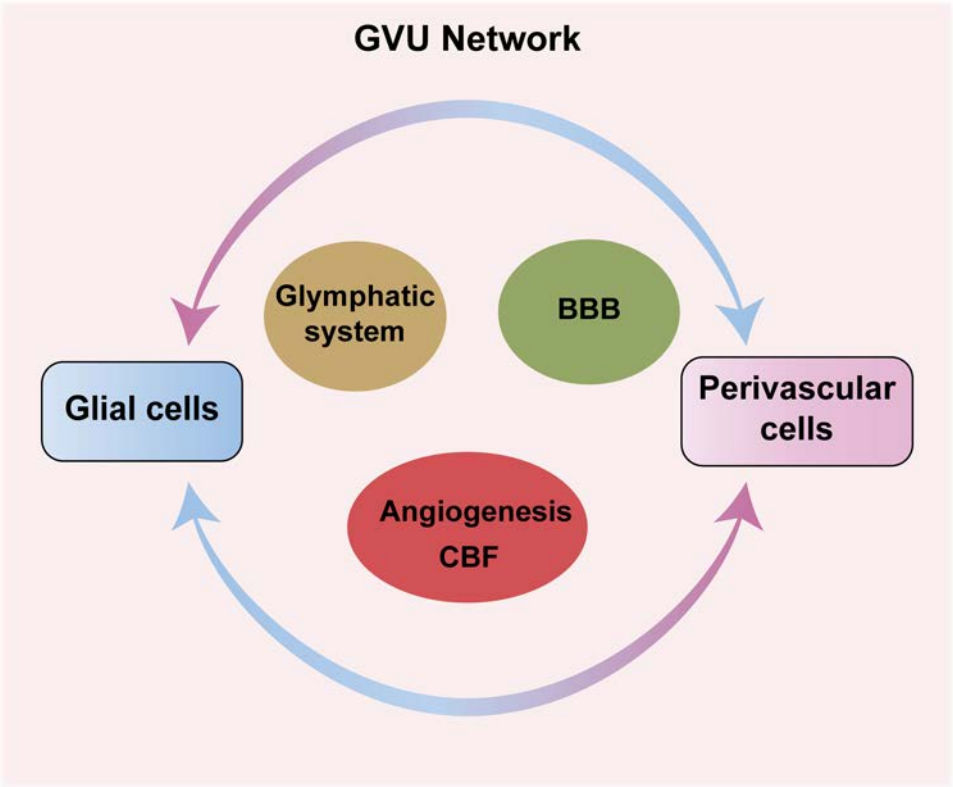


**Fig. 2** Schematic of the GVU functional network. The GVU is composed of glial cells including astrocytes and microglia, and perivascular cells including ECs, pericytes, and PVMs. The crosstalk of glial cells and perivascular cells in the PVS niche contributes to the successful fulfilment of diverse functions including the modulation of CBF and angiogenesis, the maintenance of BBB integrity, and the elimination of neurotoxic waste-products *via* the glymphatic system.

Subsequently, more detailed mechanisms of this astrocyte-mediated CBF response have been explored. Neurotransmitters released from active neurons evoke $Ca^{2+}$ increases in astrocytes, leading to the release of vasoactive substances from the astrocytic endfeet onto blood vessels. Prostaglandin E2 and epoxyeicosatrienoic acids dilate blood vessels, whereas 20-hydroxyeicosatetraenoic acid (20-HETE) constricts vessels. The release of $K^+$ from astrocytic endfeet may also contribute to vasodilation. Whether vasodilation or vasoconstriction occurs depends on the oxygen level. When the oxygen supply is normal, astrocytes prefer to mediate vasodilation, whereas, under hyperoxic conditions, vasoconstriction is favored. Besides, astrocytes also generate vascular tone by the tonic release of both 20-HETE and ATP onto SMCs [9, 44]. However, a recent study demonstrated different mechanisms of neurovascular coupling at the capillary and arteriole levels. The signaling to capillaries comes from the influx of $Ca^{2+}$ *via* the ionotropic ATP receptor P2X1. In contrast, the signaling to arterioles is mediated by the activation of N-methyl-D-aspartic acid receptors and the production of nitric oxide (NO) [45]. Since the astrocytic regulation of CBF is involved in different vasculature levels, the underlying mechanisms are controversial. The $Ca^{2+}$-dependent signaling may be a common pathway to control the CBF in neurovascular coupling.

While astrocytes play a role as critical regulators of CBF, microglia have also been increasingly implicated in CBF modulation during glial-vascular coupling. Recent studies have shown that microglia processes dynamically contact different levels of the vascular tree *in vivo*, and ~30% of resident microglia in adult mice are CAMs, indicating an indispensable role of microglia in regulating blood flow [19, 46, 47]. Indeed, the absence of microglia or blockade of the microglial P2Y12 receptor substantially impairs glial-vascular coupling in mice. The depletion of microglia reduces brain PH and impairs hypercapnia-induced vasodilation. The loss of CAMs also has a remarkable effect on vascular tone and CBF [19, 47]. While these studies shed light on the previously underestimated roles of microglia in the regulation of CBF, it remains open whether these resident immune cells modulate SMCs or pericytes directly to change the reactivity of vasculature and/or interact with astrocytes to exert joint or opposed roles in CBF regulation.

## GVU Modulates Angiogenesis

The GVU is also responsible for angiogenesis. Since astrocytes directly contact and ensheath blood vessels by their perivascular endfeet, they are necessary for the structure and function of ECs [6, 48]. By releasing high levels of factors such as VEGF or transforming growth factor 1β (TGF-1β), they play a pivotal role in facilitating vascular angiogenesis [49]. Furthermore, microvascular interactions are present in the brain in the early development stage and adulthood, thus

supporting the vasculature by participating in the formation of new blood vessels or angiogenesis. Depletion of microglia using genetic approaches deconstructs the vasculature complexity [11]. In summary, glial-vascular interaction in the GVU plays a vital role in regulating CBF and facilitating angiogenesis under physiological conditions to maintain CNS homeostasis.

Since pericytes are perivascular mural cells in brain capillaries, they likely control the CBF within the microcirculation just like vascular SMCs. A study using loss-of-function pericyte-deficient mice has shown that pericyte degeneration diminishes the capillary CBF responses to neuronal stimuli, resulting in neurovascular uncoupling and reduced oxygen supply to the brain. These pericytes-induced vascular deficits are regarded as the key mechanisms that participate in various neurological disorders such as stroke and Alzheimer's disease (AD) [50]. Later, the same group used diphtheria toxin to induce acute pericyte loss, further verifying that pericyte loss leads to reductions in stimulus-induced CBF responses and neurovascular uncoupling [51].

## GVU Modulates Neurogenesis

Glial cells are an essential part of all neurogenic niches in the embryonic period as well as in the adult CNS. Thus, glial cells play crucial roles in the production and control of neural stem cells (NSCs) from the neurogenic niches. Embryonic radial glia cells are not only the primary source of neurons and glia but also the adult NSCs in the subependymal zone, while in the subgranular zone (SGZ), astrocytes promote neuronal fate commitment and proliferation of SGZ NSCs through factors such as Wnt and EphrinB2. Strikingly, astrocytes outside the neurogenic niches may experience proliferation and plasticity when invasive injuries occur, acquiring NSC features through Shh signaling [52]. Besides, the role of astrocytes in modulating neuronal circuits within synapses has also been emphasized [53]. Substances secreted by astrocytes play a role in the formation of synapses in the CNS. For example, immature astrocytes express thrombospondins that promote synaptogenesis both *in vitro* and *in vivo* [54]. Involved in phagocytic activity and immune response, microglia may also affect synaptic activity by phagocytosis and the complement cascade [12, 55]. Microglia express high levels of complement components q (C1q) and C3, and the binding of these complement components triggers the elimination of complement-tagged synapses. Lack of C1q and C3 leads to the failure of refinement of synaptic connections [12, 55, 56]. This phagocytic phenomenon also occurs in the adult hippocampus. During the transition from neuroprogenitor cells to neuroblasts in the SGZ, phagocytosis by microglia is essential for maintaining the homeostasis of the neurogenic cascade [57]. In all, glial cells are actively involved in neurogenesis.

## GVU Participates in the Formation of the BBB

In the early 20th century, it was discovered that intravenously-injected trypan blue cannot enter the brain and spinal cord, although it distributes widely in peripheral tissues, suggesting the existence of a unique barrier protecting the CNS [58]. Now it is clear that this special barrier is the BBB, consisting of astrocytes and perivascular cells such as tightly-sealed ECs, pericytes, and basement membrane. The main functions of the BBB include supplying the brain with essential nutrients and preventing the movement of circulatory ions and fluids into the brain parenchyma [6].

GVU components participate in the formation of the BBB. Cellular junctions between ECs include tight junctions (occludin, claudins, and membrane-associated guanylate kinases such as ZO-1), adherens junctions (cadherins and PECAM-1), gap junctions (the connexin family of hemichannels), the cytoskeleton (dystrophin), and other junctional molecules [37]. ECs interact with the ECM in the vascular basement membrane *via* integrin receptors [59]. ECM proteins include laminin, collagen, and perlecan [60]. Collagens, the most abundant proteins in mammals, are deposited in the ECM and take part in the formation of the BBB [61]. Collagens in the ECM crosslink and form a network of supramolecular assemblies, such as a triple helix [62]. Just as stated by Hynes for the ECM, "collagens are not just pretty fibrils" [63]. They function mainly as structural support for vessels. Besides, collagens are also crucial for maintaining the integrity of the BBB [63]. Matrix metalloproteinases (MMPs), zinc-dependent endopeptidases belonging to the metzincin superfamily, cleave collagens and thus play an essential role in BBB breakdown [64]. As noted above, PVS exists just between the ECM and astrocytic endfeet.

## GVU Takes Part in Brain Waste Clearance

The GVU plays a conducting role in the process of brain-waste clearance, as revealed by the research group of M. Nedergaard, who proposed the novel hypothesis of the glymphatic system [40, 41]. In their studies, CSF was labeled by fluorescent tracers of different molecular sizes injected through the cisterna magna. By applying two-photon microscopy, labeled CSF was observed circulating from the cisterna magna into the PVS of the penetrating arteries. Here, CSF is transported from the PVS into the parenchymal interstitial space by aquaporin-4 (AQP4) proteins expressed on the astrocytic endfeet. CSF enters the parenchyma and forms bulk flow, removing the waste produced by neural activity such as amyloid-β. Bulk flow then

transports the waste into perivenous spaces and finally out of the brain [40]. The impaired glymphatic function in AQP4-null mice confirms this hypothesis [40]. The driving force of CSF in the PVS is mainly arterial pulsation [65, 66]. However, this viewpoint has recently been challenged by a study conducted on awake mice. According to the study, this clearance of solutes from the brain is mainly driven by ultra-slow arteriolar SMC oscillations [67]. The glymphatic system has attracted increasing interest due to its unique role in neurotoxic waste-clearance and its functional connection to the meningeal vessel, which is an immune structure in the CNS first discovered in 2015 [68, 69]. Numerous studies have been carried out to explore the detailed pathological functions of the PVS in the frame of the glymphatic system in different neurological disorders.

Besides astrocytes regulating glymphatic function *via* AQP4 polarity expressed on the endfeet, pericytes may also take part in the regulation of glymphatic system function. It has been shown that the density of AQP4 is higher on the side of astrocytes facing pericytes than on the side facing endothelial cells [70]. Deficiency of pericytes results in the abnormal polarization of AQP4 [25]. In addition, AQP4 expression is dramatically reduced and depolarized in old pericytic laminin-deficient mice, suggesting that pericyte laminin is associated with the distribution of AQP4 [71]. A recent study has shown that the development of the glymphatic system is delayed in PDGFβ$^{ret/ret}$ mice without normal platelet-derived growth factor subunit β (PDGFRβ) function. The functional deletion of PDGFRβ reduces AQP4 polarization, which indicates that pericytes play a vital role in the development of the glymphatic system, although other cells expressing PDGFRβ or disruption of the BBB cannot be excluded [72].

In addition to the widely-accepted glymphatic system outlined above, there is also an "intra-mural perivascular drainage pathway" hypothesis; this might also be one of the clearance pathways in the GVU [73]. Tracers injected into brain parenchyma diffuse through the extracellular space and enter the basement membranes of capillaries to drain out of the brain along the basement membranes in the tunica media of arteries [73]. Unlike the glymphatic system, this pathway is claimed to flow in a reverse direction to that of the blood, and drains through penetrating arteries into the subarachnoid spaces [74, 75]. Due to the limited resolution of the two-photon microscope, these two types of fluid transport cannot be distinguished [40, 76]. Besides, it has also been reported that β-amyloid stimulates ECs, recruiting monocytes from the circulation into the PVS. Recruited monocytes eliminate β-amyloid by endocytosis [77]. Since PVMs are located precisely in PVS and are capable of phagocytosis, there may also be a place for PVMs here. Further research is needed to verify the existence of the reverse intra-mural perivascular drainage pathway model and other possible clearance patterns in the GVU.

## Functional Changes of the GVU in Neurological Disorders

Beyond the roles under physiological conditions, the GVU may experience dramatic dysfunction under pathological conditions, contributing to neurological disorders. In general, glial cells and perivascular cells are activated and mutually interact, all contributing to CBF dysregulation and reduction and BBB breakdown, as well as a decrease in waste clearance, the three most evident alterations during the initiation and progression of pathology (Fig 3). Here, we introduce these pathological alterations of the GVU in various major neurological diseases.

### GVU and Ischemic Stroke

Ischemic stroke is defined as an acute neurological deficit due to decreased CBF. It is the third-leading cause of death and disability combined and the second-leading cause of death in the world, causing a tremendous social burden [78, 79]. As the population ages, the incidence of ischemic stroke is continuously increasing [80]. The primary pathological lesion in ischemic stroke is cerebral infarction caused by embolism or vessel diseases [81].

Abnormal CBF and reduced angiogenesis have been reported in ischemic stroke patients. The dysfunction of the GVU exerts a great influence on them. First, pericytes residing in the microvessels constrict the vessels and impede capillary blood flow during arterial obstruction, developing a no-reflow phenomenon [82–84]. Aside from these detrimental roles, pericytes also play beneficial roles *via* promoting angiogenesis. They migrate to the infarcted area and promote angiogenesis and blood vessel stabilization [85]. However, insufficient angiogenesis may lead to leaky blood vessels and brain hemorrhage [86, 87]. Second, in the infarct region, swollen astrocytes compress cerebral vessels, leading to a reduction in CBF, while VEGF is upregulated in the penumbra after cerebral ischemia. VEGF-A is associated with EC proliferation and VEGF-B mediates embryonic angiogenesis. Besides, the upregulation of VEGF increases CBF that maintains the penumbral blood supply, while it can also activate MMP-9, disrupting tight gap junctions and leading to BBB breakdown [88]. Emerging evidence indicates that microglia can also influence the process of angiogenesis. Through the expression of CD68, microglia disintegrate blood vessels, while VEGF released by microglia promotes the reconstruction of cerebral blood vessels [13].

It has been demonstrated that the structure and function of the BBB are impaired after ischemic stroke. First,

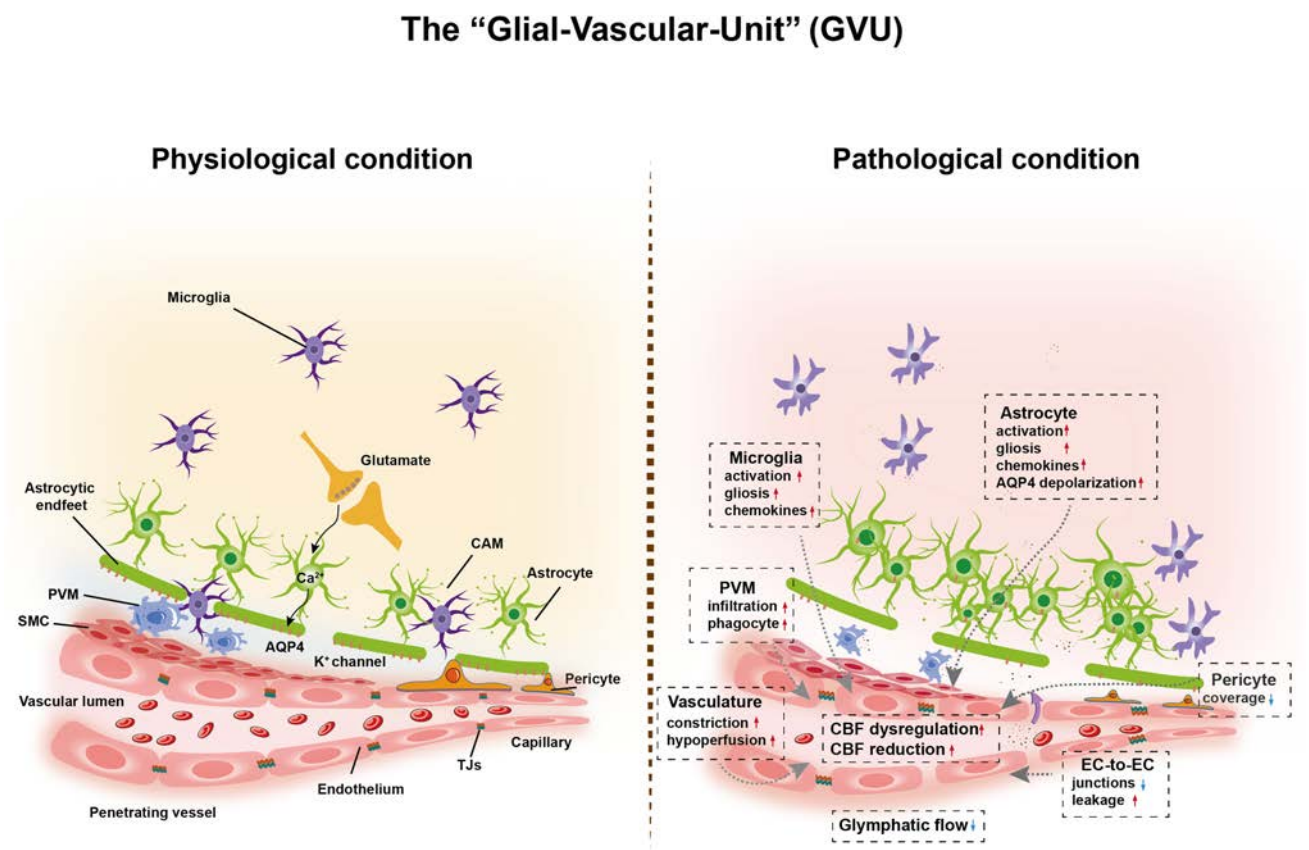


**Fig. 3** Comparison of the GVU under physiological and pathological conditions. The individual components of the GVU work in concert to execute normal functions under physiological conditions (e.g., tripartite synapses). To facilitate glial-neuronal-vascular transport and signal transduction, microglia maintain CNS homeostasis by phagocytosis and immune modulation, pericytes cover the capillary surface to modulate CBF, and the PVS and PVMs participate in the brain-waste clearance system). Under pathological conditions, responses to stimuli have been reported, including neuronal death and synapse degeneration, gliosis, release of inflammatory chemokines, BBB leakage, and impaired waste clearance.

comorbid conditions of ischemic stroke such as hypertension and hyperglycemia can lead to BBB dysfunction [89]. Second, glial cells respond quickly to several stimuli soon after the insult, including reactive oxygen species, necrotic cell debris, and impaired tissues [13]. Reactive and proliferating astrocytes and microglia crosstalk with each other, releasing cytokines and chemokines, exacerbating neuroinflammation, and impairing the integrity of the BBB. For example, astrocytes crosstalk with microglia *via* lipocalin-2 [90]. In turn, reactive microglia and their fragmented mitochondria also enable the activation of astrocytes [91]. This process further exacerbates neuroinflammation, which forms a vicious cycle leading to negative neurological outcomes [90–92]. Third, dysfunction of perivascular cells also occurs after a stroke. During ischemia, the production of ATP in ECs is severely reduced, affecting the function of ion pumps, resulting in enhanced anaerobic glycolysis, and eventually causing tissue damage [93]. Early loss of pericytes after stroke has been reported in both murine models and human samples [94]. Considering the significant roles pericytes play in the maintenance of BBB integrity, it is unsurprising that the loss of pericytes leads to leakage of the BBB. A recent study has revealed that transplantation of human pluripotent stem cell-derived pericyte-like cells promotes the BBB and functional recovery in mice with ischemic stroke, also suggesting a causal relationship between pericytes and the BBB. Besides, PVMs promote vascular leakage by recruiting granulocytes and increasing the expression of chemokines such as VEGF [95].

The ability of the GVU to clear waste-products is impaired after ischemic stroke. Glymphatic dysfunction is found both in mice and humans after ischemic stroke [96, 97]. However, most of the initial studies in this relatively new area are limited to simply uncovering the phenomenon of glymphatic change in the stroke, but do not fully explain the mechanisms of how an altered glymphatic system affects neurological functions. Considering the significant role the glymphatic system plays in brain waste-clearance such as neuroinflammatory factors and β-amyloid, it is believed that its dysfunction may contribute to both neuroinflammation

and cognitive deficits in the long term. It has been demonstrated that impairment of the glymphatic system leads to cognitive decline in chronic cerebral ischemia [98]. A recent study has revealed that CSF enters the parenchyma through the PVS within minutes after an ischemic attack. Subsequent vasoconstriction induced by ischemic spreading depolarization enlarges the PVS, which in turn increases the inflow of CSF, eventually leading to post-stroke edema [99]. This suggests that unbalanced CSF flow may exacerbate the outcome after ischemic insult.

## GVU and Traumatic Spinal Cord Injury (SCI)

Traumatic SCI is regarded as an acute disorder caused by sudden insults (for example, a motor vehicle accident, fall, or sports-related injury) that acutely damages the spinal cord, resulting in temporary or permanent neurological deficits [100]. The pathophysiological process of SCI is initiated by the sudden death of neurons and glial cells, which is followed by the formation of glial scar and cystic cavities, concomitant with severe inflammatory responses [100].

Dysfunction of the GVU causes inflammatory cascade responses, gliosis, and the release of inhibitory molecules, all contributing to the exacerbation of disease progression. The close interaction between components in the GVU actively participates in SCI [101]. Elucidating their roles in the SCI environment is crucial to harness GVU-targeted therapeutic potential for repair after SCI.

Gliosis and the inhibitory environment are mostly attributable to the activation of astrocytes in the GVU during different phases of the disease [102]. In the early phase after injury, the processes and soma of astrocytes undergo hypertrophy, proliferation increases, and intermediate filaments Glial fibrillary acidic protein, vimentin, and nestin are upregulated [8, 49]. These changes are known as reactive astrogliosis. Reactive astrogliosis is triggered and modulated by various key molecular signaling pathways. For example, activation of the STAT3, TGF-β, and NF-κB transcription factors trigger downstream signaling pathways involved in astrogliosis after SCI [102]. Reactive astrogliosis has beneficial effects on the repair of SCI, so this process is considered to be a defense mechanism of astrocytes against injury. In addition, astrocytes reconstruct the damaged blood-spinal cord barrier (BSCB) and limit the infiltration of peripheral leukocytes and the activation of resident microglia. Reactive astrogliosis also encircles the injury epicenter, preventing the normal tissue from being obstructed. Aside from being a physical barrier, reactive astrocytes also upregulate the expression of FGF-2 and S100β to promote tissue repair and regeneration [101–103]. Furthermore, a study has uncovered a new role for astrocytes. It is implied that injury induces an autologous astroglia conversion towards a neuronal lineage that may be a therapeutic strategy to replace neuronal loss after SCI [104].

At later time points after injury, astrocytes are known to play detrimental roles in axon regeneration and inflammatory responses. Astrocytic scars form barriers that prevent CNS axons from regrowing and attenuating astrocyte-formation enables spontaneous axon regrowth. However, instead of preventing axon regeneration, astrocyte scar formation may serve as a bridge to aid axon regrowth in the CNS [105]. Reactive astrocytes also contribute to the release of inhibitory ECM components like chondroitin sulfate proteoglycans (CSPGs) after SCI. These inhibitory molecules pose physical and chemical barriers to axon regeneration [102]. CSPGs also inhibit the differentiation of OPCs into mature oligodendrocytes, thus preventing myelination *in vitro*. The degradation of CSPGs by chondroitinase ABC enhances axonal regeneration through the glial scar after SCI [102]. Besides the detrimental effects of CSPGs released by astrocytes, recent studies have also reported that astrocytes secrete factors such as insulin-like growth factor-binding protein 6 and decorin that inhibit adult neural stem cell differentiation into neurons after SCI [102].

Microglia, as immune cells in the CNS, also play a vital role in SCI. After the injury, microglia are responsible for the phagocytosis of cell debris, and they also participate in forming the glial scar near the injury center together with astrocytes to isolate the injured area [101]. Most importantly, the effects of microglia on ECs greatly influence the integrity of the BSCB, a special endothelial structure that is a continuation of the BBB, selectively separating the blood circulation from the parenchyma. Microglia may lead to the increased permeability of the BSCB. Activated microglia release reactive oxygen species (iNOS and NO), resulting in oxidative damage of ECs, which increases the permeability of the BSCB [106]. IL-6 derived from activated microglia also leads to STAT3 activation in ECs, resulting in increased endothelial permeability through downregulation of tight gap junctions like occludin and ZO-1 [107]. However, hypoxia-induced microglia also play protective roles by releasing IGF-1 to induce angiogenesis. The anti-inflammatory cytokines secreted by microglia facilitate angiogenesis and neurogenesis, leading to better locomotor recovery [5, 108–110]. In brief, microglia-derived inflammatory cytokines increase the permeability of the BSCB, while anti-inflammatory cytokines contribute to angiogenesis by various signaling pathways. Microglia also have an intense interaction with astrocytes by affecting their proliferation and differentiation. Pro-inflammatory microglia secrete IL-1α, TNF, and C1q that induce neurotoxic A1 astrocytes, and these abnormal astrocytes have the detrimental effect of killing neurons and mature oligodendrocytes [111]. However, anti-inflammatory microglia induce neuroprotective A2

astrocytes that support the growth of neurons [111, 112]. Conclusively, GVU exerts vital and multifaceted functions in SCI, and they depend on both the active states of the components in the GVU and the different phases of the disease.

## GVU and AD

AD is a neurodegenerative disease that brings progressive cognitive decline and memory loss, characterized by neurofibrillary tangles (NFTs) and aggregates of amyloid-β as neuritic plaques [113, 114]. According to the two-hit hypothesis of AD, vascular dysfunction including vascular disruption with CBF reduction, and BBB breakdown with neurotoxic waste accumulation, are actively involved in the early pathogenesis of AD [115, 116].

The glial components of the GVU contribute to the regulation of CBF and functional hyperemia. Abnormal astrocytic activity may cause vasculature instability. Two-photon *in vivo* imaging of astrocytic $Ca^{2+}$ signaling reveals that animals with abnormal astrocytic activity also display unstable vascular tone as well as reduced or absent vasodilation in response to stimulation [117]. Microvascular amyloid-β deposition reduces the expression of astrocytic $K^+$ channels, compromising the neurovascular coupling [118]. Moreover, the change of astrocytic morphology alters the normal coverage of astrocytic endfeet around the microvascular network, thus adversely affecting the neurovascular coupling, CBF regulation, and glymphatic system function [118, 119].

The clearance of neurotoxic waste is markedly impaired in AD. Several mechanisms contribute to the removal of soluble interstitial amyloid-β from the brain, including degradation by cells of the GVU such as astrocytes and PVMs, transport across the BBB, and elimination *via* the glymphatic system [120]. Remarkable cognitive decline occurs in AQP4-null mice that lack a glymphatic pathway [121]. Besides, AQP4 the polarity inhibitor TGN-020 clearly exacerbates glymphatic CSF–interstitial fluid exchange and tau protein clearance [122]. This evidence suggests that the glymphatic system plays an irreplaceable role in AD.

That the glymphatic system is damaged in AD is based on the following evidence. First, postmortem AD brains have revealed the perivascular mislocation of AQP4, which is essential to the glymphatic pathway [123]. Second, the accumulation of amyloid-β in the vessel wall leads to a decrease in vascular pulsation and PVS dimensions, thus reducing the influx to the glymphatic pathway. Third, AD occurs in the aged with sleep disturbance as a common comorbidity [124, 125]. Sleep disturbance and ageing are reported to affect the directional movement of CSF in the PVS [126, 127].

Dysfunction of the brain-waste clearance system results in an overburden of noxious products. Under such circumstances, microglia are activated, leading to neuroinflammation, while astrocytes undergo lysis and release their cytoplasmic contents, forming astrocytic amyloid plaques. These small plaques, together with large neuron-derived plaques, constitute the amyloid plaque formation during AD pathogenesis [113].

BBB breakdown also participates in the progression of AD. Both astrocytes and microglia contribute to the increased permeability of the BBB, driving secondary injury mechanisms [114]. Reactive astrocytes result in the decoupling of astrocyte endfeet and ECs, which compromises BBB integrity [128]. Astrocytes also secrete VEGFs to activate endothelial MMP-9, leading to the reduced expression of claudin-5, which also increases BBB permeability [129]. The activated MMP-9 also degrades the astrocytic endfeet, thus impairing the $K^+$ buffering, increasing neuronal excitability, and ultimately leading to cognitive deficits [130]. In addition, the APOE4 carried by astrocytes can cause BBB dysfunction, and is the strongest genetic risk factor for AD [131]. Besides, astrocytes are one of the main contributors to cerebral amyloid angiopathy (CAA). The harmful cytokines secreted by astrocytes induce BBB leakage in the early stage of CAA [132]. Also, the overexpression of transforming growth factor-β1 in astrocytes results in excessive amyloid-β accumulation in microvessels, aggravating CAA [133]. Accumulating evidence has shown that microglia influence the integrity of the BBB in AD. Activated microglia engulf ECs and astrocyte endfeet, which damages the BBB by releasing various inflammatory and neurotoxic mediators. MMP-9 is released to destroy the basement membrane and induce reactive oxygen species (ROS) causing a dramatic loss of tight junctions [134]. In addition, the specifically-expressed TREM2 gene is also involved in late-onset AD. The loss of TREM2 leads to a failure of the microglial transition to disease-associated microglia, impairing their ability to engulf harmful substances [135], and the overexpression of TREM2 upregulates the expression of phagocytic genes in microglia to enhance their phagocytosis of amyloid-β [136]. Collectively, dysfunction of the GVU and individual GVU components results in the initiation and persistence of AD pathology, compromising brain functions.

## GVU and Major Depressive Disorder (MDD)

Depression is a worldwide mental disorder characterized by a persistent feeling of sadness, anhedonia, and associated deficits in daily functioning [137]. The overall lifetime risk of depression is 15%–18%, and the 12-month prevalence of MDD is ~6% [138]. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), MDD is defined as the daily presence of five or more of the key symptoms for at least two weeks including depressed mood, anhedonia, feelings of worthlessness or guilt, suicidal attempt, sleep disturbance, and weight and appetite loss [138, 139]. Currently,

the complex pathogenesis of MDD is not yet understood, but proposals include the biogenic amine hypothesis, the receptor hypothesis, and the neuroendocrine hypothesis [140]. Recent studies have also suggested that glial-vascular coupling is implicated in MDD. Clinical MDD is characterized by abnormal CBF. CBF is normally maintained and regulated by CBF self-regulation (CBFSR), which is significantly impaired in depressed patients. The underlying mechanisms include EC dysfunction and the overactivation of microglia. Dysfunction of ECs is mainly attributed to their activation and injury, weakening endothelium-mediated vasodilation [141]. The amount of NO produced by ECs is also reduced, impairing the vasodilation and adversely affecting CBFSR. Hypertrophy of SMCs also decreases the capacity for vascular dilation [142]. The over-activation of microglia affects CBFSR through the production of pro-inflammatory cytokines like IL-6 [143].

Both preclinical and clinical studies have confirmed that BBB integrity is damaged in depressive disorders. In a mouse model of chronic social defeat stress, BBB leakage has been reported in the nucleus accumbens, and this is mediated by loss of the tight junction protein claudin-5 [144, 145]. In addition, stressed animals exhibit a greater BBB permeability to 40-kDa dextran by using two-photon microscopy *in vivo*, also suggesting BBB leakage in the MDD mice. In a cross-sectional study of a small population of elderly women, in whom BBB integrity was measured using the CSF/serum albumin ratio, it was revealed that the ratio in women with MDD is higher than in those without MDD, suggesting that BBB dysfunction also occurs in humans [146]. Since the presence of BBB leakage in MDD has been verified [144–146], further studies are needed to investigate how BBB breakdown occurs in MDD, how to prevent it, and how to use it to make an early diagnosis and eventually eliminate this intractable disease.

The mechanisms underlying the disruption of the BBB in MDD remain to be fully explicated. The pathogenesis is now assumed to concern epigenetic regulation in neurological diseases [147]. Dudek et al. found that down-regulation of claudin-5 expression in MDD mice is associated with the permissive epigenetic and transcriptional regulation of the claudin-5 gene promoter, hdac1 [145]. Intriguingly, the increase of BBB permeability leads to the infiltration of stress-induced circulating cytokines such as IL-6. In turn, it has been reported that IL-6 treatment promotes the phosphorylation of tight junction proteins, increasing the BBB permeability in cultured brain ECs [148]. Therefore, MDD-initiated BBB dysfunction may lead to a vicious cycle to exacerbate MDD, possibly associated with neuroinflammation.

As the old saying goes, “every coin has two sides”. Disruption of the BBB in MDD also provides an opportunity to make an early diagnosis using biomarkers with high accuracy as well as sensitivity. It is claimed that the CSF levels of amyloid-β-42, neurofilament protein light, and glial fibrillary acidic protein are increased in patients with MDD [149], suggesting the existence of a CSF biomarker profile associated with depression. However, obtaining CSF is a relatively invasive procedure, which restricts its clinical utility. In contrast, the acquisition of peripheral blood samples is much more convenient. The serum level of S100β, a protein expressed and secreted by CNS glial cells such as astrocytes, is reported to be higher in MDD patients than in healthy controls, and decreases slightly after medical treatment [150]. Further research is needed to explore more specific biomarkers of MDD for diagnosis.

Several independent studies have confirmed that the function of the glymphatic system is suppressed in depression. In the mouse model of chronic unpredictable mild stress, it has been revealed that the impairment of the glymphatic system is related to the depolarization of AQP4 [151, 152]. Besides, reduced arterial pulsation and compliance may be another reason for glymphatic dysfunction [151]. In addition, the capacity for waste clearance is dramatically enhanced during sleep, especially in the NREM phase [126]. Therefore, sleep disturbance, a common concomitant and prodromal symptom of depression, is supposed to regulate glymphatic functions [153]. Our recent unpublished work also suggests that melatonin seems to relieve the symptoms of depression by regulating the structure of sleep phases, among which the glymphatic system may act as a bridge.

It is worth noting that impaired glymphatic function is associated with cognitive deficits in depression. Although both a polyunsaturated fatty acid (PUFA) diet and escitalopram ameliorate the symptoms of depression, only supplements of PUFA rescue glymphatic dysfunction and eventually improve cognitive performance in depression [151]. These studies suggest that the glymphatic system is a potential therapeutic target for depression-associated cognitive decline.

## Therapeutic Strategies Focused on the Regulation of GVU Functions

As described above, GVU plays a key role in maintaining sufficient CBF and promoting angiogenesis, thus therapeutic strategies targeting vascular functions in the GVU are thought to be reasonable. To prevent capillary constriction and maintain sufficient CBF, ROS/reactive nitrogen species suppression and removal of external $Ca^{2+}$ [83, 154], as well as the administration of adenosine and sodium nitroprusside, are reliable [155]. Furthermore, a RAGE (receptor for advanced glycation endproducts)-specific inhibitor can mediate the recovery of CBF [154]. The vasodilator C-type natriuretic peptide is also regarded as a potential therapeutic

target for reversing CBF reduction [156]. To promote angiogenesis and vascular stability, increasing VEGF signaling could be therapeutic by facilitating proper angiogenesis. Targeting TGFβ signaling could also promote pericyte proliferation and attachment to the vessels, promoting vascular stability [157, 158]. In addition, drugs like edaravone (a free radical scavenger) and cilostazol (a phosphodiesterase-3 inhibitor) have been shown to promote angiogenesis and maintain vascular integrity [159, 160].

The GVU participates in the maintenance of BBB integrity, thus targeting the components of the GVU may be a feasible means of protecting the BBB during neurological insults. In clinical practice, steroids are frequently applied to reduce the inflammatory response, breaking the vicious cycle between BBB leakage and neuroinflammation in the GVU after insults. For instance, dexamethasone was claimed to reduce BBB permeability and brain edema after stroke in a preclinical study [89, 161]. However, few of them have been successful in clinical trials, perhaps due to the benefits as well as the detrimental effects of inflammation after stroke [162]. Therefore, it is necessary to explore the possibility of regulating the GVU with high specificity. One potential approach is inhibiting the secretion of factors harmful to tissue by GVU components. As noted above, glia-secreted MMP-9 plays a crucial role in BBB degeneration. A series of studies uncovered that TPPU [1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea] protects BBB integrity by inhibiting the secretions of MMP-9 in the GVU following ischemic and hemorrhagic stroke [163, 164]. Another approach is GVU component replacement therapies. As mentioned above, transplantation of stem cells derived from pericyte-like cells, which express most markers of pericytes, ameliorates neurological dysfunction by reconstructing BBB integrity in a mouse stroke model [165]. In addition, transplantation of mesenchymal stem cell-derived pericytes into amyloid model mice reduces the level of amyloid-β in the brain, which might be promising for AD treatment [166].

Considering that the glymphatic system plays an important role in preventing the accumulation of β-amyloid, and maintaining the homeostasis of the microenvironment, it may serve as a therapeutic target in diverse neurological disorders. Several studies have demonstrated that ameliorating the function of the glymphatic system is beneficial for recovery from the primary disease and comorbid cognitive deficits in the long term. For example, PUFA supplementation ameliorates both depressive behaviors and cognitive impairment in mice under chronic unpredictable mild stress *via* repairing the glymphatic system dysfunction [151]. Glibenclamide treatment improves the function of the glymphatic system, alleviating brain edema and cognitive deficits after status epilepticus [167]. Digoxin rescues white matter injury and cognitive impairment after chronic cerebral ischemia through improving glymphatic system function [98]. However, the above interventions are not glymphatic-specific, which means that they may have a wide range of targets, limiting their utility in clinical practice. Since polarized AQP4 expressed on astrocytic endfeet is a key protein in fulfilling glymphatic function [40], strategies aimed at regulating AQP4 polarization may be promising in the future. Recent preclinical studies reveal that some microRNAs have effects on the polarized expression of AQP4, but further research is needed to explore the possibility of their clinical application [168].

## Conclusions

In this review, in order to gain a more precise understanding of glial cells, we regard the GVU as a complex multi-cellular structure including glial cells and perivascular cells. The functions of the GVU are summarized as modulating CBF and angiogenesis, maintaining BBB structure, and transporting brain wastes. Therapies targeting the GVU may be promising in diverse neurological diseases such as ischemic stroke, SCI, AD, and MDD.

Many issues concerning the structure and function of the GVU remain to be explicated. First, previous studies on neuro-vascular coupling have focused more on the concept of the NVU, namely the interaction between neurons and vessels, ignoring the essential roles of glial cells. Therefore, we update the concept of NVU with a specific "gliocentric" view to emphasize the central and significant role of glial cells. Second, knowledge of the essential functions of glial-vascular coupling in maintaining CNS homeostasis is limited [2, 11, 21, 48]. The functions of the GVU are summarized: CBF regulation and angiogenesis, BBB integrity support, and brain waste clearance. Third, for the first time, the PVS niche is introduced into the concept of the GVU. The novel glymphatic pathway is structurally highly dependent on glial cells and the PVS niche, and is attracting increasing attention for its unique role in maintaining microenvironmental homeostasis [169]. This is a useful extension of the concept of the GVU.

Meanwhile, we propose some potential research orientations as follows. Emerging studies show that pericytes are multipotent stem cells that play an important role in tissue injury and regenerative processes [170, 171]. Brain pericytes in regions of injury can produce ischemia-induced multipotent stem cells [172]. Besides, pericytes can also differentiate into cells expressing biomarkers of astrocytes, neurons, and oligodendrocytes in the presence of basic fibroblast growth factor [173]. Reactive astrocytes also share many characteristic hallmarks with NSCs [174]. For instance, reactive astrocytes isolated from the stroke peri-infarct can differentiate into NSCs [175]. Reactive astrocytes can also form self-renewing and multipotent neurospheres *in vitro* [176].

Therefore, the multipotent potential of GVU components has implications for regeneration strategies for CNS injury. In addition, the individual component in the GVU may play dual roles, protective or detrimental, *via* different mechanisms. Therefore, approaches that modulate GVU functions are still controversial. More work is needed to investigate approaches targeting the GVU in clinical applications in the future.

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REVIEW

# Roles of NG2 Glia in Cerebral Small Vessel Disease

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**Abstract** Cerebral small vessel disease (CSVD) is one of the most prevalent pathologic processes affecting 5% of people over 50 years of age and contributing to 45% of dementia cases. Increasing evidence has demonstrated the pathological roles of chronic hypoperfusion, impaired cerebral vascular reactivity, and leakage of the blood–brain barrier in CSVD. However, the pathogenesis of CSVD remains elusive thus far, and no radical treatment has been developed. NG2 glia, also known as oligodendrocyte precursor cells, are the fourth type of glial cell in addition to astrocytes, microglia, and oligodendrocytes in the mammalian central nervous system. Many novel functions for NG2 glia in physiological and pathological states have recently been revealed. In this review, we discuss the role of NG2 glia in CSVD and the underlying mechanisms.

**Keywords** NG2 glia · Oligodendrocyte precursor cell · Cerebral small vessel disease · White matter injury

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## Introduction

Cerebral small vessel disease (CSVD), which represents a cluster of pathologies affecting small cerebral arterioles, venules, and capillaries with diameters of 50–400 μm, is characterized by a variety of clinical manifestations and neuroimaging features [1]. CSVD is generally used to describe the pathological effects of small vascular lesions on the brain parenchyma, rather than lesions within the blood vessels. The primary lesions of CSVD are in subcortical gray matter and deep white matter in the distributional areas of the small cerebral blood vessels. The most common current view is that CSVD-related white matter lesions are caused by chronic hypoperfusion, impaired cerebral vascular reactivity, and leakage of the blood–brain barrier [2–5]. Recently, the role of glial cells in the pathological mechanism of CSVD has received increasing attention [6–9]. NG2 glia, or oligodendrocyte progenitor cells (OPCs), are characterized by their expression of chondroitin sulfate proteoglycan (NG2 antigen) and platelet-derived growth factor receptor α (PDGFαR). They are evenly distributed throughout the gray and white matter and are the fourth type of glial cell in addition to astrocytes, microglia and oligodendrocytes in the mammalian central nervous system. Although NG2 glia were initially identified as the precursor cells of oligodendrocytes, many new physiological and pathological functions of NG2 glia are gradually being revealed [10, 11]. In this review, we discuss the role of NG2 glia in the white matter injury of CSVD and the underlying mechanisms.

## Clinical Features of CSVD

Based on the Standards for Reporting Vascular Changes in Neuroimaging (STRIVE), CSVD can be divided into 6

types: arteriosclerosis/age-related, amyloid-related, genetic CSVD distinct from amyloid angiopathy, inflammatory/immunologically-mediated, venous collagenosis, and other types [1, 3]. CSVD does not necessarily present with clinical symptoms in the early stages, but signs of disease progression such as cognitive impairment, stroke, and mobility problems may occur [2].

The diagnosis of CSVD principally depends on neuroimaging, and brain magnetic resonance imaging and computerized tomography are the most important means used to detect CSVD. The typical neuroimaging features of CSVD include white matter hyperintensities (WMHs) in the vascularly dominated area, lacunar intensities, perivascular space expansion, microbleeding, and new subcortical microinfarcts, and WMHs are the most easily observed and often show the earliest changes [1, 5]. Biomarker studies of CSVD are based on biochemical changes caused by neuroinflammation, myelin degeneration, and disruption of the blood–brain barrier. For example, an elevated cerebrospinal fluid–blood albumin ratio reflects an impaired blood–brain barrier, and an increased level of matrix metalloproteinases in cerebrospinal fluid designates extracellular matrix decomposition, while neurofilaments in cerebrospinal fluid are used as biomarker of axonal injury [12]. These biomarkers are of auxiliary value in the diagnosis of CSVD. Currently, controlling risk factors for CSVD is the sole treatment aim, including blood pressure control, anti-platelet aggregation, and anticoagulation [2]. Some drugs approved for other indications may also be applied to improve the clinical symptoms of CSVD. For example, the phosphodiesterase inhibitor cilostazol (which is used to prevent stroke) has been shown to reduce the cognitive decline, endothelial dysfunction, and inflammatory response, and to enhance myelin repair. In addition, modifying an unhealthy lifestyle (such as smoking cessation) can also delay the progression of CVSD. Overall, our lack of in-depth understanding of the pathological mechanisms underlying CSVD limits the treatment of this disease.

## Pathological Alterations in CSVD

### WMHs and White Matter Injuries

WMHs, defined as high-signal dispersion areas on T2-weighted or fluid-attenuated inversion recovery sequences (FLAIR), are roughly equivalent to leukoaraiosis seen on computerized tomography scans, and essentially represent increased water content of the hydrophobic white matter fiber bundles [13]. Although used as a proxy for CSVD in clinical practice, WMHs are not specific to CSVD, as they are common in magnetic resonance images of the elderly brain.

WMHs reflect not only a difference in imaging parameters but also pathologic changes in the white matter. By reviewing studies that directly correlated postmortem MRI and histopathology, Gouw *et al*. pointed out that WMHs are heterogeneous in terms of histopathology. Damage to the white matter ranges from slight disentanglement of the matrix to varying degrees of demyelination, oligodendrocyte cell death, axonal loss, glial responses, and microvascular alterations [14]. Spot-like WMHs are considered to depict mild white matter changes associated with myelin injury, gliosis, and perivascular space enlargement, while extensive and fused WMHs represent serious pathological changes, including myelin loss and axonal breakdown [15, 16]. WMHs around the lateral ventricle angle also reflect disrupted transport of cerebrospinal fluid and periventricular edema at these sites [15]. Punctate abnormalities in subcortical and deep white matter show a low tendency for progression, while early confluent and confluent changes progress rapidly [16]. Based on a combination of diffusion tensor imaging and myelin water imaging to examine WMHs and surrounding normal-appearing white matter, Ferris *et al*. revealed varying levels of demyelination in the core region of WMHs, while WMHs have penumbra-like effects in perilesional white matter that specifically reflect increased interstitial fluid, with no changes in the myelin level [17]. Progression in white matter hyperintensity volume has been shown to be associated with a decline in cognitive function in the elderly [18], and WMHs have been suggested to serve as a predictor of mild cognitive impairment and future non-Alzheimer's dementia [19]. Moreover, a semi-automated magnetic resonance imaging segmentation analysis of a 3-year longitudinal cohort of 99 participants with symptomatic CSVD demonstrated that the rates of white matter hyperintensity expansion and grey matter atrophy are strongly correlated, and significant grey matter loss and whole-brain atrophy occur annually, suggesting a linkage between the progression of WMHs, and brain atrophy and consequent functional morbidity [20].

### Cerebrovascular Reactivity

The coupling between neural activity and cerebral blood flow is the prototypical function of the neurovascular unit (NVU), in which neurons, astrocytes, endothelial cells, smooth muscle cells, pericytes, and matrix, as well as a perivascular compartment, are comprehensively integrated [21]. Indeed, the increased neuronal activity requires large amounts of energy, and glucose and oxygen demand can be achieved by increasing cerebral blood flow. Meanwhile, an increase in flow is needed to clear away the toxic metabolites of brain activity. Cerebrovascular reactivity, which is defined as a change in cerebral blood flow in response to vasodilatory stimulation, represents the diastolic capacity

of blood vessels in the brain [5]. The presence of vascular sclerosis and chronic hypertension reduces the regulation of cerebral blood flow, increases the velocity and pulsatility of blood flow, and leads to vascular endothelial injury [22, 23]. Chabriat *et al*. used acetazolamide, a potent vasodilator, to evaluate cerebral vascular reactivity in the brains of patients with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), and found that the reactivity decreased in the WMH regions but remained unchanged in normal white matter [24]. The reactivity in WMH regions has been shown to be lower than in normal white matter regions [25]. Moreover, the diminution in reactivity occurs earlier in WMHs, suggesting that the attenuation in reactivity is an important factor in white matter lesions [25]. By using phase-contrast magnetic resonance imaging, Shi *et al*. further demonstrated that CSVD patients exhibit lower reactivity in white matter regions and that this is related to changes in cerebrovascular pulsation but not to resting cerebral blood flow [26].

**Blood–Brain Barrier (BBB) Integrity**

Beyond neurovascular coupling, the NVU is crucial for the maintenance of the BBB, which encompasses endothelial cells, a basement membrane, pericytes, and astrocyte endfeet, and damage to any of these components may cause BBB damage [27, 28]. Leakage of the BBB is considered to be the core pathologic mechanism underlying white matter injuries in CSVD [29]. The failure of BBB integrity causes the activation of microglia, NG2 glia, and astrocytes, recruits peripheral immune cells, and promotes inflammatory responses, thereby inducing lesions in the brain parenchyma. Data reflecting albumin ratios in cerebrospinal fluid and serum have shown a significant increase in the permeability of the BBB in patients with vascular dementia [30]. Moreover, accumulation of albumin in brain tissue has been found to trigger seizure activity [31, 32]. Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) is the most widely used non-invasive imaging technique for evaluating BBB breakdown [33]. DCE-MRI analysis of the BBB includes both model-free parameters and quantitative parameters using pharmacokinetic modeling, and these techniques are capable of delineating between large permeability changes, such as brain tumors and multiple sclerosis, and the subtle vascular leakage in CSVD [33]. Enhanced magnetic resonance imaging studies have demonstrated significant changes in the BBB of white matter in CSVD patients, and that the leakage of the BBB has been closely linked to increased WMH load [33–36]. Recently, Stringer *et al*. used Patlak tracer kinetic analysis, considered optimal in low-permeability states, to quantify the permeability-surface area product and blood plasma volume in CSVD patients who undergo dynamic contrast-enhanced MRI scans [37]. Their results suggested that BBB leakage is an important mechanism in CSVD.

Recent studies have shown that the perivascular space occupies a key position in the pathogenesis of white matter in CSVD [38, 39]. The primary drainage pathway for brain metabolites in cerebrospinal fluid is the perivascular space that surrounds the microvessels of the brain, and which is part of the glymphatic system. When the arteriolar pulsation index is increased (such as when blood pressure is elevated), the cerebrospinal fluid flow slows and becomes irregular, resulting in fluid retention in the perivascular space, which in turn impedes the clearance of tissue metabolites [40]. Aging may also affect cerebral artery pulsation as well as ectopic aquaporin 4 expression on astrocyte endfeet, thus perturbing perivascular drainage pathways and resulting in the expansion of the perivascular space and abnormal stagnation of the cerebrospinal fluid [41]. These accumulated metabolites may disturb the glial cell–vascular unit, and then cause dysfunction of the BBB, leading to white matter lesions. However, the specific metabolites involved in affecting the integrity of the BBB in CSVD need to be further investigated.

**Glial Reactions**

In CSVD-induced white matter lesions, glial cells respond to pathological processes in different ways, with the most significant changes including loss of myelin and oligodendrocytes and the glial activation of astrocytes, microglia, and NG2 glia [42]. Simpson *et al*. [42] examined the alterations in glial cells at the focal site of white matter injury by combining postmortem magnetic resonance imaging with histochemical staining, and established that myelin is significantly reduced in lateral paraventricular lesions (PVLs) particularly, and also in deep subcortical lesions (DSCLs). PVLs and DSCLs show many similarities, including severe myelin loss and similar levels of astrocyte reactivity as well as increases in microglial immunoreactivity compared to white matter in non-lesion elderly controls [42]. These changes may reflect pathologic features consistent with dysfunction of the BBB. There are also differences in pathology between PVLs and DSCLs, as the loss of ependyma leads to reverse diffusion of cerebrospinal fluid that may trigger specific pathological reactions in the PVL region [42]. These results suggest that white matter lesions in different brain regions reflect distinct pathological characteristics and biological responses.

Recently, by using different animal models of CSVD, the glial reactivity in white matter lesions has been intensively studied. Magami *et al*. showed pathologic changes in white matter in a model of chronic cerebral hypoperfusion in which bilateral common carotid artery stenosis was induced with microcoils in male mice (10 weeks old)

[43]. These authors demonstrated that the number of NG2 glia and astrocytes increased while the number of oligodendrocytes decreased 28 days after hypoperfusion. Mice deficient in endothelial nitric oxide synthase are a model of age-dependent, spontaneous CSVD, as these mice develop cerebral hypoperfusion and BBB leakage at a young age and progressively worsen with advancing age [44]. Luxol fast blue staining revealed white matter lesions in the mice at 12 months of age, and immunostaining confirmed significant myelin loss in the cortex and corpus callosum. The numbers of mature oligodendrocytes were reduced at the onset of the disease (12 months of age), whereas NG2 glia were significantly increased. Moreover, immunostaining of both oligodendrocyte transcription factor 1 and the mature somatic marker CC1 were reduced, suggesting the inhibition of oligodendroglial differentiation [44]. By using high-pressure freezing and freeze substitution to assess the early pathological changes of the corpus callosum in the CADASIL mouse model (TgNotch3R169C), Rajani *et al*. revealed a significant splitting of myelin layers and an enlargement of the inner tongue of small-caliber axons from the age of six months, and vesiculation of the inner tongue and myelin-sheath thinning at 15 months of age [45]. Immunohistochemistry further showed an elevation in the number of NG2 glia as well as microglia in older CADASIL mice, but not in younger mice. These results indicate that early white matter lesions in CADASIL affect the myelin sheath (first and foremost) and the inner tongue, suggesting a primary myelin injury.

## Roles of NG2 Glia in CSVD

### Impediment of Oligodendrocyte Differentiation and Remyelination

An important role for NG2 glia is to serve as precursors of oligodendrocytes. Although the proliferation of NG2 glia in CSVD white matter lesions is posited to be a compensatory reaction to demyelination and oligodendrocyte loss, many studies have shown that their differentiation and maturation are markedly inhibited in the lesions. For example, although PDGFαR-positive NG2 glia are significantly elevated in the deep white matter lesions of CSVD, there is no significant difference in the number of MAP-2+13-positive myelinating cells [46]. Why do the oligodendrocyte progenitors have such difficulty in differentiating and maturing in the white matter lesions? As shown in Figure 1, oligodendrocyte progenitors in the lesions face a hostile microenvironment created by hypoxia, activated glial cells, and extravasated blood components, and this microenvironment may thus constitute the principal reason for impediments to oligodendroglial maturation and myelination.

The abnormal activation of astrocytes caused by the destruction of the BBB may contribute to the inhibition of oligodendrocyte progenitor differentiation and maturation in the white matter lesions of CSVD. Post-stroke survivors with a heavy burden of WMHs have an increased number of clasmatodendritic astrocytes with swollen and vacuolated cell bodies and aberrant localization of aquaporin 4 in the lesions [47]. Clasmatodendritic astrocytes positive for glial fibrillary acidic protein are abundant in the deep white matter of CADASIL patients [48]. These cells are co-localized with autophagy markers, suggesting autophagy-like cell death of astrocytes with CADASIL. Miyamoto *et al*. conducted an in-depth study of the interaction between astrocytes and OPCs, and found that A1-like astrocytes dominate the elevation in astrocyte number in the white matter lesions of chronic hypoperfusion model mice (male, 10 weeks old), while the number of A2-like cells is reduced [49]. The differentiation of OPCs in cell culture is blocked when a non-lethal concentration of $CoCl_2$ is added to simulate chronic ischemia but improves with the addition of astrocyte-conditioned medium. However, administration of the conditioned medium from injured astrocytes fails to promote the differentiation of OPCs. Sozmen *et al*. implemented RNAseq on OPCs that were specifically isolated from a partially injured region adjacent to the ischemic site using a mouse model of white matter ischemic injury (male, 18–24 months old) induced by white matter injection of N5-(1-iminoethyl)-L-ornithine, a potent vasoconstrictor [50]. They identified a series of factors involved in regulating the differentiation of oligodendrocyte progenitors. Inhibin-A, secreted by reactive astrocytes, has been shown to down-regulate the expression of matrilin-2 in OPCs, and to inhibit their differentiation and remyelination.

The activation of microglia has been described as a functional dichotomy: pro-inflammatory (M1) and anti-inflammatory (M2) [51, 52]. Therefore, activated microglia may have beneficial or detrimental effects on CSVD. It has been reported that transiently-proliferating perivascular microglia induce M1-type activation that precedes cerebrovascular changes in an adult male rat model of chronic hypertension [53]. Many studies have shown that M1-activated microglia secrete a large number of inflammatory factors such as interferon-γ and tumor necrosis factor α, and that these inflammatory factors inhibit the differentiation and maturation of oligodendrocyte progenitors [54–56]. By examining the pathologic changes in white matter injury during chronic hypoperfusion in adult male rats with bilateral common carotid artery occlusion, white-matter impairments were shown to be correlated with aberrant microglial activation and an increase in the number of reactive microglia adhering to phagocytosed myelin in the hypoperfused area [57]. These reactions were accompanied by the up-regulation of complement C3 and its receptor C3aR, suggesting

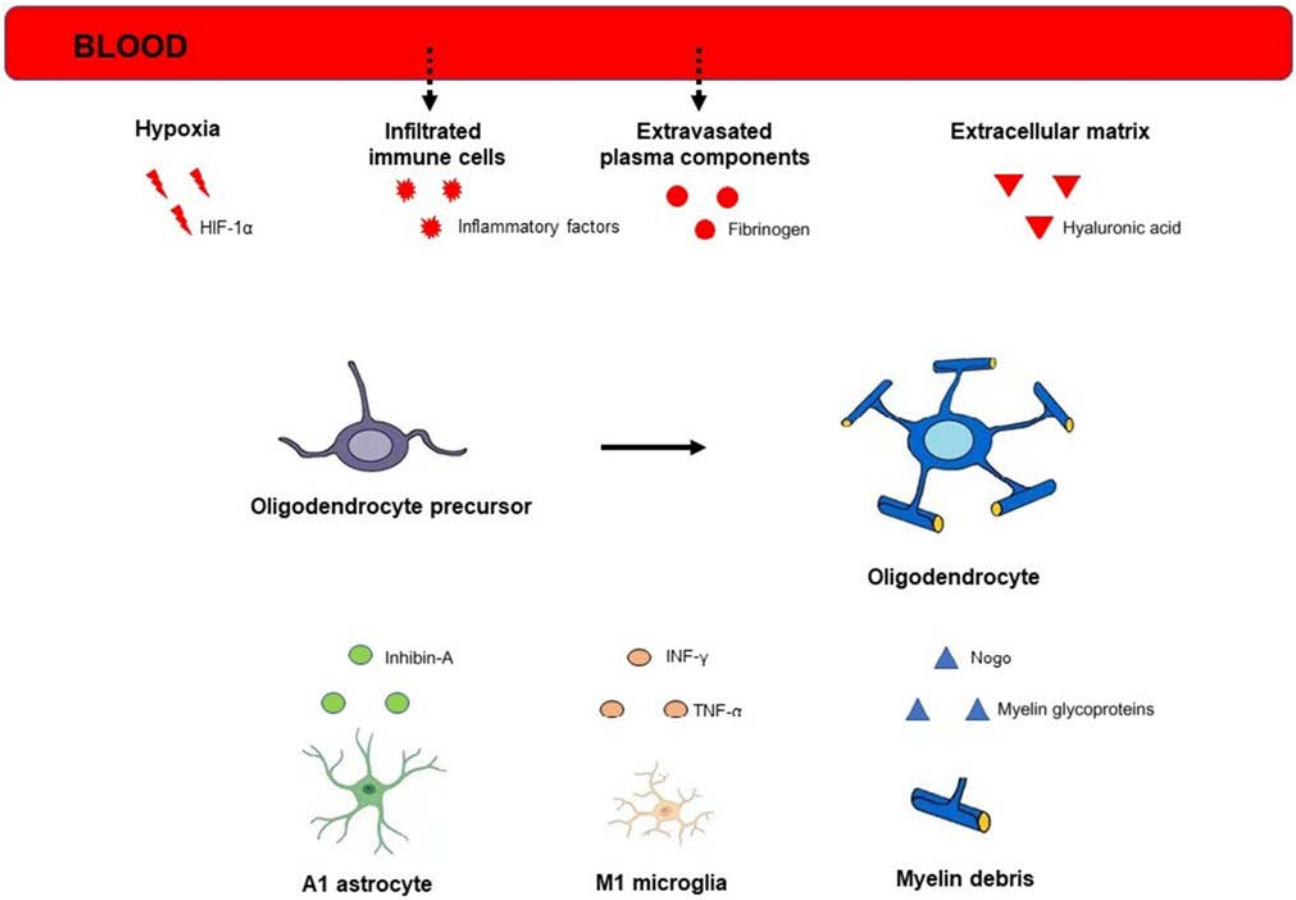


**Fig. 1** Hostile microenvironment for oligodendroglial maturation and myelination in white matter lesions of CSVD. The differentiation and maturation of oligodendrocyte progenitors to oligodendrocytes is impeded in the inhibitory microenvironment contributed by hypoxia, infiltrating immune cells, extravasated plasma components, extracellular matrix, myelin debris, and toxin-activated astrocytes and microglia.

that aberrant microglial activation aggravates white matter injury through the C3-C3aR pathway. On the other hand, M2-activated microglia have been reported to promote the differentiation and maturation of oligodendrocyte progenitors and enhance remyelination [58]. Indeed, Fingolimod has been shown to protect against white matter damage by modulating microglia toward M2 polarization after chronic cerebral hypoperfusion in mice [59].

Chronic hypoperfusion provokes hypoxia in white matter. Severe white matter damage has been reported after prolonged cerebral hypoperfusion [60]. In addition, oxidative stress has been shown to interfere with white matter repair by hindering the differentiation of oligodendrocyte precursors. Although hypoxia-inducible factor (HIF) is produced transiently to promote cellular survival with hypoxia, prolonged HIF activity drives distinct pathological responses [61]. Studies have revealed that bilateral common carotid artery stenosis induces the up-regulation of HIF-1α in the white matter lesions of mice (male, 10 weeks old), and that the HIF-1α-sphingosine kinase / sphingosine-1-phosphate signaling pathway mediates the blockade of oligodendrocyte progenitor differentiation [62]. In a mouse model of neonatal hypoxia (3 days old) that reproduced diffuse white matter injury, the histone deacetylase Sirt1 was demonstrated to serve as a Cdk2 regulator in the proliferation of oligodendrocyte precursors, and hypoxia enhanced Sirt1 and Sirt1/Cdk2 complex formation through HIF-1α activation [63]. Inhibition of Sirt1 can promote oligodendroglial differentiation after hypoxia. Moreover, by using a model of chronic HIF-1α accumulation in pluripotent stem cell-derived oligodendrocyte progenitors, HIF-1α has been shown to interact with OLIG2 to activate genes such as Ascl2 and Dlx3 and to inhibit Sox10, thereby blocking the differentiation of oligodendrocyte progenitors [61]. Therefore, under chronic hypoxia, prolonged expression of HIF-1α inhibits the differentiation and maturation of OPCs through various pathways, thus affecting myelin regeneration.

Disintegrating myelin sheaths and extracellular matrix in white matter lesions produce substances that inhibit the differentiation and maturation of oligodendrocyte progenitors. For example, myelin glycoprotein acts on the Nogo receptor on OPCs to hinder their differentiation [64]. A specific bioactive hyaluronic acid fragment has been shown to down-regulate myelin-related gene expression through the TLR/AKT/FoxO3 pathway, thereby inhibiting the differentiation and maturation of oligodendrocyte progenitors [65]. Conversely, due to injury of the BBB in the white matter, components in the blood that infiltrate into the brain parenchyma directly act on the recruited OPCs and affect their differentiation. For example, pathologic examinations have found abundant extravasated fibrinogen in the lateral paraventricular and deep subcortical white matter lesions of CSVD [46]. Studies have also revealed that fibrinogen is able to activate the signaling pathway of bone morphogenetic protein in OPCs, making them more likely to differentiate into astrocytes and not into oligodendrocytes [66].

## Contributions to Blood–Brain Barrier Integrity

The BBB maintains a highly specific microenvironment in the brain by limiting the entry of substances from the blood into the brain parenchyma. NG2 glia have been shown to coincide with cerebral vessels at the sprouting tips of cells or along the vessel walls in the embryonic brain, which are critical for vessel network formation during brain development [67]. Moreover, NG2 glia have been demonstrated to promote postnatal white matter angiogenesis and endothelial cell proliferation *via* paracrine HIF [68]. There is increasing evidence that BBB integrity requires interactions between cerebral endothelial cells and adjacent cells, including NG2 glia [69, 70]. Kimura *et al*. uncovered reduced permeability of the endothelial cell barrier to fluorescein sodium by co-culture of rat cerebral endothelial cells with NG2 glia and reported a blockade of augmented barrier function by PDGFRα inhibitors [71]. These results suggest that NG2 glia enhance the integrity of the BBB through the PDGF-BB/PDGFRα-signaling pathway. In line with this, conditioned medium from OPC culture has been reported to elevate tight-junction protein expression and restrict the permeability of the cerebral endothelial cell barrier *in vitro* by activating the transforming growth factor-β receptor-MEK/ERK- signaling pathway [70]. These results suggest that NG2 glia support BBB integrity by secreting soluble factors such as transforming growth factor-β.

White matter demyelination occurs in mice that experience prolonged cerebral hypoperfusion stress. Prior to demyelination, increased expression of matrix metalloproteinase 9 (MMP9), BBB leakage, and neutrophil infiltration have been reported in the impaired white matter of mice (male, 10–12 weeks old) [72]. MMP9 is mainly derived from oligodendrocyte progenitors, and the MMP inhibitor GM6001 reduces early BBB leakage and neutrophil infiltration. Further experiments on cultured endothelial cells have confirmed that OPCs are able to induce the secretion of MMP9 under the stimulation of interleukin-1, leading to the degradation of tight-junction protein ZO1 in endothelial cells [72]. These studies revealed that OPCs respond rapidly to white matter injury and produce MMP9 to disrupt the BBB, indicating a detrimental effect of OPCs on barrier integrity under disease conditions.

OPC migration is an important feature of embryonic neurodevelopment. In adulthood, OPCs retain the ability to migrate, which allows their rapid recruitment to the site of nerve injury. OPCs require the vasculature as a physical substrate for migration during development, and Wnt-chemokine receptor 4 signaling is crucial to regulating the interaction between OPCs and endothelial cells [73]. Further studies have revealed that large numbers of OPCs cluster around blood vessels in the active lesions of the multiple sclerosis brain due to an inability to properly detach from vessels following perivascular migration [74]. These perivascular OPCs then interfere with the integrity of the tight junctions between astrocyte endfeet and endothelial cells, resulting in altered vascular permeability. Further mechanistic studies have shown that abnormal Wnt tone in OPCs mediates their dysfunctional vascular detachment and also leads to OPC secretion of Wnt inhibitory factor-1, which then interferes with Wnt ligand functioning in endothelial tight-junction integrity [74]. It would be intriguing to determine whether similar pathologic mechanisms also exist in the white matter lesions of CSVD patients [9].

The pericyte is an important component of the BBB. Montagne *et al*. [75] investigated the relationship between changes in the microcirculation and white-matter integrity in pericyte-deficient mice with a PDGFβ point mutation, and ascertained that pericyte degeneration disrupts white-matter microcirculation. This leads to the accumulation of blood-derived fibrinogen deposits and a reduction in blood flow, triggering a loss of myelin, axons, and oligodendrocytes. By analyzing the oligodendrocyte lineage of the mouse central nervous system with single-cell RNA sequencing, a new OPC subtype that exhibits PDGFR-α but low levels of NG2 has been identified [76]. This particular subtype of oligodendrocyte progenitors is located in the vascular wall and expresses markers of pericyte lineage, suggesting that it may be directly involved in BBB formation.

## Immunomodulation of NG2 Glia

BBB disruption and chronic perivascular inflammation are typical pathologic features of CSVD [77]. In white matter lesions, microglia and astrocytes are activated and produce significant numbers and amounts of inflammatory factors.

Meanwhile, peripheral immune cells such as macrophages and T cells infiltrate into brain parenchyma with increased BBB permeability, enhancing perivascular inflammatory responses and aggravating tissue damage. NG2 glia may play multiple roles in the neuroinflammatory pathology of CSVD.

NG2 glia quickly sense chemical and physical stimuli in the brain [78]. Upon central nervous system injury, NG2 glia, like microglia, are activated and recruited to the lesion site. Injury-related factors that include myelin debris and ATP significantly promote the migration of NG2 glia [78–80]. White matter stroke has been shown to stimulate adjacent NG2 glia to divide and migrate to the lesion [50]**.** Recently, von Streitberg *et al* examined the lesions from stab wound injury to the brain of mice (3–4 months old) by *in vivo* two-photon live imaging [81]. They found that NG2 glia migrate rapidly into the injury site together with proliferation, leading to a strong increase in their numbers around and within the lesion core [81]. Thus, it would be interesting to visualize NG2-glial reactivity and migration *in vivo* using CSVD animal models.

NG2 glia express a wide range of immunomodulatory molecules that include cytokines, chemokines, and complement, as well as their receptors [82]. By using gene expression profiling on purified OPCs from mice after cuprizone-induced demyelination, studies showed that interleukin-1 beta, a cytokine involved in innate immune system functions, and chemokine CCL2, also known as monocyte chemoattractant protein-1, are significantly increased [80]. In cultured OPCs treated with interferon-γ, interleukin-1β, and tumor necrosis factor, the increased inflammatory and immune activation have been shown to be accompanied by metabolic changes and dysregulation of their proliferation and differentiation programming, suggesting neuroinflammation results in OPCs shifting towards an immunomodulatory phenotype while reducing their capacity for proliferation and differentiation [83]. In accordance with this *in vitro* study, the pro-inflammatory factor interferon-γ was demonstrated to directly act on OPCs to induce pathological quiescence following spinal cord demyelination [84]. In a mouse model that mimics inflammation-mediated white matter injury of preterm born infants and consists of intraperitoneal injection of interleukin-1β from P1 to P5, marked up-regulation of toll-like receptor-3, interleukin-1β, interferon-β, Ccl2, and Cxcl10 in OPCs were reported [85]. Moreover, the components secreted by the reactive OPCs play an important role in shaping the response of microglia during inflammation. These data, mostly assessed in the context of traumatic or demyelinating injury, strongly support the hypothesis that NG2 glia function as immunomodulatory cells [10, 82, 86]. However, the expression and secretion spectrum of reactive NG2 glia in CSVD need to be cautiously examined to understand their immunomodulatory roles.

Interleukin-17, the signature cytokine of Th17 cells, participates in the development and pathogenesis of experimental autoimmune encephalomyelitis (EAE). Interleukin-17 induces the expression of specific inflammatory mediators in NG2 glia, and deletion of NF-κB activator 1 in NG2 glia significantly reduces the severity of EAE [87]. These results indicate that NG2 glia constitute a critical target of interleukin-17 in EAE. Moreover, interleukin-17 has been shown to induce NOTCH1 activation in OPCs and enhance proliferation and inflammatory gene expression [88]. Interleukin-17 production by monocytes has been noted to be closely associated with the progression of CSVD [89]. However, it remains unclear as to whether interleukin-17 secreted by infiltrating peripheral immune cells such as Th17 cells acts on NG2 glia to participate in the regulation of inflammatory responses in the focal white matter injury of CSVD.

NG2 glia also exhibit major histocompatibility complex classes I and II (MHC-I and MHC-II), which are important in antigen processing and presentation. NG2 glia expressing MHC-II have been shown to activate memory and effector CD4+ T cells [90]. Moreover, interferon-γ has been shown to induce high levels of expression of MHC-I molecules in NG2 glia and present antigen to cytotoxic T cells [91]. Postmortem MS tissue analysis has revealed the presence of immunoproteasome-expressing NG2 glia in white matter lesions, further suggesting their role in perpetuating the autoimmune response. Low-density lipoprotein receptor-associated protein 1 (LRP1) is a multifaceted phagocytic receptor that is strongly expressed in several CNS cell types that include OPCs, and LRP1 is a involved in the polarity transformation of M1/M2 microglia [92]. LRP1 is negative regulator of oligodendroglial differentiation in adult mice [93]. Deletion of LRP1 from adult OPCs increases the number of newborn and mature myelinating oligodendrocytes in the corpus callosum and motor cortex [93]. Interestingly, other studies have revealed that specific knockout of LRP1 in oligodendrocytes does not affect myelin development, but rather mitigates the demyelination in EAE and cuprizone-treated adult male mouse models [94]. The further mechanistic study has demonstrated that LRP1 deletion does not affect the differentiation and maturation of OPCs but impairs MHC1-dependent antigen cross-presentation, thus inhibiting the propagation of the inflammatory response and promoting faster myelin repair and neuroprotection.

NG2 glia perform a dual role in immunomodulation, executing both pro-inflammatory and anti-inflammatory activities (Fig. 2). Many growth factors with neurotrophic activity, such as brain-derived neurotrophic factor, leukemia inhibitory factor, platelet-derived growth factor-A, insulin-like growth factor-2, and basic fibroblast growth factor, have been shown to promote oligodendrogenesis, survival, and differentiation [10, 82, 86, 95–98]. Studies have reported that selective ablation of NG2 glia in adult mice induces

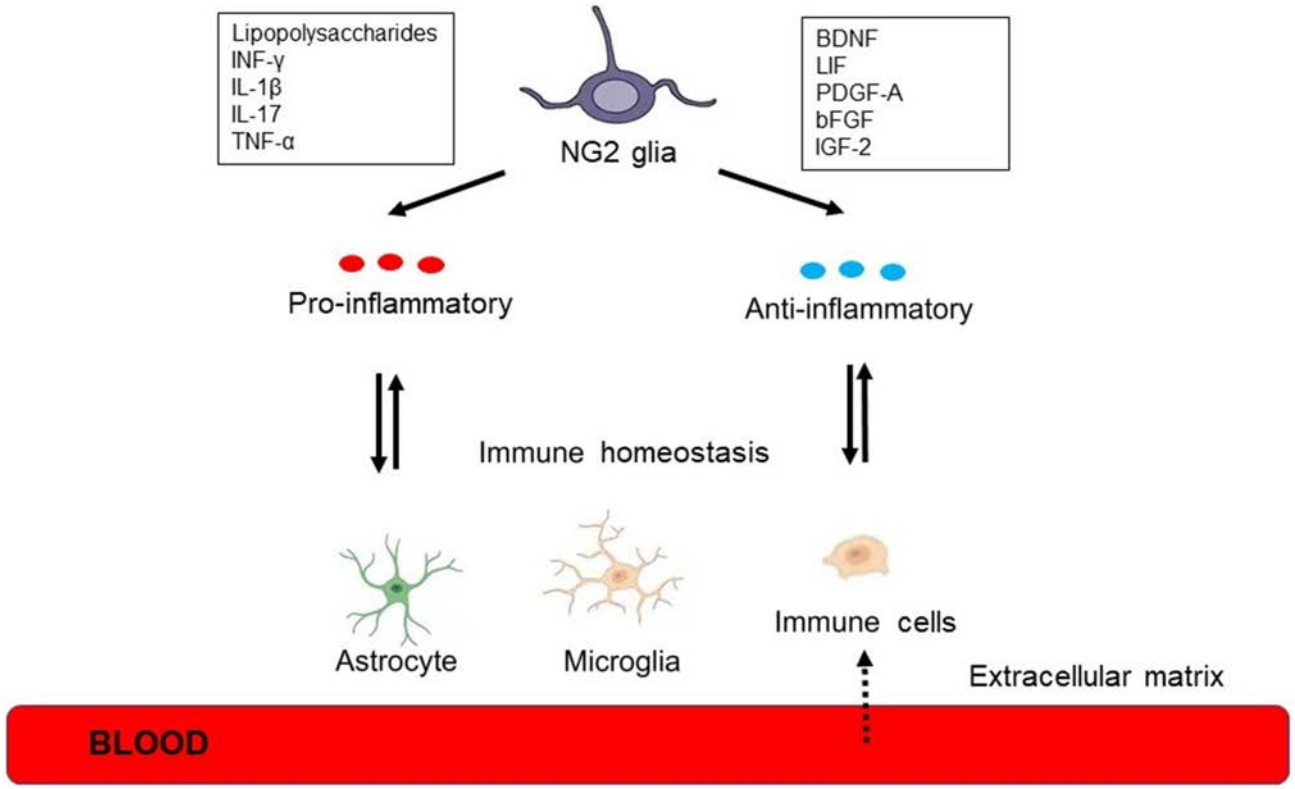


**Fig. 2** NG2 glia play a dual role in immunomodulation. NG2 glia produce both pro-inflammatory and anti-inflammatory factors, which may act on microglia, astrocyte, and immune cells, to regulate neuroinflammation in white matter lesions. An inflammatory microenvironment drives NG2 glia to produce pro-inflammatory factors, whereas a neurotrophic microenvironment promotes NG2 glia to produce anti-inflammatory or pro-regenerative factors. BDNF, brain-derived neurotrophic factor; LIF, leukemia inhibitory factor; PDGF-A, platelet derived growth factor-A; IGF-2, insulin-like growth factor-2; bFGF, basic fibroblast growth factor.

more severe neuroinflammation under stimulation by the endotoxin lipopolysaccharide, suggesting that NG2 glia are important in maintaining immune homeostasis [99]. Consistent with this, mice with ablated NG2 glia display an aberrant activation of microglia and a marked inflammatory response in the brain following lipopolysaccharide exposure, suggesting that NG2 glia exert a powerful immunosuppressive action on the brain's innate immunity [100]. Further studies of the underlying mechanism(s) of action have revealed that NG2 glia-derived TGF-β2 signaling is involved in the regulation of microglial activation. As microglia and NG2 glia are continuously activated in the white matter lesions of CSVD [46], it would be intriguing to decipher the roles of NG2 glia in its neuroinflammation.

## Conclusions and Future Directions

In summary, accumulating evidence strongly indicates that NG2 glia play a crucial role in the pathological process of CSVD. Impairment of NG2 glial differentiation and maturation leads to the failure of remyelination in demyelinating lesions in CSVD. The dysfunction of NG2 glia in the immune and vasculature systems contributes to BBB disturbance, resulting in white matter lesions in CSVD.

CSVD affects ~5% of individuals older than 50 years of age and almost 100% of people older than 90 years. Current prevention and treatment principally focus on the control of risk factors such as elevated blood pressure and blood lipids, and platelet aggregation [2]. Therefore, exploring novel therapeutic strategies is greatly encouraged. White matter injury is the common pathological feature of CSVD. Demyelination affects neural circuitry and synaptic activity and thus affects cognitive function, as myelin not only enables action potentials to spread rapidly along axons but also supports and protects axonal metabolism [10]. As CSVD is closely associated with cognitive dysfunction [101], it would be interesting to investigate whether promoting myelin repair would benefit cognitive improvement in patients with CSVD.

As precursors of oligodendrocytes, OPCs have been extensively studied with respect to differentiation and maturation during development. However, the mechanisms underlying the hindrances to oligodendroglial differentiation and

maturation in white matter lesions of CSVD remain elusive. Revealing the unique features of CSVD white matter injury compared with autoimmune demyelination and traumatic myelin damage merit consideration in developing therapeutic interventions for CSVD.

Emerging evidence suggests that NG2 glia play crucial roles in immunomodulation and the maintenance of NVU integrity. However, the roles and the underlying mechanisms of NG2 glia on the vasculature and immune system in the pathological process of CSVD remain largely unknown. Further study in this direction would therefore not only contribute to an in-depth understanding of the pathogenesis of white matter injury in CSVD but also provide novel targets for the diagnosis and treatment of CSVD.

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REVIEW

# The Structure and Function of Glial Networks: Beyond the Neuronal Connections

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**Abstract** Glial cells, consisting of astrocytes, oligodendrocyte lineage cells, and microglia, account for >50% of the total number of cells in the mammalian brain. They play key roles in the modulation of various brain activities under physiological and pathological conditions. Although the typical morphological features and characteristic functions of these cells are well described, the organization of interconnections of the different glial cell populations and their impact on the healthy and diseased brain is not completely understood. Understanding these processes remains a profound challenge. Accumulating evidence suggests that glial cells can form highly complex interconnections with each other. The astroglial network has been well described. Oligodendrocytes and microglia may also contribute to the formation of glial networks under various circumstances. In this review, we discuss the structure and function of glial networks and their pathological relevance to central nervous system diseases. We also highlight opportunities for future research on the glial connectome.

**Keywords** Glia network · Gap junction · Calcium coupling · CNS diseases

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## Introduction

Over the past few decades, accumulating evidence has suggested that glial cells are much more than supporting cells in the brain [1]. Glial cells, including astrocytes, microglia, oligodendrocytes, and neural glial antigen-2 (NG2) glia (also known as oligodendrocyte progenitor cells, OPCs), constitute >50% of the total number of cells in the rodent and human central nervous system (CNS) [2, 3]. These cells are vital for the maintenance of CNS homeostasis and are responsible for the regulation of a variety of physiological and pathological processes. Astrocytes form tripartite synapses with neurons and take part in synaptic pruning, $K^+$ buffering, and neurotransmitter release [4]. Astrocytes also contribute to the formation of the blood-brain barrier and participate in tissue-specific immune responses to injury *via* astrogliosis. Oligodendrocytes wrap around axons to form myelin, which allows rapid signal transmission and the metabolic support of neurons. Microglia are the guards of the CNS; they rapidly respond to injury and infection by secreting various pre- or anti-inflammatory cytokines [5]. NG2-glia are proliferative cells in the brain parenchyma that can differentiate into mature oligodendrocytes throughout their lifetime [5].

Brain functions have long been believed to be executed *via* neural circuits where information is interpreted as the temporal-spatial transmission of electrophysiological events or action potentials within one neuron and transferred from one neuron to another through rapid and precise neurotransmission. On top of this, neural circuits are further connected to form a large-scale brain network, which is the basis of cognitive function and intelligence [6]. To the same extent, glial cells also form networks both structurally and functionally. They exchange information with each other in various forms [5, 7]. Channels formed by connexins allow glia-glia communication *via* substrate exchange [8]. Moreover, glial

cells communicate with one another *via* secretory vesicles, especially when activated or stimulated [9]. Finally, $Ca^{2+}$ waves may also carry information and influence both glial and neuronal activity [10–12].

Glial networks resemble neural circuits to some extent. The functions of neural circuits rely on synaptic structures formed between pre- and post-synaptic neurons through the release of neurotransmitters. Likewise, glial cells, in particular astrocytes and oligodendrocytes, form specific structures, termed gap junction channels, between each other; these facilitate a relatively stable spatial relationship between the two types of glial cells. In addition, glial cells regulate the state of neighboring neurons by releasing gliotransmitters or other biological substances. However, glial networks also possess distinct characteristics compared to neuronal networks. The links between cells in the glial network are not highly specific but are much more diverse, and this may ensure the redundancy of glial network functions. The signal transduction between glial cells is not as fast as an action potential, but it lasts longer and can have a long-term influence on brain homeostasis [13, 14].

In this review, we discuss the structural basis and function of glial networks focusing on the following two aspects: (1) glial networks in the healthy brain and (2) the pathological relevance of glial networks in the CNS.

## Glial Networks in the Healthy Brain

### Cell-to-Cell Contact *via* Connexins

Neurons communicate through synapses, whereas glial cells form relatively stable intercellular positional relationships with each other through gap junctions. Connexins (Cxs) are structurally-related transmembrane proteins that are assembled to form gap junctions in vertebrates and are pivotal for maintaining normal brain function [15, 16]. Many tissues and cell types express two or more members of the Cx family. For instance, Cx29, Cx32, Cx36, Cx37, Cx43, and Cx47 are expressed in the mouse brain, mainly on oligodendrocytes, neurons, endothelial cells, and astrocytes [15]. As a mediator of intercellular communications, Cxs first form hetero- or homo-hemichannels and insert into the plasma membrane. Then, with the aid of specific proteins, mainly cadherins, the hemichannels can dock with the hemichannels of adjacent cells to form gap junctions [8]. Since not all channels are the same, they usually share the property of allowing molecules up to 1–1.5 kDa to pass; for example, ions ($K^+$, $Ca^{2+}$, $Na^+$), second messengers, and small metabolites [8, 17].

### Astrocyte Network

Functional tests and electrophysiological recordings have demonstrated that astrocytes are extensively coupled to each other by gap junctions in cortical, cerebellar, and hippocampal tissue slices [18]. Astrocytes mainly express three Cx isotypes, namely Cx43, Cx30, and Cx26 [19]. Cx43 is specifically expressed in astrocytes in CNS white matter [19]; despite that, it is the most abundant Cx in the mammalian brain. In the developing nervous system, astrocytes may directly arise from radial glia, from an astrocyte-restricted progenitor population, or from the proliferation of newborn astrocytes [20, 21]. Astrocytes migrate to a specific region in the CNS during development and stay quiescent in adults. These astrocytes are primarily connected by Cx43/Cx43 homo-channels to establish a syncytial system, which enables electrical coupling to rapidly and mutually minimize membrane potential differences among interconnected cells. Thus, syncytial isopotential is a physiological mechanism that efficiently coordinates astrocytic network activity at the network level [17, 22].

It is well known that neurons are circuity- and region-specifically connected. Interestingly, glial cells share some of these features in their connections. For example, in the olfactory glomeruli, thalamus, and anterior hypothalamus, Cx30 prevails, while in the hippocampus, Cx43 is dominant [23]. In contrast, Cx26 expression is restricted to certain sub-cortical regions, including the reticular thalamic and subthalamic nuclei, as well as the hypothalamus and meninges [24]. Regional heterogeneity in gene expression of Cx30, Cx43, and Cx26 may be correlated with their distinct functions. Previous studies have shown that Cx30 deficiency attenuates A2 astrocyte responses and induces severe neurodegeneration [25]. Cx43 mediates adhesion, energy metabolism, and neurodegeneration. In contrast, Cx26 is responsible for neurotoxic signaling [26], demonstrating the complexity of their functionality and regulation.

Furthermore, adult astrocytes arise from clonal divisions of early differentiated astrocytes and these clones may specify domains of distinct classes of astrocytes and perform diverse functions in different brain regions or even in the same brain region [27, 28]. For example, Martin and coworkers found that subpopulations of astrocytes selectively respond to activity in the specific medium spiny neuron subtype in the dorsal striatum [28], indicating that astrocytes can form functionally distinct subpopulations within the same brain region, where they potentially form circuitry-specific connections between each other.

### Oligodendrocyte Connection

Oligodendrocytes are differentiated from OPCs and enwrap myelin around axons in response to several intrinsic and extrinsic cues after birth [29]. Myelin sheath gaps, a structure known as the node of Ranvier, enable the increases of both the speed and energy efficiency of action potential propagation and nerve conduction [30].

Oligodendrocytes express various connexin isotypes, such as Cx29, Cx32, and Cx47, which play a crucial role in ensuring effective communications within the oligodendrocyte network internally and with different types of glial cells externally. Oligodendrocytes are electrically and metabolically coupled *via* intercellular gap junctions with other oligodendrocytes (O/O junctions), as well as with astrocytes (O/A junctions) [24, 31]. O/O coupling is facilitated by gap junctions in homotypic configurations with homomeric hemichannels containing Cx32 or Cx47 [32]. Cx32 is mainly expressed in the white matter and is localized in the myelin sheath of large-diameter fibers forming intracellular gap junctions within the myelin sheath. Cx47 is expressed at an early developmental stage in all oligodendrocytes throughout the CNS and is mainly localized on the perikaryal and proximal processes of myelinating cells as well as on OPCs [32]. Cx32 and Cx47 may be localized in the same gap junction plaques on the oligodendrocyte soma. But they do not appear to form heteromeric channels. A deficiency of *Cx32* or *Cx47* in animals only leads to a modest reduction of myelin volume without behavioral defects. In contrast, *Cx32* and *Cx47* double knockout results in severe demyelination, massive apoptotic oligodendrocyte death, and early mortality [33–36].

In addition to O/O interaction, O/A gap junction channels are formed through heteromeric hemichannels of Cx47 on oligodendrocytes and Cx43 on astrocytes [37]. Previous studies on astrocyte-oligodendrocyte crosstalk have focused mostly on glial development, the regulation of gap junctions, myelination, and cellular response to CNS injury [38]. It was a popular idea in earlier times that astrocytes and oligodendrocytes share a common precursor [39–41], though recent studies using mice lacking transcriptional factors which determined cell fate disproved that theory and suggested that they have different precursors [42]. Coupling between oligodendrocytes and astrocytes constitutes a more stable glial network than the astrocyte network described before, which promotes $K^+$ re-distribution and ensures normal axonal activity [32]. Other than $K^+$ clearance, gap junctions between oligodendrocytes and astrocytes may also serve as metabolic support channels [22].

Neurons have a characteristic of clear orientation. Cell-to-cell communications between glial cells also show directional flow based on unique structures. Earlier work has implied that heterotypic O/A gap junctions exhibit a directional diffusion barrier for the movement of ions and larger negatively-charged molecules from cells expressing Cx47 to those with Cx43 [43]. Ions and small molecules pass unidirectionally from astrocytes into neighboring astrocytes and oligodendrocytes through gap junctions [44]. These studies indicate a potential control hierarchy between interconnected glial cells. It is worthy of note that the directional flow of ions/small molecules has only been demonstrated between astrocytes and oligodendrocytes *in vitro* in one study. Extensive investigations on this topic are required in the future.

Intriguingly, some molecules have been found to be involved in the physical connections between glial cells. For example, in the mouse brain, the PDZ domain-containing protein occludens-1 interacts with several Cxs in astrocytes and oligodendrocytes and helps to anchor signal molecules with the gap junction [45], revealing complex machinery that is a key for stabilizing the spatial relationship between glial cells.

## Microglial Connections

It appears that microglia do not form gap junctions with each other or with other types of cells *in vivo* under both physiological and pathological conditions. However, a recent study demonstrated that microglia do have membrane-to-membrane contacts between neighboring microglia. The microglial network has been found to rely on F-actin-based tunneling nanotubes that facilitate the transfer of α-synuclein from overloaded microglia to naïve cells [46]. These findings indicate that even the most dynamic glia are able to link with each other to form a functional intercellular connection. It is worth noting that tunneling nanotubes were first discovered as a new type of transmission in cultured cell systems by Gerdes and co-workers [47]. Tunneling nanotubes can result in the formation of dynamic syncytial cellular networks among different cells in a variety of cell types [48]. The structure is used for intercellular communications by neurons and astrocytes in the CNS [49, 50]. Further research on how tunneling nanotubes are formed between microglia under physiological and pathological conditions and their regulation is required.

## Functional Coupling Relies on Calcium Waves

Upon stimulation, neurons generate and conduct action potentials along their axons to affect target cells. Likewise, astrocytes are known to generate $Ca^{2+}$ transients in their processes and sometimes they propagate along the process into the soma, and even between cells [12, 51]. Subsequently, the $Ca^{2+}$ signals regulate the expression of genes involved in the target cells, such as oligodendrocytes and microglia.

### *Calcium Signaling in Astrocytes*

Unlike neurons, astrocytes do not generate action potentials. However, these cells can communicate with each other *via* $Ca^{2+}$ signaling. $Ca^{2+}$ is a major player in astrocytes in encoding and transmitting information; this can regulate the release of gliotransmitters, such as glutamate and ATP, as well as gene expression [52, 53]. There are two types of $Ca^{2+}$ signaling in astrocytes: $Ca^{2+}$ transients and $Ca^{2+}$ waves.

Both propagate in the intracellular milieu. However, $Ca^{2+}$ waves may reflect long-range signaling that can propagate hundreds of micrometers and activate hundreds of cells [12].

Astrocytes intensively express receptors for most neurotransmitters, and respond to them through intracellular $Ca^{2+}$ oscillations, followed by the propagation of intercellular $Ca^{2+}$ waves. The mechanism by which intracellular $Ca^{2+}$ rises is believed to start with phospholipase C activation and $IP_3$ production. The latter then triggers endoplasmic reticulum-dependent $Ca^{2+}$ release [54].

The $Ca^{2+}$ elevation can be restricted to a single cell, but it can also spread across adjacent non-activated cells as intercellular $Ca^{2+}$ waves (ICWs). Astrocytes derived from cell culture, brain slices, and whole retina preparations are all capable of propagating ICWs [10, 55–57]. Although the velocities by which the $Ca^{2+}$ waves travel vary according to different sample preparations and types of stimuli. Gap junction channel-mediated ICWs were the first pathway identified in astrocytes [58]. Many early studies support the idea that gap junction channels play an important role in the rapid propagation of $Ca^{2+}$ waves, even though the gap junction blockers used may not be highly specific [59, 60]. The extracellular pathway of the spread of intercellular $Ca^{2+}$ waves involves the activation of hemichannels and gliotransmitter release, first described by Osipchuk and Cahalan [61] in non-coupled mast cells. Later, Guthrie and colleagues [62] found that ATP is the extracellular molecule released by activated astrocytes. ATP or glutamate acting on plasma membrane receptors mediates the extracellular mechanism, and most brain regions are more responsive to ATP than glutamate except for the striatum [11, 58].

Whether the association of $Ca^{2+}$ waves with behavioral performance and astrocytic networks can be computed remains a challenge. The computational model of $Ca^{2+}$-mediated astrocyte function, which was well described in detail by Manninen and Linne [63], provides their thoughts for future studies. One recent study showed that sleep and wakefulness are accompanied by state-dependent changes in astroglial activity [64]. Using miniature microscopy, they found that astroglial $Ca^{2+}$ signals reach a peak when awake and are lowest when asleep. Notably, $Ca^{2+}$ signals are most pronounced in astroglial processes [64]. These data suggest that glial cell networks regulate animal behavior in an enduring and large-scale manner that is different from neural circuits.

*Calcium Signaling in Oligodendrocytes*

Since communications between astrocytes are partially dependent on Cx-based $Ca^{2+}$ waves, oligodendrocytes communicate with astrocytes through gap junctions as well. Indeed, using laser photo-stimulation and $Ca^{2+}$ imaging in primary cultures, Parys and co-workers demonstrated the presence of bi-directional $Ca^{2+}$ waves from astrocytes to oligodendrocytes that are sensitive to gap junction blockers [65]. Oligodendrocytes do not communicate with each other *via* $Ca^{2+}$ waves. However, they do experience an increase in $[Ca^{2+}]_i$, which is mediated by numerous $Ca^{2+}$ channels on the plasma membrane. Oligodendrocytes express voltage-gated $Ca^{2+}$ channels [ligand-gated $Ca^{2+}$ channels (i.e. AMPARs, NMDARs, and P2X7 channels), and GPCRs (i.e. mGluRs and mAChRs)], $Ca^{2+}$-sensing receptors, and $Ca^{2+}$ release-activated $Ca^{2+}$ channels, which make oligodendrocytes a perfect effector of glial $Ca^{2+}$ signaling [66–68]. Cx abnormality in both oligodendrocytes and astrocytes induces demyelination [69].

*Calcium Signaling in Microglia*

Microglia are distributed across the entire brain; they account for 5–12% of the total number of cells in the mouse brain and 0.5–16.6% in the human brain depending on where they are located [2]. Under physiological conditions, microglia are characterized by a ramified morphology with a small soma and long and highly motile processes constantly surveying their territory.

Once activated by pathogen-associated molecular patterns or damage-associated molecular patterns (DAMPs), microglia have elevated intracellular free $Ca^{2+}$ concentrations mediated by ionotropic and metabotropic receptors in the plasma membrane [70, 71]. These elevations, in turn, trigger effector functions of microglia, such as phagocytosis, chemotaxis, and the release of pro- and anti-inflammatory cytokines [72–74]. For example, ATP is a typical DAMP that can activate $Ca^{2+}$-permeable ligand-gated ion channel P2X receptors [75]. Microglia release ATP in response to glutamatergic AMPAR activation and subsequent PKC activation. This process causes $Ca^{2+}$ release from internal ER stores and acts as positive feedback to allow microglia to rapidly shift states, recruit surrounding cells, and proliferate in response to stimuli [75].

In addition to ligand-activated $Ca^{2+}$ signaling, microglia also undergo spontaneous transient elevations in intracellular $Ca^{2+}$ [70]. Spontaneous $Ca^{2+}$ transients can be recorded in both quiescent and activated microglia *in vivo*, *in situ*, and *in vitro*. Furthermore, these $Ca^{2+}$ transients are not triggered by astrocytic $Ca^{2+}$ waves and the frequency is increased in the absence of neuronal activity. However, in healthy young animals, spontaneous $Ca^{2+}$ transients in cortical microglia measured *in vivo* are relatively rare and their physiological function remains unclear.

## Pathological Relevance of Glial Networks in the CNS

Glia in the CNS are activated in response to pathological conditions, such as neurodegenerative diseases, psychiatric

disorders, brain tumors, and pain. Activated glia display a hyper-functional state [76]. The disturbed astrocytic network can exacerbate the progression of CNS disease and clinical symptoms, highlighting its potential for therapeutical intervention.

### Neurodegenerative Diseases

Neurodegeneration is a chronic process with gradual loss of the structure and function of neurons, which are essential for mobility, coordination, strength, sensation, and cognition. Neurodegenerative diseases, including amyotrophic lateral sclerosis, Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease, occur as a result of neurodegenerative processes [77–79]. The deregulated glial network has been implicated in these diseases. Targeting dysfunctional glial networks, instead of the direct intervention in neuronal activity, could be a new therapeutic strategy against neurodegenerative diseases [80].

As previously described, glial network scaffolds and $Ca^{2+}$ waves mediated by gap junctions have multiple functions in regulating glial and neuronal activity and cell-to-cell communication. For example, *Cx30*-deficiency attenuates A2 astrocyte responses and induces severe neurodegeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride-induced mouse model of PD [25]. Moreover, analysis of GJA1 (Cx43) expression across 29 transcriptomic and proteomic datasets from post-mortem AD and normal control brains has revealed that GJA1 is strongly associated with AD pathology and cognitive decline [81]. Astrocytes lacking GJA1 exhibit reduced ApoE levels and impaired A $\beta$ phagocytosis [81], suggesting the importance of *GJA1* in AD pathogenesis. At the network level, A $\beta_{25\text{-}35}$ has been reported to increase the intracellular pool of Cx43 but impairs the assembly of functional gap junctions between astrocytes [82]. This study provided a novel insight into the intercellular communication between astrocytes in AD and provided a new therapeutic strategy for AD intervention.

Besides a deficiency of gap junctions, they also mediate the systemic pathology of neurodegeneration. It has been reported that gap junctions can contribute to β oscillations in the basal ganglia of parkinsonian animal models and PD patients, and administration of a gap-junction inhibitor attenuates β oscillations and improves left forepaw akinesia [83].

Although currently there is no direct evidence showing connections between microglia, a recent study has revealed that, in the context of PD, microglia establish an "on-demand" functional network allowing the transfer of the burden of aggregated α-synuclein to neighboring microglia. Moreover, in addition to the a-synuclein burden, microglia also share mitochondria through tunneling nanotubes consisting of F-actin [46]. This process is crucial for PD pathogenesis, as its disruption leads to increased inflammatory profiles and cell death in PD [46].

### Psychiatric Diseases

Astrocytic Cx dysfunction is closely associated with depressive-like behavior. In a mouse model of social defeat stress, the frequencies of sEPSCs in the medial prefrontal cortex (mPFC) and hippocampus were significantly reduced along with a decrease in astrocytic Cx30 and Cx43. And this reduction only occurred in the mPFC and hippocampus but not in the amygdala and ventral tegmental area, indicating that the region-specific function of the astrocyte network is pivotal [84].

### Glioblastoma

Glioblastoma is an aggressive type of cancer that begins in astrocytes in the brain or spinal cord. Glioma cells are reported to form gap junctions with surrounding astrocytes and microglia *in vitro*. The hetero-cellular gap junctions between glioma cells and astrocytes/microglia are increased after a longer incubation period with a higher number of glioma cells, supporting glioma invasion, adhesion, and migration [59, 85].

### Pain

Unlike many other neurological diseases, neuropathic pain has a rapid onset. As mentioned previously, the gap junction communication between astrocytes is mediated by Cx43. Nerve injury induces the expression of Cx43 in astrocytes and switches the function of Cx43 from forming gap junction to paracrine modulation, which causes increases in the release of glutamate, ATP, and chemokines [86, 87]. Peripheral nerve injury is characterized by mechanical allodynia, a pain evoked by normally innocuous stimulation, such as light touch. This causes remarkable microgliosis in the spinal cord. Microgliosis in response to nerve injury is elevated by ATP and the ATP receptor subtype P2X4 is upregulated [88]. Tsuda *et al*. demonstrated that spinal injection of ATP-activated microglia was sufficient to evoke rapid mechanical allodynia within one hour of injection [89]. Together, these results suggest that activation of microglia and astrocyte induces neuropathic pain *in vivo*.

## Challenges in Glial Network Research

### Glial Heterogeneity

It is now well established that astrocytes are not a physiologically homogenous population; they differ between brain

regions and even within the same brain region. For example, Martín and co-workers found that striatal subpopulations of astrocytes release glutamate that selectively activates NMDRs in homotypic, but not heterotypic, medium spiny neurons [28]. Their finding raises the possibility that cell-specific astrocytic mini-networks regulate information flow in certain brain regions, providing a new perspective on circuit assembly and dynamics in the brain. Moreover, in the past few decades, reactive astrocytes have been identified in nearly all diseased conditions, such as CNS injury, neurodegeneration, and brain tumors. Two different types of reactive astrocytes, termed A1 and A2, may be induced by neuroinflammation. Each type displays distinct responses and properties in different injuries [27, 90, 91]. Furthermore, the soma and processes of astrocytes may have different responses to the same stimuli [92]. For example, the $Ca^{2+}$ signaling pathways we know so far are mainly derived from studies on astrocyte somas. In contrast, our understanding of $Ca^{2+}$ signaling in the processes remains rather limited. Altogether, these features of astrocytes further highlight the complexity of astrocyte networks in the brain.

Like astrocytes, microglial heterogeneity has also been investigated [93]. In healthy mouse and human brains, unbiased clustering has revealed the presence of nearly 10 subclusters of microglia [94]. The current understanding of cell markers and the biological functions of these subclasses of microglia in the healthy brain remains limited. Further, in disease states microglia are classified into several different subtypes, such as M1 and M2 subtypes, DAMs (disease-associated microglia), plaque-associated microglia, dark microglia, and human Alzheimer's microglia [95] Both healthy and disease-associated microglial subclusters could exist in the CNS at the same time. How these different microglial subtypes influence each other and interact with other glia in response to different stimuli is still a very challenging question.

In addition to temporal and spatial heterogeneity, species heterogeneity is also a very important aspect. Morphological analysis of astrocytes in the rodent and human neocortex shows marked differences between the two species. In contrast, the functional properties of astrocytes and NG2 glial cells in these species are strikingly similar [96]. The difference in glial biology between experimental animal models and humans may substantially impact our data interpretation.

### The Glial Network is not Quiescent but Highly Dynamic

Unlike neurons, glial cells, in particular microglia, often change their morphology to a more mobile type in response to stimuli. Using two-photon imaging microscopy, researchers have revealed that microglial processes make brief, repetitive contacts with synapses at a frequency of about once per hour in the somatosensory and visual cortex [97]. And this microglial behavior is modulated by neuronal activity. For instance, light deprivation reduces motility while re-exposure to light reverses it. Depending on the timing and type of the disease, microglia can be multifaceted, such as the cause, contributor, bystander, protector, or consequence of neuronal dysfunction [98, 99]. This comes up with a new question of how the glial network changes its characteristics during disease progression.

### The Glial Network is much more Complex than Glia Themselves.

Given that Cx-based gap junctions serve as links connecting oligodendrocytes (O/O junctions), as well as with astrocytes (O/A junctions), it is reasonable to suspect that each glial networks are not entirely independent of each other. Instead, they may function collaboratively, depending on the context and the specific needs. Interestingly, different types of glial cells influence each other. For instance, astrocytic gap junctional communication and Cx43 expression are inhibited by microglia-derived IL-1β and tumor necrosis factor-α (TNF-α) [100]. Conversely, activated astrocytes facilitate microglial activation *via* ATP, which acts on purinergic receptors in microglia. It is plausible that astrocytes control the activation of microglia not only in the local region but also in a broader area since during the propagation of $Ca^{2+}$ waves in the astrocyte network, distant microglia can be activated in response to ATP released by astrocytes [101]. On the other hand, under certain circumstances, activated astrocytes can also inhibit microglia by suppressing the production of pro-inflammatory mediators, such as nitric oxide, reactive oxygen species, and TNF-α [102]. These findings add new layers of complexity to glial networks.

## Perspective: Mapping the Glial Connectome

Glia cells are wired together through gap junctions or tunneling nanotubes, demonstrating a potential for glial cells to function as a whole at the system level in the modulation of neuronal activity. It remains to be determined how glial cells are connected and how their connections are functionally regulated under various conditions. Therefore, it is important to investigate the glial connectome focusing on the structural and/or functional connections between a variety of types of glial cells, in addition to neuronal-level connectomes to which much attention is currently being paid.

We hypothesize that astrocytic $Ca^{2+}$ is at the core of glia-to-glia interactions. Astrocytes receive stimulatory inputs from neurons or neighboring astrocytes, which leads to the generation and propagation of $Ca^{2+}$ waves. The astrocytic $Ca^{2+}$ wave likely encodes information about glial activity

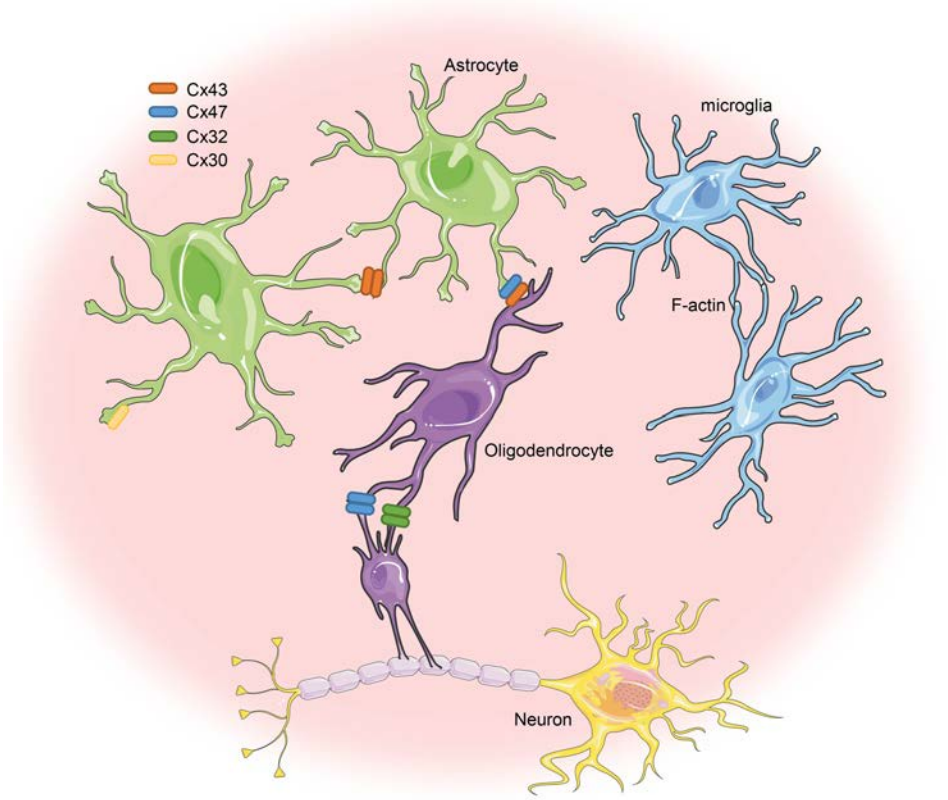


**Fig. 1** Astrocytes and oligodendrocytes are connected mainly through connexin-based gap junction channels. Astrocytes form A/A gap junction through Cx43 and hemichannels through Cx30. Astrocytes form A/O gap junction through Cx43 on astrocytes and Cx47 on oligodendrocytes. Oligodendrocytes form O/O gap junctions through Cx47 and Cx32. Microglia wire together through F-actin-containing membrane-membrane connections.

and synaptic transmission, and it could be shaped and altered to some extent by neuronal activity during its propagation. $Ca^{2+}$ signals also reach oligodendrocytes and microglia but have much more enduring effects on diverse biological processes. Given its quantifiable and propagative nature, astrocytic $Ca^{2+}$ activity may represent an embodiment of higher-level brain functions. Decoding glial $Ca^{2+}$ signaling may be an important step for understanding glial communication and the regulation of brain activity as a whole in the future(Fig. 1; Table 1).

**Table 1** Glial network in CNS diseases.

| | Glia-glia connections | Glial $Ca^{2+}$ signaling |
|---|---|---|
| Alzheimer's disease | GJA1 is strongly associated with AD amyloid and tau pathologies and a decline in cognitive function | β-amyloid peptide stimulates L-type voltage-gated $Ca^{2+}$ channels. |
| Parkinson's disease | Cx30 deficiency induces severe neurodegeneration in a PD animal model | A53T mutation in α-synuclein upregulates and hyperactivates N-type voltage-gated $Ca^{2+}$ channels |
| Brain injury and Neuroinflammation | Microglia may be capable of forming homo-cellular syncytia mediated by gap junctions in the context of CNS bacterial infections | LPS challenge significantly elevates the baseline of spontaneous microglial $Ca^{2+}$ activity. |
| Brain tumor | Glioma cells form gap junctions with surrounding astrocytes and microglia *in vitro* | |
| Pain | Cx43 is up-regulated in astrocytes and switches function from forming gap junctions to paracrine modulation | |

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REVIEW

# Interactions Between Astrocytes and Oligodendroglia in Myelin Development and Related Brain Diseases

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**Abstract** Astrocytes (ASTs) and oligodendroglial lineage cells (OLGs) are major macroglial cells in the central nervous system. ASTs communicate with each other through connexin (Cx) and Cx-based network structures, both of which allow for quick transport of nutrients and signals. Moreover, ASTs interact with OLGs through connexin (Cx)-mediated networks to modulate various physiological processes in the brain. In this article, following a brief description of the infrastructural basis of the glial networks and exocrine factors by which ASTs and OLGs may crosstalk, we focus on recapitulating how the interactions between these two types of glial cells modulate myelination, and how the AST-OLG interactions are involved in protecting the integrity of the blood-brain barrier (BBB) and regulating synaptogenesis and neural activity. Recent studies further suggest that AST-OLG interactions are associated with myelin-related diseases, such as multiple sclerosis. A better understanding of the regulatory mechanisms underlying AST-OLG interactions may inspire the development of novel therapeutic strategies for related brain diseases.

**Keywords** Astrocyte · Oligodendroglia · Glial network · Myelination · Synaptogenesis · Neuroinflammation

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## Introduction

Astrocytes (ASTs) and oligodendrocytes (OLs), the two types of macroglial cells abundant in the central nervous system (CNS), are derived from the same radial glia or neural stem cells. Both were thought to be passive supporters of neurons due to their electric non-excitable characteristic [1]. In recent years, studies have shown that ASTs can transmit information over long distances in the brain by means of the intercellular spread of $Ca^{2+}$ waves [2]. Moreover, ASTs can regulate synaptic development and plasticity through tripartite synapses, and actively participate in brain functions such as learning and memory [3].

Oligodendroglial lineage cells (OLGs) are a series of developing cells that mature progressively from oligodendrocyte precursor cells (OPCs) into post-mitotic myelinating OLs. Besides ensuring the rapid conduction of neuronal action potential by myelin, OLs provide metabolic support to axons through the monocarboxylic acid transporter 1 (MCT1)-mediated lactate shuttle [4]. Recent studies have demonstrated that the proliferation and differentiation of OLGs are highly dynamic and plastic, as the newly-formed myelin sheath is not only necessary for motor learning, but also contributing to the enhancement of fear memory consolidation and synaptic plasticity [5–7]. Moreover, OLGs can adopt an immune phenotype by expressing specific genes previously thought to be unique to immune cells and act as an initiation factor of immune inflammatory diseases such as multiple sclerosis (MS) [8, 9].

Notably, as essential components of the CNS glial microenvironment, ASTs and OLGs communicate and interact with each other through Cx-mediated glial networks, and regulate each other in a paracrine manner [10]. Increasing evidence suggests that the interactions between ASTs and OLGs play important roles in modulating

various physiological processes in the brain, myelination in particular. For instance, the proliferation and differentiation of OLGs are regulated by factors or extracellular vesicles (EVs) derived from ASTs. In addition, ASTs and OLGs are capable of regulating synaptogenesis and synaptic transmission, suggesting their interaction around synapses (Fig. 1). The interactions between ASTs and OLGs are also involved in the pathogenesis of brain disease, especially demyelinating diseases such as MS, in which both ASTs and OPCs secrete factors to modulate the permeability of the BBB. Moreover, the evidence of OLGs adopting an immune phenotype suggests that ASTs and OLGs co-regulate glia the activation and immune inflammation process (Table 1). In this review, we recapitulate the communication basis of the glial network and mainly focus on the role of AST-OLG interactions in myelin development and related diseases.

## Correlations of ASTs and OLGs during CNS Development

Derived from the same progenitor cells (radial glia/ neural stem cells) in neural epithelia, the first step in the genesis of ASTs and OLGs is specialization, which is determined by a switch of the external glial signals in the niche, such as bone morphogenic protein (BMP) or Notch signaling, activation of which is crucial for AST genesis [11]. Normally, the differentiation of each glial lineage is controlled by a special transcriptional program with the expression of a series of specific genes. For example, in astroglia, glutamate aspartate transporter (GLAST) and glutamine synthetase (GS) are expressed in the immature stage [12], while glial fibrillary acidic protein, S100 β, and Cxs are mainly expressed during or after maturation. During OLG development, platelet-derived growth factor alpha receptor and Neuron-glial antigen 2 (NG2) are mainly expressed in OPCs while myelin

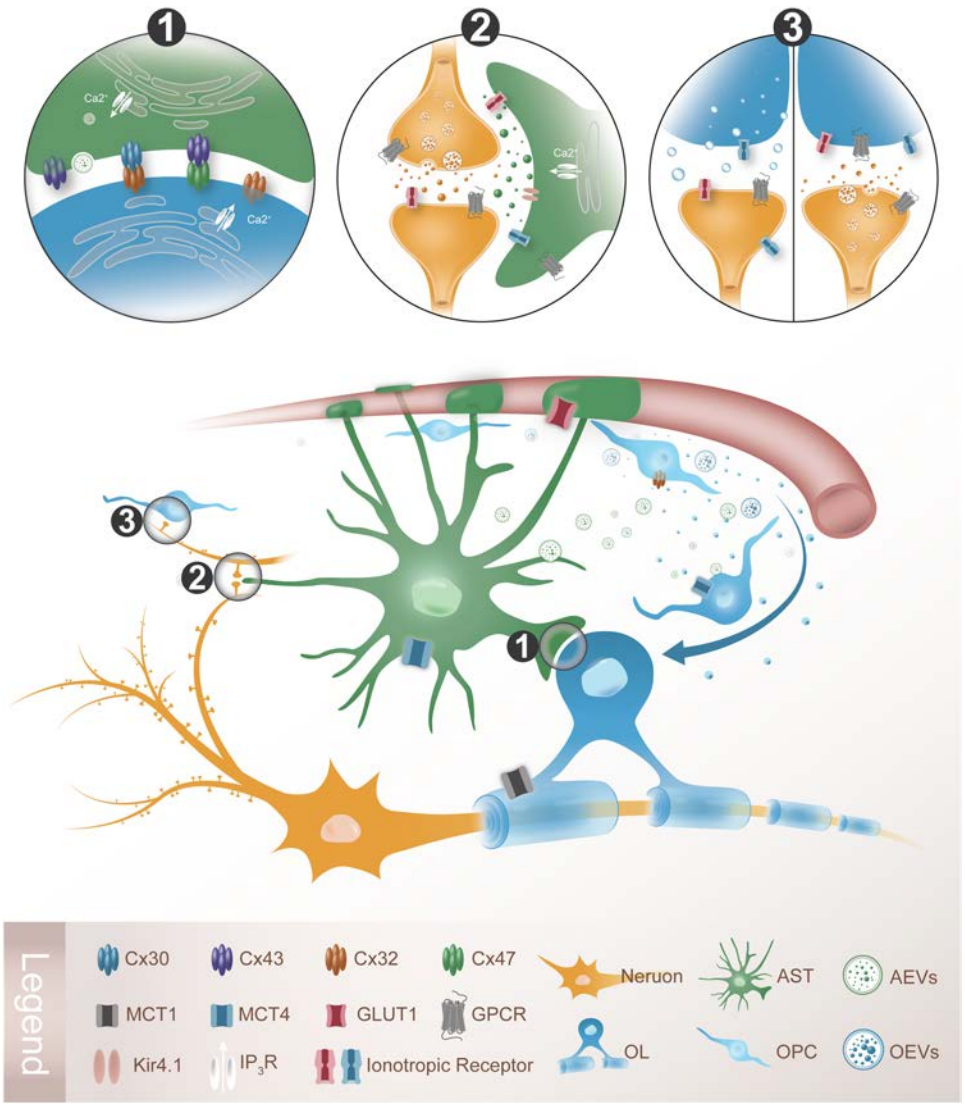


**Fig. 1** Schematic of AST-OLG interactions at three domains: associated with blood vessels, interacting at the synaptic level, and communicating *via* the Cx-mediated network. ASTs obtain their energy substrate (glucose) from blood vessels through GLUT1 to supply neurons, and the distribution of MCTs among ASTs and OLGs implies a lactate shuttle; OPCs migrate along the blood vessel and differentiate into mature OLs to form myelin; this is regulated by AST secretory factors or AEVs. In the enlarged view: ① ASTs and OLs form heterotypic gap junctions (A:O) or hemichannels by specific Cxs (Cx43/Cx47, Cx30/Cx32) for cell communication and substance exchange. The opening of hemichannels depends on signaling by Ca2+ released from the ER. ② The AST-neuron tripartite synapse: AST regulates synaptic transmission *via* neurotransmitter receptors such as GPCRs located on the AST membrane or gliotransmitters. ③ Proposed patterns of the OPC-neuron synapse: OPCs acts as presynaptic membrane (left) or as postsynaptic domain (right), by which OPCs modulate synaptic transmission.

**Table 1** ASTs interplay with OLGs in demyelinating diseases.

| Detrimental roles | Ref. | Functional factors | Beneficial roles | Ref. |
|---|---|---|---|---|
| ASTs secrete IFN-γ to switch OPCs to immunophenotype and reduce OPCs | [8] | Exocrine molecules | IL-1β，CCL2, and CXCL1 released by ASTs recruit OPCs | [113] |
| Co-recruit T cells and cross-present antigens to amplify inflammation | [8] | | FGF and PDGF secreted by ASTs promote OPC survival or proliferation | [33] |
| TNF-α released by ASTs induces OPC cell death | [113] | | CNTF and IGF produced by ASTs promote OPC proliferation and remyelination | [10] |
| MMP9 produced by OPCs destroys the BBB | [53] | Architecture | OPCs up-regulate claudin-5, occludin, and β-catenin to increase BBB tightness | [54] |
| OPCs dislocate the end-feet of ASTs | [57] | | Glial scars provide a complex of regulating factors for OLG remyelination | [111] |
| Double knockout of Cxs in ASTs | | Cx network | O:A gap junctions and hemichannels support OPC survival and | [18] |
| and OLs induces myelin breakdown | [24] | | proliferation | |
| | | | Cx43 deletion in ASTs promotes OPC differentiation and remyelination | [27] |

Demyelination

Remyelination

basic protein (MBP), myelin proteolipid protein, and myelin oligodendrocyte glycoprotein (MOG) are used to identify OLs [11].

In some cases, such as in culture, OPCs are considered to potentially differentiate into OLs or ASTs [13]. After CNS injury, neurons are rarely replaced, but ASTs and OLGs are highly regenerated through homologous differentiation or replacement by each other. It has been reported that OPCs can produce ASTs by trans-differentiation [14]. Using pedigree tracking in OL-specific presenilin enhancer 2 (Pen-2) knockout mice, it was found that newly-generated ASTs are produced by labeled OPCs. Further studies have shown that deletion of Pen-2 in OPCs promotes the formation of ASTs by the Notch-STAT3 signaling pathway [15]. Recently, it was reported that overexpression of DLX2 by injecting lentivirus (e.g. Lv-gfap-dlx2) into the mouse striatum induces adult ASTs to turn into Achaete-scute homolog 1 (ASCL1) neural progenitor cells (NPCs) within two weeks, and subsequently differentiate into multi-lineage cells such as neurons, ASTs, and OLGs. The reprogramming process is closely similar to endogenous neurogenesis, which requires activation of the Distal-less homeobox (DLX) family and inhibition of the Notch signaling pathway [16]. Although the underlying regulatory mechanism is not clear and the exocrine factors derived from either ASTs or OLGs remain to be identified, it could be speculated that exocrine mechanisms account for this process.

## Infrastructure and Mechanisms of AST-OLG Interactions

### Cx-mediated AST-OLG Communication

In the CNS, a typical feature of glial cells is the strong expression of Cxs, which form gap junctions or hemichannels on the cell surface, enabling the exchange of ions and small molecules between adjacent cells and the direct communication of signals and metabolites [17, 18]. Moreover, these gap junction channels play a pivotal role in the control of the surrounding ionic homeostasis, such as buffering $K^+$. Mature ASTs strongly express Cx43 and Cx30 by which they form a complex glial network [19], while Cx47, Cx32, and Cx29 (also known as GJC2, GJB1, and GJC3, respectively) are present in OLs [20, 21]. Besides the homologous gap junction among the same glial type, heteromorphic gap junctions can form between different cell types, including ASTs and OLs (Fig. 1). For instance, OLs are specifically coupled

to ASTs through heteromorphic gap junctions composed of Cx47:Cx43 or Cx32:Cx30 (O:A coupling) [22]. $Ca^{2+}$ and glucose can be delivered through these gap junction channels, forming a pan-glial metabolic route between OLGs and ASTs [18, 23, 24]. It has been experimentally demonstrated that Cx47 is more important than Cx30 in the formation of O:A couplings [20]; it is asymmetrical since it shows electrical and metabolic rectification activity [25].

Moreover, hemichannel activity depends on intracellular $Ca^{2+}$ elevation, which is involved in OLG differentiation [26]. It has been found that there are no functional gap junctions between ASTs and OPCs. However, deletion of Cx43 in ASTs inhibits OPC proliferation by decreasing matrix glucose levels without impacting OPC hemichannel properties, which mediate glucose uptake for OPC proliferation [27]. Under pathological conditions, for example, oxidative stress and inflammation in ischemic stroke, the stability of OL gap junctions relies on Cx43 expression in ASTs [28]. A deficiency of Cx43 causes the internalization and degradation of Cx47, hampering the transport of ions and nutrients and spreading inflammatory mediators [29]. As a result, the inflammatory response after loss of glial cells and myelin break-down in ischemia is severely aggravated [30].

The activity of the inflammasome and/or hemichannels of OLGs can be increased by prenatal stress, which is mimicked by urocortin II [31]. Recently, in a mouse model of maternal isolation monitoring early life stress, a decrease in the number of OPCs in the hippocampus was observed, accompanied by developmental disturbance of ASTs and the functional AST network. Furthermore, in the PDGFRαCreER:DTA mouse model, in which OPCs are eliminated by tamoxifen, the morphology of ASTs displayed an atrophic state. Importantly, both expressions of Cx30 and Cx43 was reduced [32], suggesting that OPCs can regulate AST development and network formation.

## Exocrine Regulation of AST-OLG Communication

Both ASTs and OLGs secrete a variety of factors, and regulate each other during development. For example, platelet-derived growth factor AA secreted by ASTs is a trigger or substance that promotes OPC proliferation and maintains their survival by activating the JAK/STAT signal pathway [33]. ASTs also secrete neurotrophic factors such as insulin-like growth factor 1 (IGF-1) and ciliary neurotrophic factor [34], which are essential for OPC differentiation and maturation through the MAPK/ERK signal pathway, while inhibiting oligodendroglial apoptosis *via* PI3K/AKT signaling. In adulthood, IGF-1 has been found to promote OPC differentiation from neural stem cell (NSCs) by inhibiting the BMP signal pathway. Moreover, ASTs release BMPs that prevent OPC differentiation. Therefore, astrocytic factors regulate OL development either positively or negatively (Fig. 1).

Similarly, OPCs can also have a paracrine effect on the development of ASTs. Our recent study, by knockout of Wnt7a/b in OPCs, demonstrated that OPC-derived Wnt7a/b, especially Wnt7b, mediates the regulation and influence on ASTs *via* the Wnt/β-catenin pathway. Then, supplementation of Wnt7a/b can remedy the naive state of ASTs by increasing the expression of Cx43 and Cx30 and enhancing the function of the astroglial network [32].

## Co-regulation of ASTs and OLGs *via* Extracellular Vesicles

EVs are nanometer-sized vesicles secreted by almost all living cells, and the predominant forms are exosomes and microvesicles [35]. Mediated by the containing proteins, lipids, and miRNA, EVs are an alternative mode of intercellular communication for many physiological and pathological functions [36]. Since EVs can even cross the BBB [37], they not only tie intercellular communications throughout the nervous system, but also work as potential biomarkers in the circulation for neurodegenerative diseases such as Alzheimer's disease [38].

With emerging research, communications between ASTs and OLGs could be achieved by EVs as an option. Recent studies have reported that AST-derived EVs (AEVs) contain fibroblast growth factor-2 and vascular endothelial growth factor (VEGF), which had been confirmed to regulate OPCs in brain development [39]. Critically, AST-derived exosomes have been reported to enhance the chemotaxis of OPCs, improving their differentiation and migration under ischemia *in vitro* and inhibiting their proliferation under severe hypoxia [40]. In addition, AEVs with aging phenotypes have negative effects on OL maturation [41]. Moreover, ASTs can augment the production of exosomes by OPCs *via* ITGB4-mediated cell adhesion and thus stimulate OPC proliferation [42]. Therefore, the secretion of EVs seems likely to be the result of the interaction between OPCs and ASTs.

Subsequently, OLs-derived EVs (OEVs) were also discovered. They generally store myelin components, such as CNPase (2',3'-cyclic-nucleotide 3'-phosphodiesterase), MBP, MOG, myelin-associated glycoprotein, cholesterol, and sphingolipids, and thus contribute to the regeneration and maintenance of myelin [43], increasing action potential promoting axonal transport [44]. Besides, intercellular delivery of OEVs containing SIRT2 are critical for axonal energy enhancement [45]. Similarly, AEVs loaded with synapsin-1 foster axonal growth, the functional maturation of synapses, and synaptic plasticity. The release of EVs by both may have special regulation patterns in synapses, but the exact mechanism remains to be explored. Moreover, OEVs can aid neuronal resistance to ischemic stress *in vitro* by transferring superoxide dismutase and catalase [46], while

Apo-D in AEVs has been found to resist oxidative stress and be essential for anti-aging and the prevention of neurodegenerative diseases. Recently, EVs have been shown to be loaded with immune factors and complement, mediating immune regulation [39, 47].

Notably, a recent study pointed out that conditional knockdown of dicer, an essential miRNA synthetic enzyme in ASTs, inhibits OPC differentiation and delays remyelination in the lysophosphatidyl-induced demyelination mouse model [48], suggested an important role of AST-derived miRNAs in OPC differentiation. It has been found that miR-302/367 induces the conversion of astrocytes to OLGs and enhances myelin repair *in vivo* [49]. Given that AST-derived EVs (AEVs) promote OPC migration and differentiation under severe hypoxic conditions [40], it is possible that miRNA-enriched AEVs may be involved in the regulation of OPC differentiation; this requires further investigation.

## AST-OLG Interactions and BBB Integrity

The BBB is a physical protective interface composed of multiple cell-types. It maintains the immune privilege and dynamic stability of the CNS. As an important component of the BBB, ASTs play a vital role in the maintenance of its integrity. In recent years, OPCs have attracted attention due to their close positional relationship with the BBB. During CNS development, OPCs use the intracephalic vascular system as a scaffold for migration [50], suggesting that they may have close contact with ASTs. By adding OPC-conditional media to endothelial cell cultures, subsequent pharmacological experiments demonstrated their ability to activate the MEK/ERK signaling pathway and enhance the expression of tight junction proteins *in vitro* [10, 51], suggesting an important role of OPCs in the permeability of the BBB. Moreover, OPC-specific transforming growth factor β (TGF-β)-deficient mice show cerebral hemorrhage and BBB damage, suggesting that OPC-derived factors regulate BBB integrity [52]. Under pathological conditions, such as demyelination in mice induced by long-term cerebral hypoperfusion stress, matrix metalloproteinase-9 secreted by OPCs induces early BBB destruction and inflammatory infiltration [53]. In the ischemic stroke model of transient arterial occlusion (90 min), OPC transplantation can activate the Wnt/β-catenin pathway to protect the BBB during the acute phase of ischemic stroke and promote the recovery of neurological function. Further studies have demonstrated that OPCs can rescue BBB leakage by increasing the expression of claudin-5, occludin, and β-catenin in endothelia [54].

Because of their close relationship with the vascular system, OPCs may not only participate in the pathogenesis of the BBB, but also be victims of its destruction. For example, after BBB breakdown, the increased vascular permeability leads to the leakage of plasma proteins, destroying the original micro-environmental homeostasis. As a consequence, the coagulation factor fibrinogen induces the activation of BMP signaling in OPCs and thus inhibits their differentiation and remyelination [10]. Indeed, at the beginning of demyelination, such as in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, it has been reported that tumor necrosis factor (TNF) secreted by ASTs or endothelial cells may cooperate with TNF receptors on the surface of OLs to damage the BBB [55]. This is accompanied by the upregulation of hypoxia-inducible factor-1 directly produced by activated ASTs and microglia, which boost VEGF and interleukin (IL)-1 and further aggravates the BBB permeability in EAE [56]. Dysfunctional Wnt signaling in MS can result in OPC aggregation along the blood vessels, which may interfere with AST end-feet on the vascular surface [57], leading to altered vascular permeability and the related inflammatory response. Thus the interaction between ASTs and OPCs is important in regulating the integrity of the BBB (Fig. 1).

Interestingly, deficits in Cxs can also induce BBB injury [58]. Compared with normal mice, Cx47-KO mice are more susceptible to EAE and exhibit more intense pathologies, including destruction of the blood-spinal cord barrier, infiltration of inflammatory cells into spinal cord tissue, and apoptosis of OLs [59, 60]. Thus, the Cx-mediated AST-OLG networks are also involved in the regulation of BBB integrity.

## AST-OLG Interactions in Nourishing Neurons

### Modes of Neuronal Energy Support

In the human brain, glucose is the major energy source for neuronal activity; alternative energy sources include ketone bodies, lactate, fatty acids, and amino-acids. As neurons consume high energy for neural transmission but lack glycogen storage, so they rely on glial cells for glucose acquisition [61, 62]. ASTs, as the main glycogen storage in the brain, provide nutritional support to neurons by taking up glucose from blood vessels through the glucose transporter GLUT1 and also take up various metabolites released by neurons, such as lactate and glutamate [63]. Lactate is not only an effective fuel for neuronal activity [64], but also acts as an intercellular messenger to shuttle between ASTs and neurons [65]. In addition, ASTs play an essential role in maintaining glutamate homeostasis by recycling glutamine, an intermediate of the tricarboxylic acid cycle critical for generating glutamate, back to neurons.

Besides transporting glucose to neurons, ASTs are the only type of cells in the brain that oxidize fatty acids and produce ketone bodies to support energy for neurons in

hypoglycemia or starvation states [1]. It should be noted that ketone bodies, as with lactate, can cross the BBB *via* MCTs in endothelial cells and ASTs. During long-term fasting (5–6 weeks) in the obese patients, ketone body levels in the brain increase significantly, providing almost 60% of the brain's energy, therefore ketone replaces glucose as the primary fuel [66].

Compared to ASTs and neurons, OLGs show the highest glucose utilization because of their requirement of lipid synthesis for myelination [67]. Due to the physical isolation of the myelin sheath, OLGs support axons by supplying energy substrates. In terms of energy substrates, OLs actually prefer lactate to glucose as the substrate for myelin production, as lactate produces more myelin in brain slices than glucose-treated slices. It has been reported that OLs use three times more lactate than ASTs and neurons [68]. Some experiments have shown that OLGs express MCT1, a selective transporter of lactate, which provides lactate to support axon integrity. It has been found that conditional knockout of MCT1 in OLs leads to myelin dysfunction and axonal degeneration [69], and impairment of MCT1-mediated lactate transport from OLs to axons is considered to contribute to the pathogenesis of amyotrophic lateral sclerosis [4].

### AST-OLG Cooperation in the Lactate Shuttle

In the CNS, the cellular distribution and physiological characteristics of MCTs suggest an energy correlation between different cell types by the lactate shuttle [70]. MCT1 is mainly expressed in OLGs and a few specific neurons [71], MCT2 is chiefly expressed in neurons, and MCT4 is principally expressed in ASTs. According to previous studies, ASTs transfer substrates for energy metabolism directly to OLs through heterotypic gap junctions. And it has been speculated that mature OLs provide lactate to axons through MCT1 as energy during high metabolic activity such as myelination, while lactate can also be shuttled back and forth between neurons and ASTs through MCT2 and MCT4 [72]. Some studies have shown that, in the middle cerebral artery occlusion mouse model, after ischemic reperfusion, the expression of MCT1 in the striatum is significantly up-regulated [73]. The vulnerability of OLGs to metabolic stress is related to their distinct MCT1 expression profiles of, the mild hypoxia-glucose deprivation caused by ischemia triggers the upregulation of MCT1 in OPCs to adapt to stress [74], and this enhances the transport of lactate from ASTs and blood to redistribute energy substrates. Moreover, due to the hypermetabolic demand for myelin generation, OLGs are the cells with the highest iron levels in the brain [75].

Since ASTs occupy an important position in obtaining nutrients including iron from circulating blood, upregulation of iron importers such as transferrin receptor, divalent metal transporter 1, and ZIP14 in ASTs in the EAE model was considered to favor iron supply for OLGs. Meanwhile, as the ferroportin-ceruloplasmin system is responsible for cellular iron efflux, the expression of ferroportin (SLC40A1) in ASTs suggests their important role in iron homeostasis [76, 77],which is relevant to OLG maturation and myelination [78] . Nevertheless, OLGs show relatively low glycolytic enzyme expression, and they communicate with ASTs *via* gap junctions, allowing the transfer of lactate and other metabolites between the two kinds of cells [79]. Therefore AST-OLG cooperation in the lactate shuttle is an important pathway for neuronal energy support (Fig. 1).

## AST-OLG Interactions in Neural Plasticity

### The AST-Neuron Tripartite Synapse

The synapse is the structural unit that mediates signal transmission between neurons. However, neuronal synapses are not only composed of presynaptic and postsynaptic neurons, but also are connected by AST processes in many cases to form tripartite synapses [3]. Similar to neurons, ASTs express a variety of neurotransmitter receptors in the presynaptic or postsynaptic membranes, such as receptors for glutamate, gamma-aminobutyric acid (GABA), endogenous cannabinoids, dopamine (DA), 5-hydroxytryptamine, ATP/adenosine, acetylcholine, and opioids. Many of the neurotransmitter receptors of ASTs are G protein-coupled receptors (GPCRs) [80]. Once activated by neurotransmitters released from the presynaptic membrane, the elevation of intracellular $Ca^{2+}$ causes ASTs to release signaling molecules, namely gliotransmitters, which in turn modulate synaptic transmission, either excitatory or inhibitory. The gliotransmitters glutamate, GABA, D-serine, and ATP may differently participate in long-term potentiation (LTP), long-term depression, and heterosynaptic facilitation/depression [81].

It has been shown that purinergic signals such as ATP and adenosine play important roles in regulating synaptic activity and function. Purinergic receptors are classified as P1 (adenosine receptors) and P2 (ATP receptors), both of which are involved in neuron-ASTs interactions. Numerous studies have shown that synaptic activity causes ATP released from ASTs to induce homosynaptic and/or heterosynaptic inhibition. Extracellular adenosine levels are largely dependent on ATP release by ASTs. Adenosine inhibits synaptic transmission by activating A1 receptors while activation of the A2A receptor enhances this process [82]. It is important to note that, due to the difference in the distribution of presynaptic receptors, AST-derived ATP or adenosine can have different effects on different neurons or neural circuits. For example, hippocampal ATP/adenosine derived from ASTs down-regulate excitatory synaptic transmission by

activating presynaptic adenosine A1 receptors and enhance inhibitory synaptic transmission by activating postsynaptic P2Y1 receptors [83], thereby effectively down-regulating the excitability of the entire hippocampal neural circuit.

Moreover, D-serine released by ASTs can act as an endogenous co-agonist of postsynaptic N-methyl D-aspartate receptors (NMDARs), enhancing NMDAR activity. For example, activation of α7n-acetylcholine receptors in ASTs results in the release of D-serine, which enhances postsynaptic NMDAR activity and fear memory [84]. Activation of cannabinoid receptor 1 (CB1) in ASTs also increases $Ca^{2+}$ activity and releases D-serine, which in turn activates NMDARs to enhance hippocampal-dependent object recognition memory. CB1-knockout in ASTs inhibits LTP in the hippocampus and reduces object recognition memory [85], effects that can be reversed by exogenous D-serine supplementation. In addition, the $K^+$ channels Kir4.1, mGluR3, and mGluR5 have been shown to monitor and regulate synaptic function, thus actively controlling synaptic transmission [86].

Although the regulatory mechanism remains unclear, IP3R2 (receptor for inositol 1,4,5-trisphosphate) has been considered the main regulator of GPCR-mediated $Ca^{2+}$ mobilization in ASTs. Consistent with this, in IP3R2 knockout mice, neurotransmitters have no impact on intracellular $Ca^{2+}$ in ASTs [87].

### Astrocytic Factors Regulating Synaptogenesis

Although most neurons are generated in the embryonic stage, synapses are not formed until birth and massively increase at the end of the first postnatal week after AST differentiation in mice. Increasing evidence indicates that signals derived from ASTs are essential for synaptogenesis and neural circuit formation, functional maturity, and improvement [88]. Experiments have shown that rodent neurons form very few synapses *in vitro*, while co-culture with ASTs greatly increases the number of synapses [89]. ASTs secrete many cytokines or gliotransmitters to regulate synapse formation, such as BDNF, TNF-α, TGF-β, thrombospondins (TSPs), glypicans, and SPARCL1. For example, TSP1/2 and SPARCL1 (secreted protein acidic and rich in cysteine)-like 1)/Hevin control the formation of glutamatergic synapses [90]. ASTs also produce a synaptic Hevin antagonist called SPARC, which negatively regulates the number and function of synapses. In addition, mediated by Wnt signaling, glial cells can affect synapse formation, pruning, and maturation by accumulating glutamate receptors in excitatory synapses [91].

Some experiments have shown that TSP promotes remodeling of the actin cytoskeleton during initial contact of synapses by activating the Rac1 pathway. With the secretion of TSP, ASTs also release the innate immune molecule pentraxin3, which promotes the maturation of excitatory synapses by accumulating AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors) in the postsynaptic membrane [92].

Moreover, to prevent excessive and inappropriate synapses, ASTs regulate synaptic elimination [93]. It has been reported that ASTs directly eliminate excess synapses in the developing brain through the MEGF10 and MERTK phagocytic pathways [94]. ASTs also indirectly regulate synaptic elimination by secreting TGF-β, which mediates synaptic elimination by microglia [95].

### OLGs Modulate Synaptogenesis and Neural Transmission

Besides forming the myelin sheath around axons, OLGs also interact with neurons and affect neural networks at the synaptic level. It has been shown that OPCs express an array of receptors (AMPARs, NMPARs, and GABARs), which are endowed with the capacity to respond to neuronal activity and thus regulate the survival, proliferation, migration, differentiation, and myelination of OLGs. However, the expression and function vary among these receptors. For example, following the discovery of excitatory synaptic transmission between neurons and NG2+ OPCs [96], the expression of AMPARs has been found in OLGs and changes with OLG development. AMPARs are highly expressed in OPCs, and down-regulate (~12-fold) upon OPC differentiation into mature OLs [97]. Sufficient evidence has shown that activation of AMPARs inhibits OPC proliferation, while blocking AMPAR activity impairs the morphological development of OPCs and promotes OPC proliferation and differentiation [98].

In addition to the AMPAR, another key ionotropic receptor mediating glutamate transmission is the NMDAR. NMDAR currents peak during the critical time of myelination, decline with sexual maturity, and completely disappear at 9 months in mice [97, 99]. The role of NMDARs in neuron-OPC synapses is controversial. Some experiments have demonstrated that NMDARs are not required for OPC proliferation and myelination, and the presence of NMDA in these synapses only accounts for the AMPAR-dependent signals in OPCs [99].

What is more, GABAA receptors were first confirmed in explant cultures of spinal cord [100], then GABAA receptor-evoked depolarization in OPCs has been described in different CNS regions. The expression of GABAA receptors is down-regulated during the differentiation process. It has been suggested that GABA plays a central role in regulating OPC proliferation, differentiation, and myelination. Stimulating GABA activity in OPCs inhibits cell proliferation but promotes differentiation, while sensitivity to GABA is largely reduced in mature OLs [101]. To date, the

understanding of OPCs has been limited to passively receiving neuron signal transmission in neuron-OPC synapses. However, a recent report demonstrated that NG2+ OPCs form presynaptic membranes with neurons in hippocampus. Photo-stimulating NG2 glia functionally drives GABA release and enhances inhibitory synaptic transmission to proximal interneurons, which may be correlated with anxiety-like behavior in mice [102]. Moreover, in a recent study, conditional ablation of Nogo-A in OLs increases the density and length of dendritic spines in motor cortical pyramidal cells [103], suggesting that the OL-specific protein Nogo-A is a regulator of events in synaptic refinement.

On the other hand, the continuous production of OPCs in the adult brain can form new myelin sheaths, which can wrap and produce a new sheath, affect the thickness of the sheath, and regulate the conduction velocity of the axon. To examine the functional significance of myelination on white matter injury (WMI), an emerging study by knocking out Olig2 (loss-of-function) or M1R (gain-of-function) in OPCs, demonstrated that hypo-myelination results in the loss of excitatory synapses and functional deficits. Enhancing myelination rescues synaptic deficits and improves motor behavior in mice after chronic hypoxia [5], suggested that myelination may facilitate excitatory presynaptic innervation.

### A Speculation: AST-OLG Interplay at the Synaptic Level

Although ASTs and OLGs are involved in the formation of and transmission by synapses by sharing the same receptors, signals and transmitters, such as AMPARs and NMDARs, no direct evidence has ever shown the interaction of both cells at the synaptic level. Given the architecture of AST-neuron tripartite synapses, and that OPCs can form GABAergic presynaptic membrane with neurons, we boldly speculate that OPCs may modulate synaptic transmission by neurotransmitter receptors or gliotransmitters the same as the AST-neuron tripartite synapse. Is it possible that OPCs surround the AST-neuron tripartite synapse with their processes to form a kind of tetragonal synapse? These interesting ideas are worthy of further study. Functionally, both glial cells were found to be able to regulate synaptic formation or plasticity in learning and memory. During this process, the communication between ASTs and OLGs, such as the glial network mediating material delivery, and the regulatory factors secreted by each cell, are likely to further promote or inhibit the formation or function of synapses. For example, the release of L-lactate by ASTs has been found necessary for long-term memory [104], while lactate metabolism involves the interactions between the two types of glial cells in previous studies [105]. In addition, gliotransmitters around the AST-neuron tripartite synapse, such as adenosine, are involved in regulating OPC development. It has been suggested that adenosine not only inhibits the proliferation of OPCs, but also stimulates the migration and differentiation of OPCs and promotes myelin formation [106]. Thus an interaction between ASTs and OLGs may exist and play an important role in synaptogenesis and plasticity (Fig. 1).

## AST-OLG Interactions in Neuroinflammation

### AST Activation in Neuroinflammation

The CNS has been considered to be immune exempted due to its unique anatomical features, including the relative absence of lymphoid drainage and specific antigen-presenting cells such as dendritic cells, the absence of secondary lymphoid organs, and the presence of mechanical barriers such as the BBB that limit the exchange of immune cells and molecules [107]. Therefore, the immune defense mechanism mainly relies on the natural immune cells residing in CNS, microglia and ASTs, which respond quickly to all kinds of insults, such as ischemia-hypoxia, injury, and infection. Activated by membrane receptors including toll-like receptors [108], purinergic receptors, and triggering receptors expressed on myeloid cells 2 (TREM2), microglia can either recruit peripheral monocytes and lymphocytes by releasing chemokines, or induce AST activation by releasing cytokines. Activation of ASTs is a common response in many pathophysiological conditions. Active ASTs are characterized by cell body hypertrophy, thickening of processes, increased branching, eosinophilia, and release a series of factors, such as cytokines (lipocalin 2, IL-1β, and TNF-α) and neurotrophic factors (BDNF and VEGF). These cytokines play either neuroprotective or neurotoxic role, ultimately triggering inflammatory responses or exacerbating CNS injury.

Taking MS as an example, microglia and ASTs have been considered to be the key players in neuroinflammation to induce OL damage and demyelination in the initial phase [109]. While the role of glial scars, one of the stuctures responsible for neural damage (such as demyelination), is controversial (Tabe1). Previously, glial scars were thought to hinder OPC survival and migration, thus inhibiting remyelination [110]. Recently, a study indicated that glial scars do not have a rigid border but rather provides a complex of regulating factors for OLG remyelination [111]. Nevertheless, recent studies have demonstrated that OPCs can transform into a disease-specific cellular state characterized by the activation of genes previously thought to be unique to immune cells, thus providing a new perspective for the study of neuron-immune diseases such as MS [8].

### OLGs Adopt an Immunophenotype in MS

MS is an autoimmune demyelinating disease in which the immune inflammatory response is activated to attack myelin and OLs [112]. Myelin repair occurs in all stages of MS, and often coexists with demyelination. However, a number of studies have shown that OPCs are abundant in the injured areas but fail to differentiate due to local inflammation [109], which may account for the bad outcome of treatment.

Recent analysis by flow cytometry and RT-qPCR of OLGs in EAE have mice revealed that EAE-specific OLGs express genes involved in antigen processing and presentation including major histocompatibility complex class I and II (MHC-I and -II), and interferon response genes, including toll-like receptor 3 and members of the serpina gene family [113]. These disease-specific OLGs or the immunological OPC state are also present in MS brain tissue. For instance, in the lesion areas, OLGs express the immunoproteasome subunit PSMB8, which is not found in normal white matter [8].

To investigate how neuroinflammation influences OLGs, researchers carried out a fate-tracing study in a mouse model of inflammatory demyelination. The study revealed that OPC differentiation is inhibited by IFN-γ released by both effector T cells and ASTs [8]. The absolute number of OPCs is significantly reduced, while immunoproteasomes and MHC class I are induced in the remaining OPCs under induction by IFN-γ. Moreover, *in vitro* studies have shown that OPCs are capable of phagocytosis and MHC-II-expressing OPCs activate memory and effector CD4-positive T cells. In EAE, OLGs co-present MHC class I-restricted MBP with Tip-dendritic cells to cytotoxic CD8 T cells [113]. In a newly-published study, the RNA sequencing of cultured OPCs exposed to inflammatory cytokines (IFN-γ, IL-1β, and TNF) revealed that OPCs are able to generate chemokines to recruit and activate microglia, and the transformation of OPCs toward an immune phenotype seems to be mediated by TNFR2 signaling [114], undermining their ability to proliferate and differentiate. Moreover, OPCs can facilitate the migration of microglia by releasing chemokines such as CCL2 [115], or participate in immunoregulation through secreting CCL5, CX3CL1, and CXCL10 [116]. Therefore, OLs and OPCs are not passive targets, but instead, they can co-operate with microglia and ASTs to perpetuate the autoimmune response in MS [117].

## Concluding Remarks

As two types of non-excitable nerve cells, ASTs and OLGs have been found to be functionally involved in brain activity and the pathogenesis of brain diseases in recent studies. In particular, these two highly homologous glial cells function closely *via* a Cx-mediated network and exocrine mechanisms. Nevertheless, the detailed patterns are not fully understood, and their interaction is more complicated because of the newly-proposed concept of glial heterogeneity [118]. Hence, we pose the following questions. Are the glial networks between ASTs and OLGs consistent or diverse in subtypes and in distinct brain regions? Are those glial networks formed by different subtypes of ASTs or OLGs affected by diseases? While biochemical molecules mediate the interaction between the two glial cells, the quantities and corresponding receptors of the related molecules released by those subtypes of glial cells in various brain regions remain undefined. These complex patterns may underlie the mechanism by which glial cells precisely modulate neural transduction of CNS.

It is desirable to explore the spatio-temporal regulation patterns between ASTs and OLGs, as well as their modulatory effect on specific neural circuits in further studies. New technologies such as *in vivo* $Ca^{2+}$ imaging, cell fate tracing, viral loop tracking, chemogenetic and optogenetic tools, and 3D organic culture may uncover the new properties of glial interactions [119]. Disclosing the regulatory mechanisms underlying AST-OLG interaction may provide novel insights into therapeutic strategies for related brain diseases.

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INSIGHT

# Sense to Tune: Engaging Microglia with Dynamic Neuronal Activity

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Microglia, the primary resident immune cells in the central nervous system (CNS), continuously survey the microenvironment *via* their ramified and motile processes to maintain brain homeostasis [1]. As immune sentinels, microglia detect exogenous pathogens, elicit inflammation, promote tissue repair, and engage in the onset and progression of brain diseases [1]. Accumulating evidence has revealed that microglia play active roles in sculpting healthy brain functions by modulating neuronal wiring and synapse formation and elimination [1–3]. They constantly interact with different elements in the brain, particularly neurons, to keep neuronal activity under tight check and ensure functional homeostasis in the brain [2]. Here, we briefly summarize the latest progress regarding how microglia sense and, in turn, respond to neuronal signals to modulate neuronal activity. For additional information on this topic, please refer to other excellent reviews [1, 2].

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## Microglial Sense

Microglia are highly sensitive to changes in neuronal activity in various physiological and pathological states [4]. Real-time imaging studies in both zebrafish and mice have reported that extended microglial processes appear to preferentially approach active neurons, contacting neuronal somas and pre-synaptic terminals in the healthy brain [2]. Changes in neuronal activity are often accompanied by changes in microglial morphology and intracellular $Ca^{2+}$ levels [4, 5], indicating that microglia actively sense and respond to neuronal activity (Fig. 1).

## Sensing Neuronal Hyperactivity

Under pathological conditions, microglial processes rapidly migrate towards and converge at the injury sites and increase their contact with local neurons [6]. The classical excitatory neurotransmitter receptors and purinergic receptors, such as N-methyl-D-aspartate receptors (NMDARs) and purinergic receptor (P2Y12) expressed on microglia [7], confer on microglia the ability to quickly sense elevated glutamate and ATP released from hyperactive neurons [2]. Application of either ATP/ADP or glutamate induces rapid extension and convergence of microglial processes towards the glutamate or ATP/ADP-releasing source in brain slices, and these are efficiently blocked by NMDAR or P2RY12 antagonists [8]. However, only ATP/ADP, but not glutamate, induces microglial process extension and movement in culture [8, 9]. Moreover, antagonizing P2RY12 also inhibits glutamate-induced microglial responses in brain slices, suggesting that glutamate-induced microglial responses likely act through ATP-P2RY12-dependent signaling [8]. Further studies have shown that NMDAR activation induces the release of ATP through the Pannexin-1

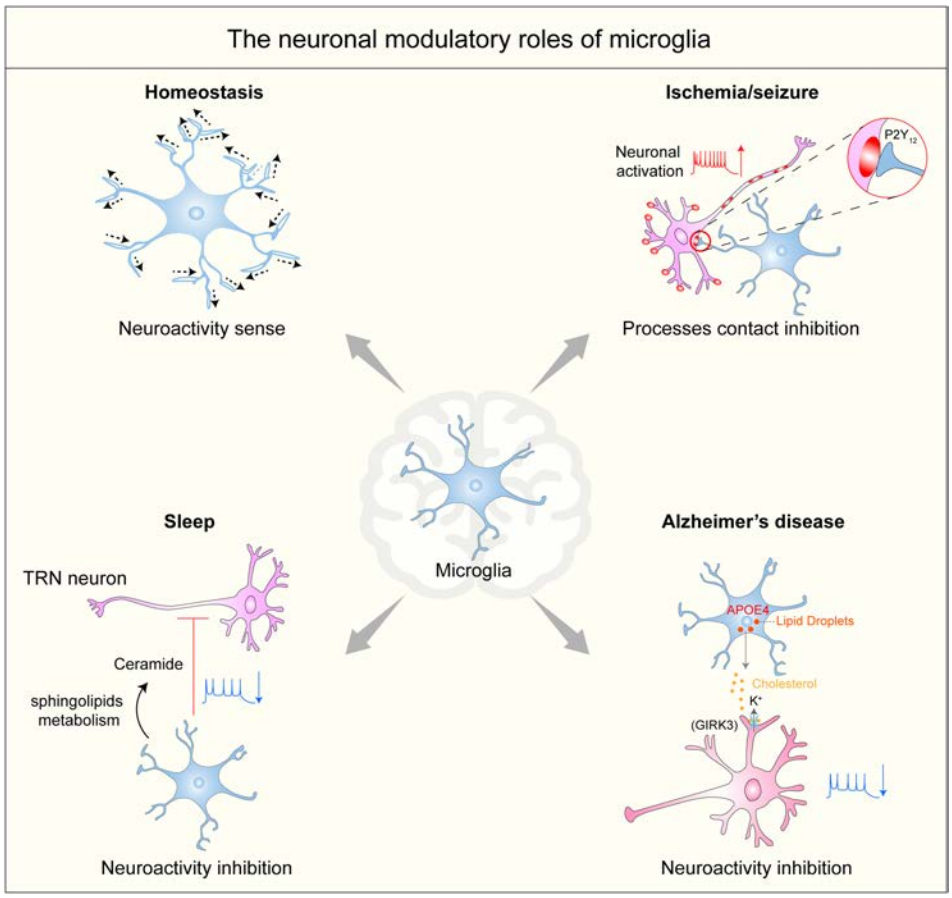


**Fig. 1** The neuronal modulatory roles of microglia. Emerging studies have revealed the neuronal regulatory roles of microglia in the brain under physiological and pathological states. The most important feature of microglia is their motile process dynamics, through which microglia rapidly sense and modulate neuronal activity. Microglial processes are in proximity to neuronal somas and synapses, where they either contact neurons or release active molecules to affect neuronal activity under different conditions including ischemia, sleep, and Alzheimer's disease.

hemichannel, and ATP acts on P2RY12 to trigger microglial responses [8]. The high affinity and sensitivity of P2RY12 to extracellular ATP thus provides an important mechanism by which microglia quickly sense neuronal hyperactivity. Moreover, microglia-neuronal somatic contacts provide a specialized nanoarchitecture to facilitate the microglial sensing of ATP released from local neurons [6]. The ATP- P2RY12 signaling thus provides an important mechanism by which microglia quickly sense neuronal hyperactivity [2].

Additional receptors at the cell surface of microglia, including complement receptors, fractalkine receptors, adenosine receptors, and other neurotransmitter receptors have also been implicated in sensing signals derived from hyperactive neurons under different states such as epilepsy, seizures, brain ischemia, and injury [1]. Together, they enable microglia to rapidly sense and respond to fluctuations in the environmental milieu and subsequently trigger a series of morphological and biochemical changes within microglia.

## Sensing Neuronal Hypoactivity

Early *in vivo* imaging studies failed to observe changes in microglial dynamics in the brain when inhibiting neuronal activity by the application of tetrodotoxin (TTX) [2], leading to the assumption that microglia may be insensitive to neuronal hypoactivity. However, these studies were carried out under anesthetic states, in which neuronal activity had already decreased to the minimal levels. For microglia to play a housekeeping role in the brain, they should be able to sense both neuronal hyperactivation and hypoactivation. By imaging in awake mice, Liu *et al.* and Stowell *et al.* recently reported that anesthesia-induced global neuronal hypoactivation robustly increases the dynamics of microglial processes and expands their surveillance territory [10, 11]. Moreover, whisker trimming and optogenetic or pharmacological manipulation-inducedlocal neuronal hypoactivation in the somatosensory or barrel cortex also enhanced the motility and surveillance of microglial processes [10]. These findings suggest that microglia sense and rapidly respond to neuronal hypoactivation.

Decreased neuronal activity is also accompanied by altered levels of neurotransmitters. Norepinephrine (NE), released by noradrenergic neurons in the locus coeruleus (LC) in awake animals, is significantly decreased during anesthesia as a result of the inhibition of noradrenergic LC neurons. Replenishing NE in the mice or optogenetically activating noradrenergic LC neurons prevents the increase in

microglial dynamics and surveillance induced by anesthesia or whisker-trimming [10]. Interestingly, microglia express high levels of $\beta_2$-adrenergic receptors (*Adrb2*), and NE application to brain slices induces the retraction of microglial processes *via* these receptors [12]. Blocking $\beta_2$-adrenergic receptors or optogenetically inhibiting noradrenergic neurons *in vivo* mimics the increase in anesthesia-induced surveillance, suggesting that reduced NE under anesthesia enhances microglial dynamics by releasing inhibition from NE-$\beta_2$ adrenergic signaling [10, 11].

## Microglia Fine-tune Neuronal Activity

The dynamics of microglial processes have been shown to be tightly associated with $Ca^{2+}$ activity [2], and neuronal activation- or suppression-induced alterations in microglial morphology and motility are also accompanied by rapidly elevated intracellular $Ca^{2+}$ levels in microglial processes [4, 8], indicating that microglia sense neuronal activity and respond with both morphological and biochemical changes.

## Microglia Modulate Sleep

Under physiological conditions, neurons in different brain areas go through different phases of activity. For example, sleep/wake cycles involve down-regulated and back-to-normal neuronal activity [13]. Hayashi *et al.* have reported longer and more complex microglial processes in the somatosensory cortex during the dark stage when mice are more active, along with upregulated P2RY12 transcripts [13]. In addition, microglial depletion by pharmacological or toxin-based approaches reduces stable wakefulness and increases non-rapid eye movement (NREM) sleep and neuronal activity during the dark stage [14, 15]. These effects are related to microglial CX3CR1 signaling and microglia-derived ceramide elevation in the anterior thalamic reticular nucleus (TRN) [14, 15], suggesting that microglia participate in the physiological modulation of the sleep/wake cycle.

Microglia also affect sleep regulation under disease conditions [16]. Pro-inflammatory cytokines released by activated microglia have been shown to increase NREM sleep. In addition, complement 1q released by microglia leads to the loss of GABAergic thalamic reticular neurons and disrupts sleep spindles after traumatic brain injury [16].

## Microglia tune down hyperactive neurons in epilepsy and ischemia

A number of disease conditions such as epilepsy and ischemia are also characterized by aberrant neuronal hyperactivity. Epilepsy is generally thought to result from synchronized hyperactivity of neurons, which release a large amount of ATP. Microglia not only sense ATP with P2RY12 but are capable of hydrolyzing ATP into adenosine, which in turn suppresses neuronal activity *via* the A1 receptor, thus creating an important negative-feedback mechanism to control neuronal activity [2]. To support this hypothesis, $P2RY12^{-/-}$ mice exhibit exacerbated seizure behaviors, with reduced microglial process extension upon kainic acid administration [8]. Therefore, microglia act as an important "brake" to prevent the over-activation of neurons.

## Perspective

Microglia continuously survey the local milieu and actively respond to environmental changes. Interestingly, whether neurons are hyperactive or hypoactive, microglia respond with apparently the same reactions: increased process dynamics/surveillance and elevated intracellular $Ca^{2+}$ levels. Although increased process dynamics enhances the ability of microglia to directly contact adjacent neuronal elements and facilitates neuronal modulation, it remains unclear exactly how microglia "calm down" hyperactive neurons or "tune-up" hypoactive ones. Do microglia release different soluble factors that selectively act on hyper- or hypo-activated neurons or directly transfer some chemicals to restore neuronal activity? While there is little evidence regarding how microglia regulate hypoactive neurons, recent studies have presented potential mechanistic links by which microglia negatively regulate neuronal activity. For example, other than hydrolyzing ATP into adenosine [2], microglia-derived platelet-derived growth factor b or ceramide are involved in suppressing neuronal activity in the paraventricular nucleus or TRN, respectively [14, 17]. In addition, accumulated extracellular cholesterol, as a consequence of impaired lipid uptake in induced pluripotent stem cell-derived microglia, due to the presence of the Alzheimer's disease-associated risk gene apolipoprotein E4, seems to also blunt neuronal activity *via* the potentiation of G-protein-gated inwardly rectifying $K^+$ currents [18]. Moreover, microglial Gi pathways also appear to be implicated in the negative regulation of neuronal networks [1]. It seems that microglia modulate neuronal activity in multiple ways and they may adopt different means of regulation under different conditions. The underlying intrinsic selection mechanism remains to be further investigated.

Drug-induced microglial depletion has been used to investigate microglial function and this has revealed different functional aspects of microglia [2]. However, this approach only reflects an extreme condition, which does not mimic either physiological or pathological states. Specific tools for temporal and spatial manipulation of microglia are

important to further uncover their roles *in vivo*. A newly-developed adeno-associated virus that is highly selective for microglia with low immune responses may provide aid [19]. Microglia-specific tamoxifen-inducible Cre-ERT2 mice [20], combined with the diphtheria-toxin receptor or microglial survival-dependent csf1r floxed mice, can be used for temporal or spatial microglia depletion [20]. In addition, the Cre-ERT2 animals can also be combined with Gq-coupled M3 muscarinic receptors, or red-activated channel rhodopsin floxed mice to generate chemogenetically- or optogenetically-responsive tools for microglia [21, 22]. Together, with the advances in imaging and manipulation tools, the mystery of microglia and its roles in the brain will be gradually unraveled.

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**Conflict of interest** The authors declare no competing interests.

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CORRECTION

# Correction: Novel Microglia-based Therapeutic Approaches to Neurodegenerative Disorders

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The author contribution was missing from this article and should have read 'Lijuan Zhang, Yafei Wang, Taohui Liu contributed equally to this review.'
The original article has been corrected.



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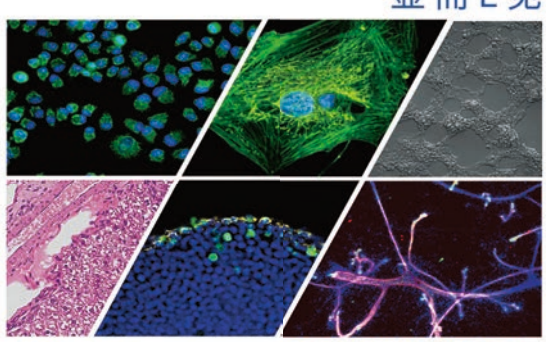

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