# ROLE OF GLIAL CELLS OF THE CENTRAL AND PERIPHERAL NERVOUS SYSTEM IN THE PATHOGENESIS OF NEURODEGENERATIVE DISORDERS

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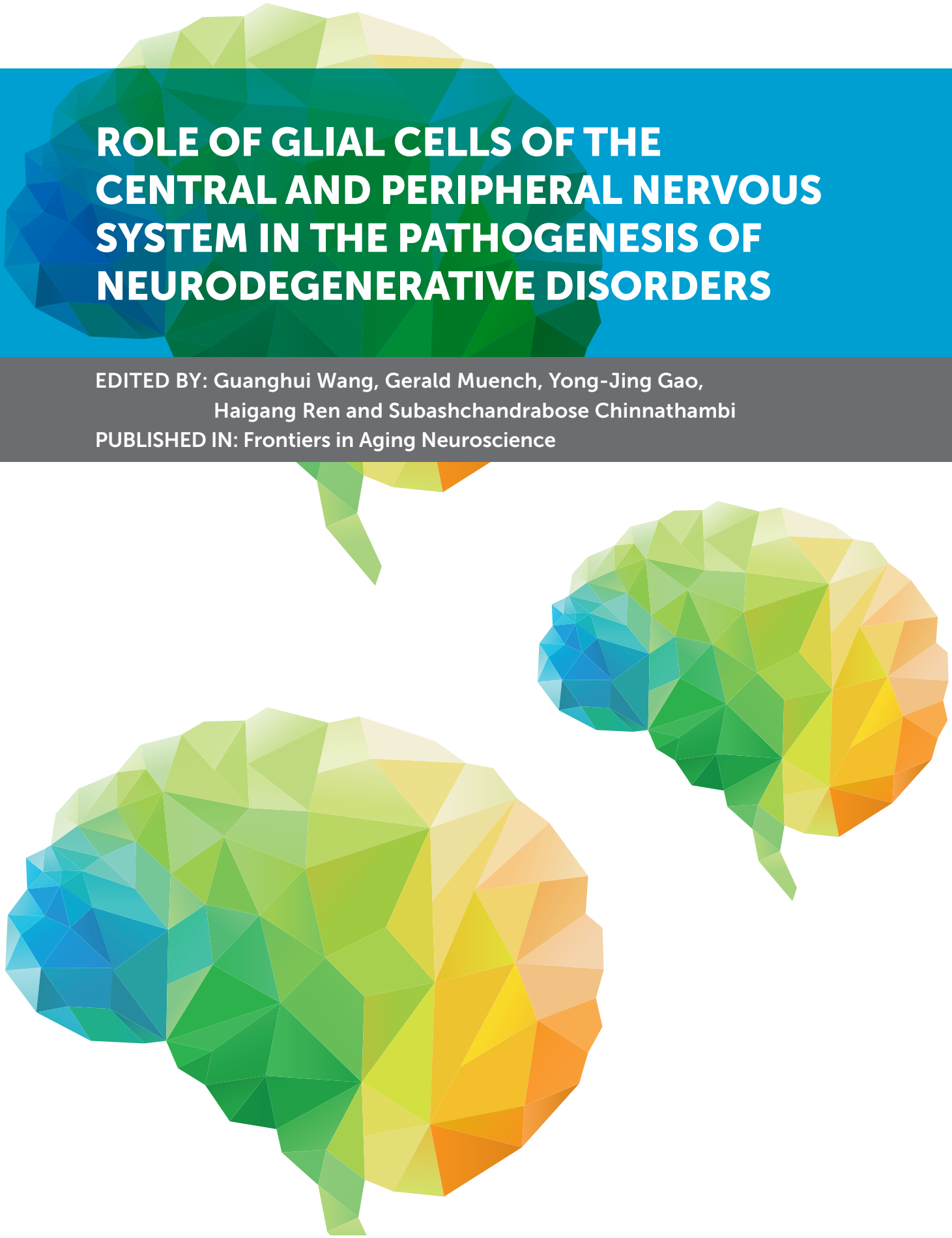

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# ROLE OF GLIAL CELLS OF THE CENTRAL AND PERIPHERAL NERVOUS SYSTEM IN THE PATHOGENESIS OF NEURODEGENERATIVE DISORDERS

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EDITORIAL
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# Editorial: Role of Glial Cells of the Central and Peripheral Nervous System in the Pathogenesis of Neurodegenerative Disorders

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**Keywords: microglia, astrocyte, neuroinflammation, neurodegenerative diseases, central nervous system**

## Editorial on the Research Topic

## Role of Glial Cells of the Central and Peripheral Nervous System in the Pathogenesis of Neurodegenerative Disorders

Glial cells are critical to maintain brain homeostasis by multiple ways, including neuronal support and immunological defense in the development of the central nervous system (CNS) and in the peripheral nervous system (PNS). However, glial cells show progressive dysfunction and damage neurons in diseases, especially in neurodegenerative diseases (NDs) (Giovannoni and Quintana, 2020). Mutations of genes *CD33*, triggering receptor of myeloid cells 2 (*TREM2*), apolipoprotein E (*APOE)*, *GBA1* and *GRN* that are mainly expressed in glial cells have been identified as various AD and PD risk factors in genome-wide association studies (GWAS), sparking insights into shed light on the roles of glial cells in pathogenesis of NDs (Bartels et al., 2020; Lewcock et al., 2020). Moreover, single-cell sequencing analyses provide clearer clues for the understanding of the temporal and spatial heterogeneity of glial cells during the progression of NDs (Colonna and Brioschi, 2020). The manuscripts in this Research Topic focuses on the roles of glial cells in the pathogenesis of NDs in the CNS and PNS. We highlight three specific themes in this topic: (1) the contributions of glia-associated neuroinflammation to the diseases; (2) the roles of the interactions between glial cells and neurons in the diseases; (3) the glia-based therapeutics through the modification of glial activation for the disease treatments.

In NDs, glia-mediated neurodegeneration involves multiple pathways, including glial activation, neuronal damage by different signaling, and immune cell response. Su and Zhou reviewed the recent studies about α-synuclein (α-syn)-induced neuroinflammation in the pathogenesis of PD. They described the signalings of microglia upon α-syn stimulation and the contributions of microglia on the transmission of α-syn pathology. Moreover, they discussed the effects of α-syn on T cells and detailed the subtypes of T cells, either inflammatory or anti-inflammatory in response to α-syn, which indicate the involvements of the autoimmune and adaptive immune responses in PD. It is well known that pattern recognition receptors (PRRs), including toll-like receptors (TLRs), are important for initiating the activation of microglia in response to extracellular stimuli (Colonna and Butovsky, 2017). Gu et al. comprehensively reviewed the roles of G protein-coupled receptors (GPCRs) in microglial activation and their potentials as the therapeutic targets in PD. They discussed the mechanisms of different types of GPCRs in microglial activation and the correlations to the progression of PD. Moreover, they also summarized the mechanisms of the

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PRRs such as TLRs and NOD-like receptors mediated microglia-associated neuroinflammation in PD. Mitochondria are important organelles that function in the degeneration in neurons and the activation in glia. Rahman and Suk discussed the alteration of mitochondrial dynamics in astrocyte activation, which links the mitochondria, astrocytes and neurodegeneration. Using a spontaneous aging mouse strain and an AD mouse model, Molina-Martinez et al. found that aging and AD promote inflammatory gene expressions in hippocampus, suggesting an increased inflammatory response in aging and AD. By analyses of the spatiotemporal specific co-expression networks in AD, Guo et al. found that more microglial and astrocyte genes are enriched than neurons, further suggesting an involvement of glia in AD.

Accumulating evidence suggests that the communications between glial cells and neurons play important role in the regulation of signal transductions and immune responses in the CNS and PNS. Lana et al. reviewed the roles of interactions among microglia, astrocytes and neurons in the hippocampus. They described the changes of the morphology and functions of glial cells during aging and acute inflammation and discussed how activated microglia and astrocytes interact each other to maintain brain homeostasis during neuronal apoptosis in hippocampus. Under pathological conditions, the release of pro-inflammatory factors from microglia and astrocytes influences glial phagocytosis and damages neurons, which aggravates neuroinflammation. In addition to the production of inflammatory factors by glial cells, the communications between glia and neurons can be mediated by the extracellular vesicles (EVs). Li et al. comprehensively reviewed the different effects of glia-derived EVs on the pathogenesis of NDs. They discussed the beneficial and/or the detrimental roles of EVs secreted by glial cells in NDs. They described the roles of glial EVs in the functional regulation through the glia-glia or glia-neuron transmission of key mediators including miRNA, molecular chaperones, signaling and inflammatory components under the physiological and pathological conditions. They also discussed the potentials of EVs in biomarker development and in therapeutic application in diseases.

To date, the studies on the mechanisms of neurodegeneration provide great insights on the pathogenesis of NDs, therapeutic strategies to slow down the progression of NDs have not yet been succeeded. Based on the importance of glial cells in the pathogenesis of NDs, it is promising to develop therapeutic strategies for modulating the functions of glial cells. Brown and St George-Hyslop reviewed the effects of soluble TREM2 (sTREM2) on the functions of microglia and the inhibition of Aβ aggregation. sTREM2 is secreted by microglia and can activate microglia. It also binds to Aβ to repress Aβ aggregation, which protects against the amyloid plaques in AD animal models. Wang Y. et al. discussed current regenerative strategies with reprogramming astrocytes to functional neurons to replace the loss of neurons in NDs. They summarized the key regulators in regenerative strategies to achieve astrocyte-to-neuron reprogramming in NDs and discussed the advantages and difficulties in regenerative strategy therapies *in vivo*. Interestingly, Kim et al. identified that the sulfonylurea drug gliquidone, a FDA approved drug for the treatment of type 2 diabetes, has strongly inhibitory effects on LPS-induced microglial activation *in vivo* and *in vitro*. Gliquidone blocks LPS-induced inflammasome activation in microglia, suggesting that it holds promise in the treatment of inflammation in NDs.

In summary, this Research Topic summarizes the diverse roles of glia in the pathogenesis of NDs and discusses the molecular mechanisms of glia-associated NDs in response to the genetic and environmental factors.

## AUTHOR CONTRIBUTIONS

RW drafted the manuscript. HR and YG provided suggestions. GW revised and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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# Satellite Glial Cells of the Dorsal Root Ganglion: A New "Guest/Physiopathological Target" in ALS

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**Introduction:** Amyotrophic lateral sclerosis (ALS) might not only be circumscribed to the motor system but also involves other neuronal systems including sensory abnormalities. In line with this notion, we aimed to assess the pathophysiology of sensory disturbances in the SOD1$^{G93A}$ mouse model of ALS, focusing on the satellite glial cells (SGCs) at the dorsal root ganglion (DRG) as a new potential target of the disease.

**Material and Methods:** The presence of sensory disturbances was evaluated using von Frey, hot plate, and hot water tail immersion tests at 75 days old, which represented the motor-pre-symptomatic stage. Cell biology analysis was performed at 75 and 95 days old and included conventional histology, immunofluorescence, and electron microscopy of sensory neuron-SGC unit dissociates as a well as western blotting from DRG lysates.

**Results:** At 75 days old, von Frey and hot plate tests demonstrated clear thermoalgesic disturbances in ALS transgenic mice. Histological studies of the SN-SGC units revealed abnormal SOD1 accumulation, which was associated with nitro-oxidative stress and biogenesis of lipid droplets in SGCs. Interestingly, these alterations led to a progressive lysosomal storage disorder and occasionally vacuolar degeneration in SGCs.

**Conclusions:** SGCs emerge as a primary pathophysiological target in the SOD1 transgenic murine model of ALS, clearly reinforcing the pathogenic role of glial cells in motor neuron disease. Presymptomatic alterations of SGCs, might not only be responsible of sensory disturbances in ALS, but due to spinal cord sensory-motor circuits could also contribute to anterior horn motor disturbances.

**Keywords: ALS (Amyotrophic lateral sclerosis), satellite glial cells (SGCs), sensory, SOD1 mouse G93A, glia**

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## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common neurodegenerative disease affecting motor neurons (MNs) with an annual incidence that ranges from 1 to 3 cases per 100,000 individuals (Logroscino et al., 2010; Riancho et al., 2016). The pathogenesis of ALS has not yet been completely elucidated. A small percentage (20%) of cases have a familial origin (fALS) that is related to mutations in specific causative genes such as SOD1, CR9ORF72, TARDBP, and

FUS (for a review, see Zufiría et al., 2016). In contrast, the vast majority of cases are thought to be sporadic (sALS) and caused by interactions between genes and environmental conditions, leading to disease onset in genetically predisposed individuals (Riancho et al., 2018). Clinically, ALS is characterized by the progressive degeneration of both upper and lower MNs typically results in progressive muscle wasting and usually lead to death within 3 years after symptom onset (Amato and Russell, 2008). Since its description, ALS has been classically considered as a "motor system-circumscribed disease"; however, other clinical manifestations, including autonomic disorders, cognitive impairment and sensory disturbances, have been reported in patients with ALS. Regarding the latter, several experimental and clinical studies support some degree of sensory system impairment in individuals with ALS (Chiò et al., 2017; Riancho et al., 2020b).

Concerning preclinical studies, most evidence has been generated using the murine SOD1$^{G93A}$ model. First, Guo et al. (2009) used this model to study the sensory system at several levels, including the dorsal roots, dorsal root ganglia (DRG), and posterior column tracts. Interestingly, the authors observed a loss of axons, cellular alterations, and demyelination. Shortly thereafter, Sábado et al. (2014) reported demyelination and a loss of axons in the posterior roots as well as loss of neurons and SOD1 accumulation in the DRG of this murine model. Consistent with this finding, researchers have postulated that sensory disorders in the SOD1 mutant murine model might be associated with the accumulation of a neurotoxic splice variant of peripherin (Sassone et al., 2016). Within this model, some investigators have noted that proprioceptive sensory neurons are particularly susceptible to pathology (Sábado et al., 2014; Seki et al., 2019). In addition, according to Vaughan et al. (2018) cultured sensory neurons harboring mutations in either TDP43 and SOD1 exhibit lower growth rates, reduced neurite branch generation, and an increased susceptibility to cellular stress, thus suggesting important roles for this neuronal population in ALS-related pathogenesis.

The DRG contains the cell bodies of neurons that transmit the sensory information (proprioceptive, light touch, vibration, thermoceptive, and nociceptive information) from the periphery to the central nervous system (CNS) through the dorsal and anterolateral tracts of the spinal cord (Haberberger et al., 2019). In addition to controlling sensory information, proprioceptive sensory neurons are key modulators of motor behavior that integrate the sensory and motor systems into the CNS. Interestingly, sensory neurons have been recently suggested as ALS targets in a *Drosophila* model of MN disease (Held et al., 2019).

Classically, rodent DRG neurons are classified into three main morphological based on their size and the distribution of their organelles: A (large), B (medium size), and C (small dark) (Pena et al., 2001). Type A neurons have thick myelinated fibers and are mainly mechanoreceptive (proprioceptive). Type B neurons exhibit thin myelinated fibers and are mechanoreceptive and nociceptive. Finally, non-myelinated, slow conducting, type C neurons are mainly involved in thermo- and nociception (Wotherspoon and Priestley, 1999; Hunt and Koltzenburg, 2005; Berta et al., 2017). Each sensory neuron (SN) is wrapped by the cell bodies and laminar processes of several satellite glial cells (SGCs), forming a morphological, and functional unit (SN-SGC unit) (Pannese, 1981; Hanani, 2005). SGCs play an important role in regulating sensory neuron function, particularly in controlling the neuronal microenvironment (Hanani, 2005; Haberberger et al., 2019).

Although the selective cell death of MNs is a key feature of ALS, other tissues, and organs may be responsible for the clinical manifestations of the disease (for a review, see Zufiría et al., 2016). In fact, as shown in our recent study, that dermal fibroblasts from patients with ALS recapitulate alterations typical of ALS motor neurons and exhibit an abnormal DNA-damage response (Riancho et al., 2020a). Therefore, the DRG in the PNS might contribute not only to the subtle sensory manifestations of patients with ALS but also to MN degeneration by impairing sensory-motor spinal networks.

The aim of this study is to determine the cellular basis of the sensory system impairment in SN-SGC units in SOD1$^{G93A}$ mice. To date, the SOD1 transgenic mouse has been the most extensively employed animal model for preclinical investigations of ALS. High-copy SOD1$^{G93A}$ transgenic mice recapitulate an important proportion of the pathophysiological features of human ALS, including progressive MN degeneration and neuromuscular function loss as well as a reduced lifespan (Gurney et al., 1994). Although this mouse model is based on a familial form of the disease, several authors have highlighted its translational value in studies of sALS (Bosco et al., 2010). The potential contribution of SGCs to the sensory component of ALS at both, late presymptomatic (75 days old) (Riancho et al., 2015) and symptomatic (95 days old) stages should receive special attention. Based on our results thermonociceptive and fine touch dysfunction accompanied motor alterations in the SOD1$^{G93A}$ mouse model of ALS. Sensory disturbances correlated with high expression of mutant SOD1 in SGCs, which appeared to increase lipid peroxidation and an aberrant storage of lysosome-related structures, preferentially in SGCs belonging to type B and C units.

# METHODS

## Animals

The transgenic mice used were B6SJL-Tg (SOD1$^{G93A}$ 1Gur/J (SOD1$^{G93A}$) obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained at the Animal Service of the University of Cantabria. The colony was maintained by mating heterozygous transgenic males with B6SJLF1/J hybrid females. Real time quantitative PCR of DNA obtained from tail tissue was used for genotyping, with specific primers detecting human SOD1 and the housekeeping mouse gene ApoB. Primer sequences were: SOD1: GGG AAG CTG TTG TCC CAA G and CAA GGG GAG GTA AAA GAG AGC; ApoB: TCA CCA GTC ATT TCT GCC TTT G and GGG AAG CTG TTG TCC CAA G. Transgenic mice and control littermates were housed under controlled temperature and humidity, with a 12-h light/dark cycle and free access to water and food. The experimental protocol was approved by the Ethics Committee of the University of Cantabria following the Spanish legislation. All

animal experiments were carried out in accordance with the EU Directive 2010/63/EU.

Male animals were distributed into four groups: two wild-type mice (75 and 95 days old) and two SOD1$^{G93A}$ mice (75 and 95 days old). In SOD1$^{G93A}$ mice these age stages correspond to the presymptomatic and symptomatic stages of the disease in this murine model. Globally, sensory tests included 23 transgenic SOD1 and 23 wildtype mice, respectively. For light microscopy and immunofluorescence 16 control and 16 SOD1 animals were used. Finally, biochemical analysis was performed in 3 transgenic and 3 wild type mice, respectively.

## Rotarod and Sensory Tests

For investigating the presence of subjacent sensory disorders and in order to not be biased by motor symptomatology, tests were performed on day 75 of life. Globally 23 wild-type and 23 transgenic mice were used. Before assessing sensory disturbances mice weight and rotarod performance and were evaluated as previously described (Riancho et al., 2015). Sensory tests included von Frey hair test, hot water tail immersion test, and hot plate test. These tests assess nociception induced by both, mechanical and thermal stimuli.

For von Frey test, mice were placed into the corresponding testing area and left there for 20 min. Once habituated, mechanical stimuli with von Frey filaments (Semmes Weinstein von Frey Aesthesiometer for Touch Assessment, Stoelting Co, Illinois EEUU) were applied on the forelimb of each animal. Limb shaking or limb licking, were considered as positive responses. Nociceptive threshold was then determined as the force evoking a 50 percent of positive responses.

The hot water tail immersion test evaluated the latency of the tail flick reaction in mice after immersing their tails in a constant temperature bath. Three centimeters of mice tail were submerged in hot water at 45, 47, and 49°C, respectively. A cut off point of 60 s was stablished in order to avoid tissue damage. For hot plate test, mice were place into 2 metallic cylinders which had been previously heated up to 50 and 52°C. Then the latency until the animal exhibited a positive response, considered as limb shaking, limb licking, or jumping, was measured. A cut off point of 120 s was considered to evade mice injuries. All sensory tests were done in quintuplicate.

## Light Microscopy and Immunofluorescence

To study histological changes, 8 mice of each group (SOD1 and control mice) were euthanized on days 75 and 95, respectively, and their dorsal root ganglia (DRG) processed for light microscopy and immunofluorescence. After deep anesthesia with pentobarbital (50 mg/kg) mice were perfused with 3.7% paraformaldehyde in PBS (pH 7.4) for 15 min. DRG were dissected and post-fixed for 2 h. Small tissue fragments were processed for mechanical dissociation of SN-SGC units following the procedure of Pena et al. (2001). Briefly, DRG fragments were transferred to a drop of PBS on a siliconized slide. Then, a coverslip was applied on top of the slide and the tissue was squashed by percussion with a histologic needle to dissociate neuronal cell bodies. The preparation was then frozen in dry ice, and the coverslip removed using a razor blade. Using this procedure most SN-SGC units remained adhered to the slide. Cell samples were processed in 96% ethanol at 4°C for 10 min and rehydrated progressively in 70% ethanol and PBS. Some preparations were stained with propidium iodide (PI), a fluorescent staining of nucleic acids. For immunofluorescence, squash preparations were sequentially treated with 0.5% Triton X-100 in PBS, 0.1 M glycine in PBS containing 1% bovine serum albumin and incubated with the primary antibody overnight at 4°C. Then, the sections were incubated with the specific secondary antibody conjugated with FITC or Cy3 and mounted with Vectashield (Vector USA). Confocal images were obtained with a LSM510 (Zeiss, Germany) laser confocal microscope using the 63x oil (1.4 NA) objective. In order to avoid overlapping signals, images were obtained by sequential excitation at 488 and 543 nm, to detect FITC and Cy3, respectively. Images were processed using Photoshop software.

To determine the relative nuclear and cytoplasmic levels of SOD1 in both sensory neurons and SGCs in wild-type and SOD1$^{G93A}$ mice confocal images were recorded by using a 63x oil (1.4 NA) objective and the same confocal settings at resolution of 1,024 × 1,024 pixels. Images were background corrected by reference regions outside the tissue and fluorescence intensities were estimated by using the ImageJ software (NIH, Bethesda, Maryland, USA; http://rsb.info.nih.gov/ij/). We use three animals per experimental group and at least 35 neurons per animal were sampled.

The following primary antibodies were used. Goat polyclonal antibody anti-cathepsin D (dilution 1:100; Santa Cruz Biotechnology, USA) and rabbit polyclonal antibodies anti-SOD1 (dilution 1:100; Enzo Life Sciences, Switzerland) and anti-ubiquitin-protein conjugates (dilution 1:50; Biomol International).

## *In situ* Determination of Lipid Peroxidation

For the determination of lipid peroxides squash preparations of DRG fixed with 3.7% paraformaldehyde in buffer phosphate 0.12 M were air dried, washed in PBS and incubated with the probe C11-BODIPY$^{581/591}$ or BODIPY$^{493/503}$ (Molecular Probes, USA) at the concentration of 1 μg/mL for 30 min at 37°C (Liu et al., 2015). Then, the samples were washed in PBS and mounted with the antifading agent Vectashield-DAPI (Vector Laboratories, USA). Images were acquired immediately with a Zeiss LSM 510 microscope.

## Electron Microscopy

For electron microscopy, wild-type and SOD1$^{G93A}$ mice were deeply anesthetized and perfused with 1% glutaraldehyde and 1% paraformaldehyde in 0.12 M phosphate buffer. Tissue samples of the DRG were postfixed with 2% osmium tetroxide dehydrated in increased concentrations of ethanol and embedded in Araldite (Durcupan, Fluka, Switzerland). Semithin sections (1 mμ thick) were stained with toluidine blue for the light microscopy examination of the DRG. Ultrathin sections mounted in copper grids were stained with uranyl acetate and lead citrate and examined with a Jeol 2011 electron microscope operated at 80 kV.

## Western Blotting

For Western blot (WB) analysis, DRG were homogenized in a lysis buffer (0.1 mol/L NaCl, 0.01 mol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, and 1 μg 7 Ml aprotinin) and the homogenates were centrifuged. Overall, 3 SOD1 and 3 control mice were used, respectively. The protein in the supernatants were subjected to SDS-PAGE electrophoresis and afterwards transferred to polyvinylidene difluoride membranes, stained with antibodies and visualized with the Odyssey system (LI-COR Biotechnology). Alpha-Tubulin was used as protein loading control. The antibodies used were rabbit polyclonal anti-SOD1 (dilution 1:1,000; Enzo, Life Sciences, Switzerland), goat polyclonal antibody anti-cathepsin D (dilution 1:2,000; Santa Cruz Biotechnology, USA) and mouse monoclonal anti-Tubulin (dilution 1:000; Abcam, Cambridge, USA). ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA) was used to quantify the density and size of the blots.

## Statistical Analysis

The significance of the differences in weight differences was tested by unpaired Students'*t*-test, while non-parametric Mann-Whitney *U*-test was used to compare sensory test scores of wild-type and SOD1$^{G93A}$ mice.

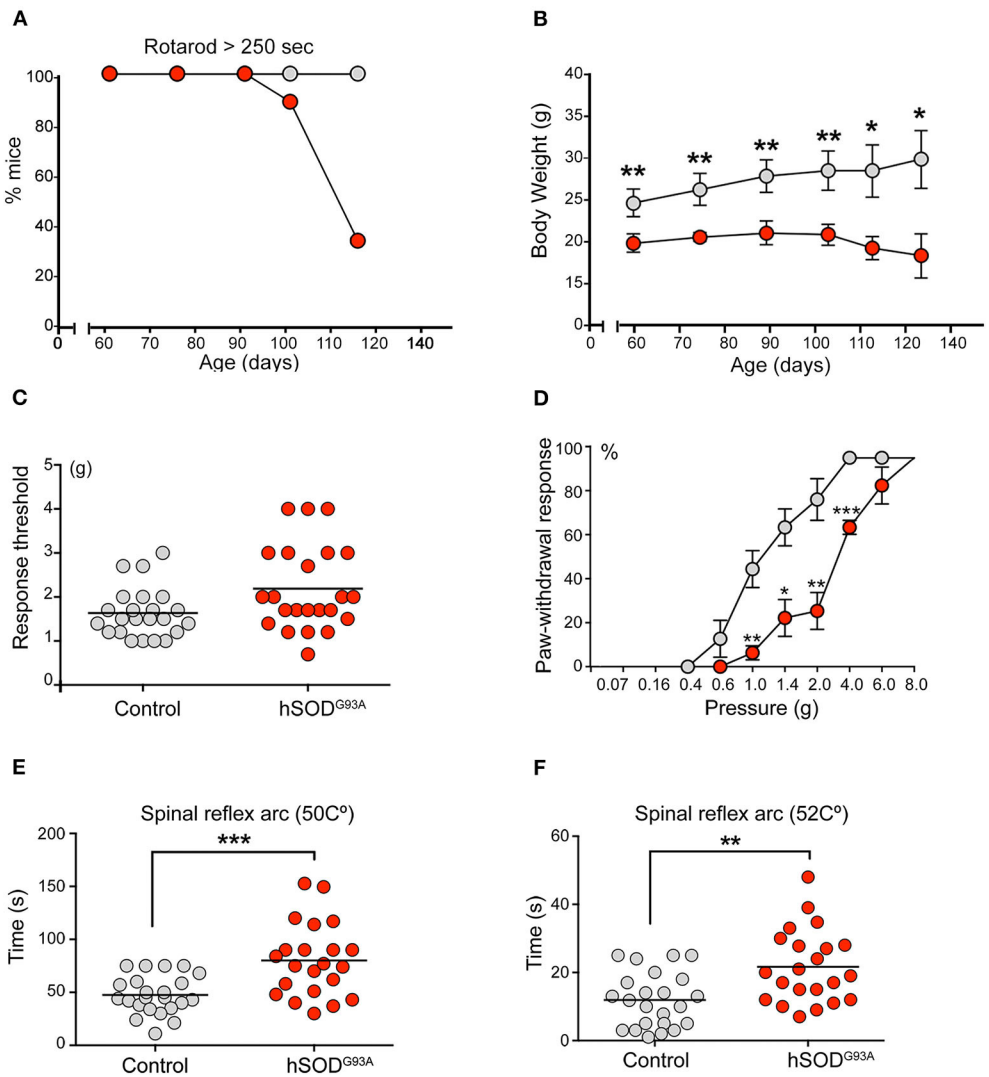


**FIGURE 1 |** Sensitive pathology in SOD1$^{G93A}$ mice. **(A)** Rotarod test showed a progressive neuromuscular deterioration in SOD1 transgenic mice from day 95 onwards. **(B)** ALS mice exhibited a significant lower weight during the whole experimental period (Students'*t*-test, $^{*}p < 0.05$; $^{**}p < 0.005$). **(C)** Globally, von Frey test evidenced a lower non-significant tactile threshold in the control group in comparison to SOD1 transgenic mice (1.65 vs. 2.14). Of note, 3 ALS transgenic mice showed a markedly higher tactile threshold, thus suggesting some degree of tactile disturbance. **(D)** When evaluating the proportion of positive responses depending on the filament force, a clear significant delayed response was evidenced in SOD1 transgenic mice from 1 g upwards (Students'*t*-test, $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$). **(E,F)** Hot plate test evidenced a significant delayed response in ALS transgenic mice when compared to control mice at both 50 Celsius degrees **(E)** 49 vs. 74 s; Students'*t*-test, $^{*}p < 0.05$) and 52 Celsius degrees **(F)** (10 vs. 21 s; Students'*t*-test, $^{**}p < 0.005$).

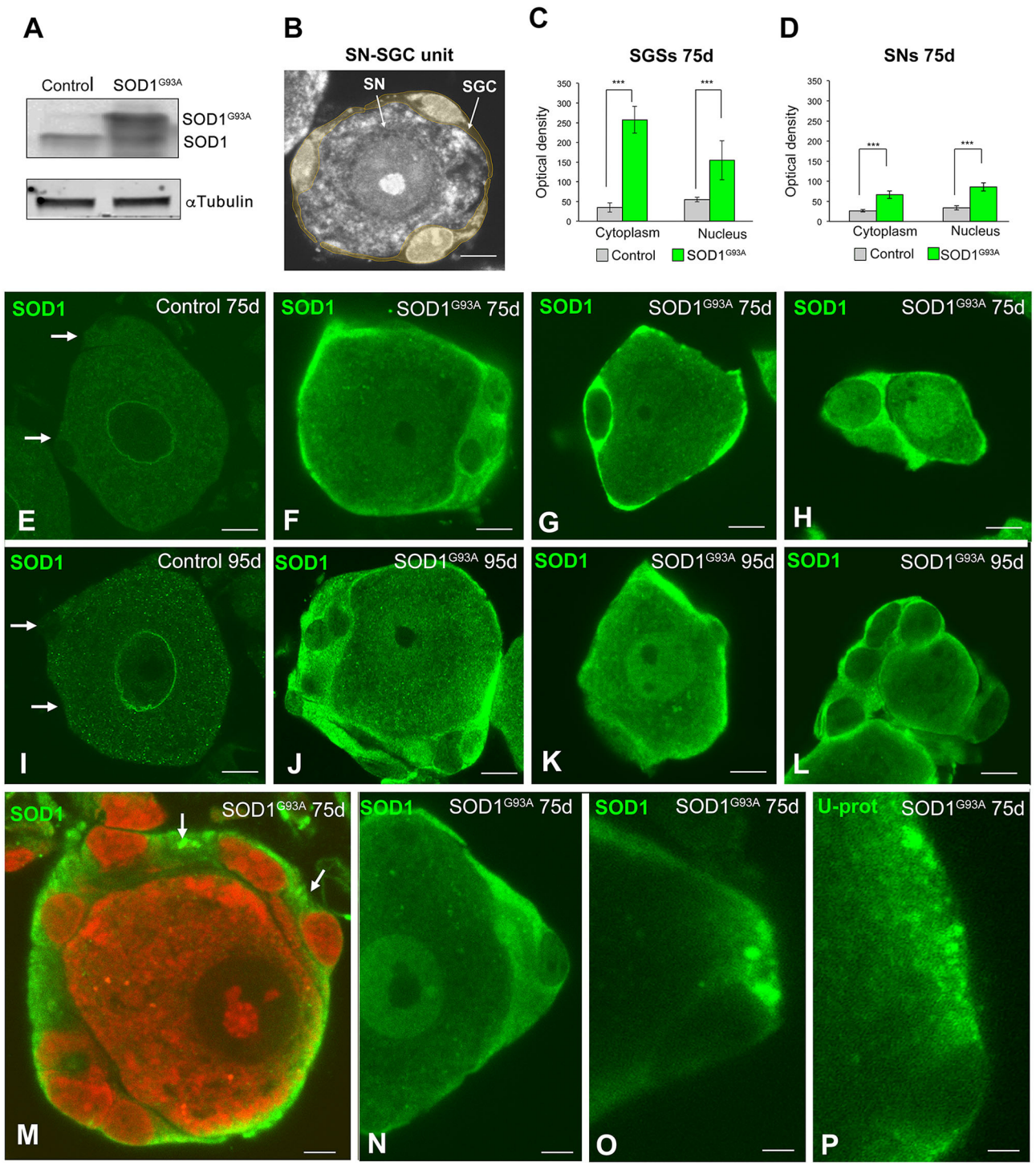


**FIGURE 2 |** Expression of SOD1 in SN-SGC units from control and SOD1$^{G93}$ mice. **(A)** Western blotting of SOD1 expression in DRG lysates from control and SOD1$^{G93A}$ mice. Note the high levels of SOD1$^{G93A}$ expression found in SOD1 transgenic mice. α-tubulin was used as load control. **(B)** Representative example of a typical SN-SGC unit in a control DRG. GSCs appear colored in pale yellow. **(C,D)** Densitometric analysis of SOD1 expression in the nucleus and cytoplasm of SGCs and sensory neurons at 75 and 95 days of age. **(E–L)** Immunodetection of SOD1 in control and SOD1$^{G93}$ SN-SGC functional units at 75 **(E–H)** and 95 **(I–L)** days of age. **(E,I)** Images of control SN-SGC units showing low levels of SOD1 expression in the nucleus and cytoplasm from both sensory neuron and SGCs (arrows). **(F–H,G–L)** Representative examples of types **(A–C)** SN-SGC units from SOD1$^{G93A}$ at 75 and 95 days of age. Note the high levels of cytoplasmic SOD1 in SGCs and the moderate expression of SOD1 in sensory neurons. **(M)** A SN-SGC unit of a SOD1$^{G93}$ mouse immunolabeled for SOD1 and counterstained with PI illustrating the

*(Continued)*

**FIGURE 2 |** high concentration of SOD1 in the glial cytoplasm and its aggregation in cytoplasmic inclusions (arrows). Note the low expression of SOD1 in the neuronal cytoplasm and the well-preserved Nissl substance and prominent nucleolus counterstained with PI. **(N,O)** Detail of SGC cytoplasm from $SOD^{G93A}$ mice showing areas with different SOD1 concentration and the presence of inclusions highly enriched in SOD1. **(P)** Immunodetection of ubiquitin-protein (Ub-prot) conjugates forming cytoplasmic aggregates in a SGC from a $SOD1^{G93}$ mouse. The cell nucleus of the SGC appears unstained. Scale bar: **(B)** and **(E–L)**: 10 μm. **(M–P)**: 5 μm. Student *t*-test in **(C,D)** $p < 0.001$, $^{***}p < 0.001$.

# RESULTS

## Presymptomatic SOD1 Transgenic Mice Exhibit Sensory Disturbances at 75 Days of Age

The presence of sensory alterations was evaluated in 75-day-old mice to avoid any motor-induced bias. Before the assessment, all mice performed the rotarod test to exclude any subjacent neuromuscular disorder. At that age, no differences in rotarod performance were observed between wild-type (hereafter referred to as control) and transgenic mice. As expected, a progressive worsening in rotarod performance was observed beginning at day 90 of life (**Figure 1A**). Consistent with our previous studies (Riancho et al., 2015), ALS transgenic mice exhibited a significantly lower weight than controls (**Figure 1B**).

The mechanical nociceptive threshold von Frey hair test evaluated the sensation of pain. This test is based on the principle that a compressed elastic column will buckle elastically at a specific constant force. The filaments (hairs) are used to provide a range of forces to the mouse forelimb and identify the force to which the animal reacts because it causes a painful sensation. Globally, the results of the von Frey test revealed a lower but non-significant tactile threshold (force at which the animal moved off its limb at least 50% of the time) in the control group than in ALS transgenic mice (mean force, $1.6 \pm 0.21$ vs. $2.1 \pm 0.09$, $p = 0.0634$, **Figure 1C**). Notably, 3 ALS transgenic mice exhibited markedly higher tactile thresholds, thus suggesting some degree of tactile disturbance. Although no significant differences were noted in the global threshold analysis, clear and significant delays were observed when forces of 1 g and greater were applied to the $SOD1^{G93A}$ group, based on the percentage of positive responses to the filament force (**Figure 1D**). These results reveal a delayed tactile response in transgenic mice.

The hot water tail immersion test and the hot plate test were performed to assess temperature-related nociception. These tests are complementary. The former mainly evaluates the spinal reflex arc (König et al., 1996), whereas the latter requires supraspinal processing (Le Bars et al., 2001). Regarding the hot water tail immersion test, no significant differences in tail withdraw latencies were not observed between transgenic and control mice at 45, 47, and 49°C. The hot plate test, which evaluates the latency of a mouse to jump off a warming plate, was performed with plates at 50 and 52°C (**Figures 1E,F**). At both temperatures, SOD1 transgenic mice exhibited a significantly delayed latency (mean latency, $47.8 \pm 2.53$ vs. $65.2 \pm 2.46$ s at 50°C, $p = 0.0217$; $12.04 \pm 0.86$ vs. $20.6 \pm 2.34$ s at 52°C, $p = 0.0024$) (**Figures 1E,F**).

## SN-SGC Units Express Mutant SOD1 at High Levels in $SOD1^{G93A}$ Mice

The levels of both endogenous mouse SOD1 and mutant $SOD1^{G93A}$ were estimated by performing Western blotting of DRG lysates using an antibody that recognizes murine and human SOD1. As expected, non-significant differences in murine SOD1 levels were detected in the DRG between control and $SOD1^{G93A}$ mice. In contrast, high levels of $SOD1^{G93A}$ were detected in the $SOD1^{G93A}$ DRG (**Figure 2A**). This finding confirms the overexpression of the mutant human SOD1 in the DRG of transgenic $SOD1^{G93A}$ mice and suggests that the high levels of the mutant human dismutase do not exert a negative dominant effect on wild-type murine SOD1 expression. Thus, the gain of function of the mutant $SOD1^{G93A}$ rather than the loss of function of the murine SOD1, might be involved in the sensory dysfunction observed in $SOD1^{G93A}$ mice.

Next, we investigated the subcellular distribution of SOD1 in SN-SGC units (**Figure 2B**) from control and $SOD1^{G93A}$ mice by performing immunofluorescence staining using a polyclonal rabbit anti-SOD1 antibody. At 75 days of age, a very weak cytoplasmic signal was detected in both neurons and SGCs from control mice (**Figure 2E**). Conversely, in SN-SGC units from the $SOD1^{G93A}$ mice, high SOD1 expression was observed in SGCs, and to a lesser extent, in sensory neurons (**Figures 2F–H**). Moreover, SOD1 expression was markedly increased in the cytoplasm of SGCs compared with the nucleus (**Figures 2F–H**). This observation was confirmed by the densitometry analysis of the fluorescent intensity of SOD1 in the nucleus and cytoplasm of SGCs, that revealed significant differences between these two compartments (**Figure 2C**). In sensory neurons from $SOD1^{G93A}$ mice, SOD1 expression was generally increased in the nucleus compared with the cytoplasm (**Figures 2D,H**). At 95 days of age, a similar pattern of SOD1 expression was observed in SN-SGC units from both control and $SOD1^{G93A}$ mice when compared to their respective 75-day-old control and $SOD1^{G93A}$ mice (**Figures 2I–L**). Interestingly, some SGCs exhibited cytoplasmic SOD1 aggregates in $SOD1^{G93A}$ mice (**Figures 2M–O**). Moreover, cytoplasmic aggregation of SOD1 was associated with the accumulation of ubiquitylated proteins as revealed by immunostaining for ubiquitin-protein conjugates (**Figure 2P**). Based on the biochemical determination of the levels of the SOD1 protein and the reduced expression of this dismutase in control SN-SGC units, the SOD1 immunofluorescence signal mainly corresponded to the transgenic expression of the mutant SOD1 protein.

## Overexpression of Mutant $SOD1^{G93A}$ Is Associated With Nitro-Oxidative Stress and Biogenesis of Lipid Droplets in SGCs

As an *in situ* marker of oxidative stress, we assessed lipid peroxidation levels using the fluorescent probe BODIPY-C11. The lipid peroxidation signal was clearly increased in dissociated SN-SGC units from $SOD1^{G93A}$ mice compared with control animals (**Figures 3A,B**). Lipid peroxides accumulated in the cytoplasm of both sensory neurons and SGCs from

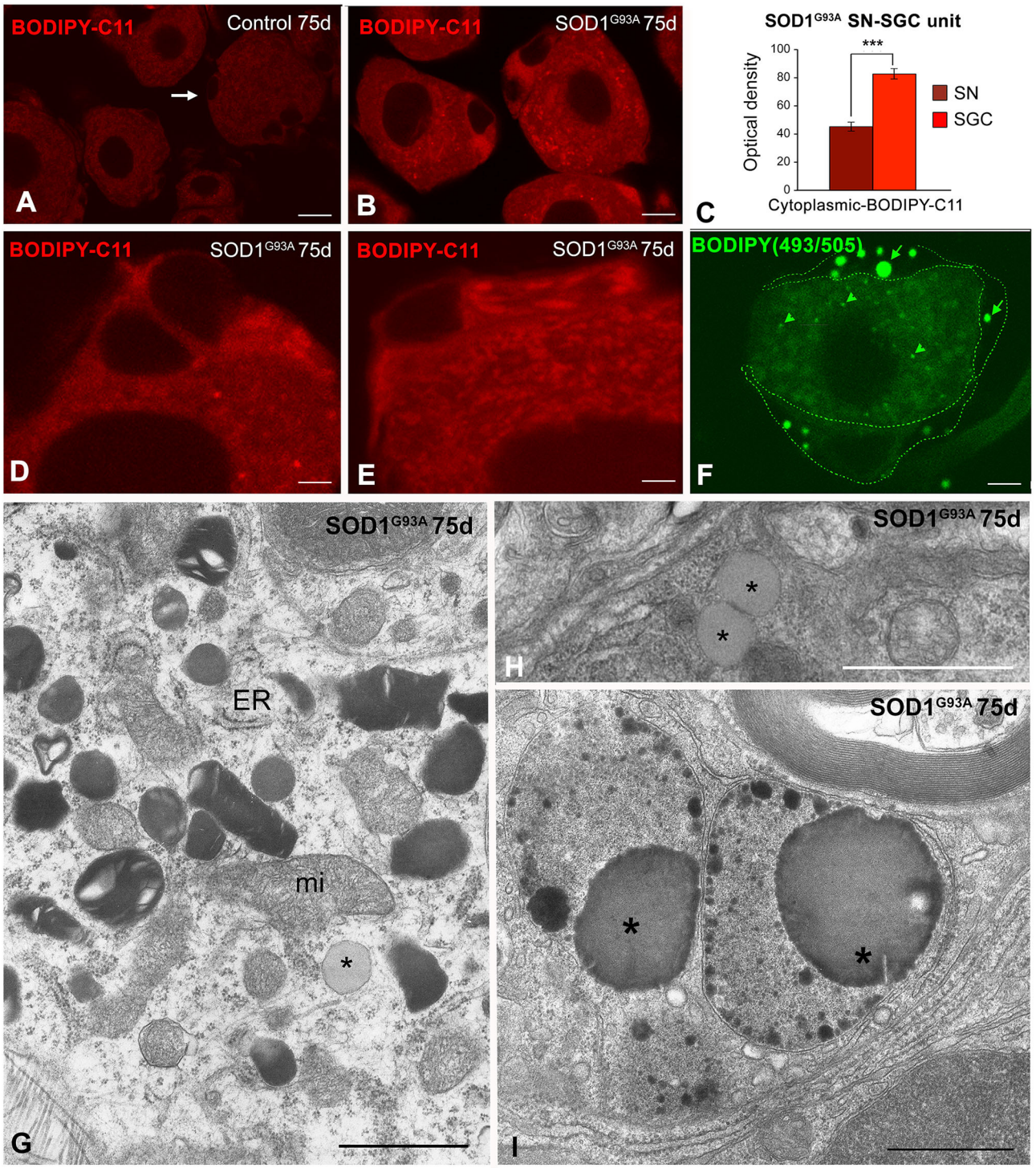


**FIGURE 3 | (A–E)** *In situ* determination of lipid peroxidation with the BODIPY-C11 probe in SN-SGC units from control **(A)** and SOD1$^{G93}$ mice **(B,D,E)**. **(A)** In control SN-SGC units both sensory neurons and SGCs (arrows) exhibit low fluorescent signal of lipid peroxidation in the cytoplasm. **(B,D,E)** Lipid peroxidation signal intensity notably increases in the cytoplasm of both sensory neurons and SGCs from SOD1$^{G93}$ mice, although BODIPY-C11 labeling was substantially higher in SGCs. Note the absence of fluorescent signal in the nucleus. **(C)** Densitometric analysis of the cytoplasmic BOBIDY-C11 fluorescent signal intensity in sensory neurons and SGCs from SOD1$^{G93}$ mouse at day 75 of age. **(F)** BODIPY-493/503 staining of a SN-SGC unit from the SOD1$^{G93}$ mouse at day 75 of age. Spherical lipid droplets of neutral lipids appear intensity labeled with the probe. Lipid droplets are larger and more abundant in SGCs (arrows) than in the sensory neuron (arrowhead). The limits of the SGC have been drawn with dashed green lines. **(G)** Ultrastructural image of the SGC cytoplasm from a SOD1$^{G93A}$ mouse illustrating the presence of numerous lysosomes with a heterogeneous morphology, isolated cisterns of endoplasmic reticulum (ER), some mitochondria (mi) and a lipid droplet (asterisk). **(H)** Detail of two spatially associated lipid droplets (asterisks) in a SGC from the SOD1$^{G93}$ mouse. **(I)** Electron micrograph showing very complex lysosomal compartments that includes large lipid droplets (asterisks). Scale bar: **(A,B,F)** 10 μm; **(D,E)** 5 μm; **(G,H)** 1 μm; **(I)** 2 μm. Student *t*-test in **(C)** $p < 0.001$.

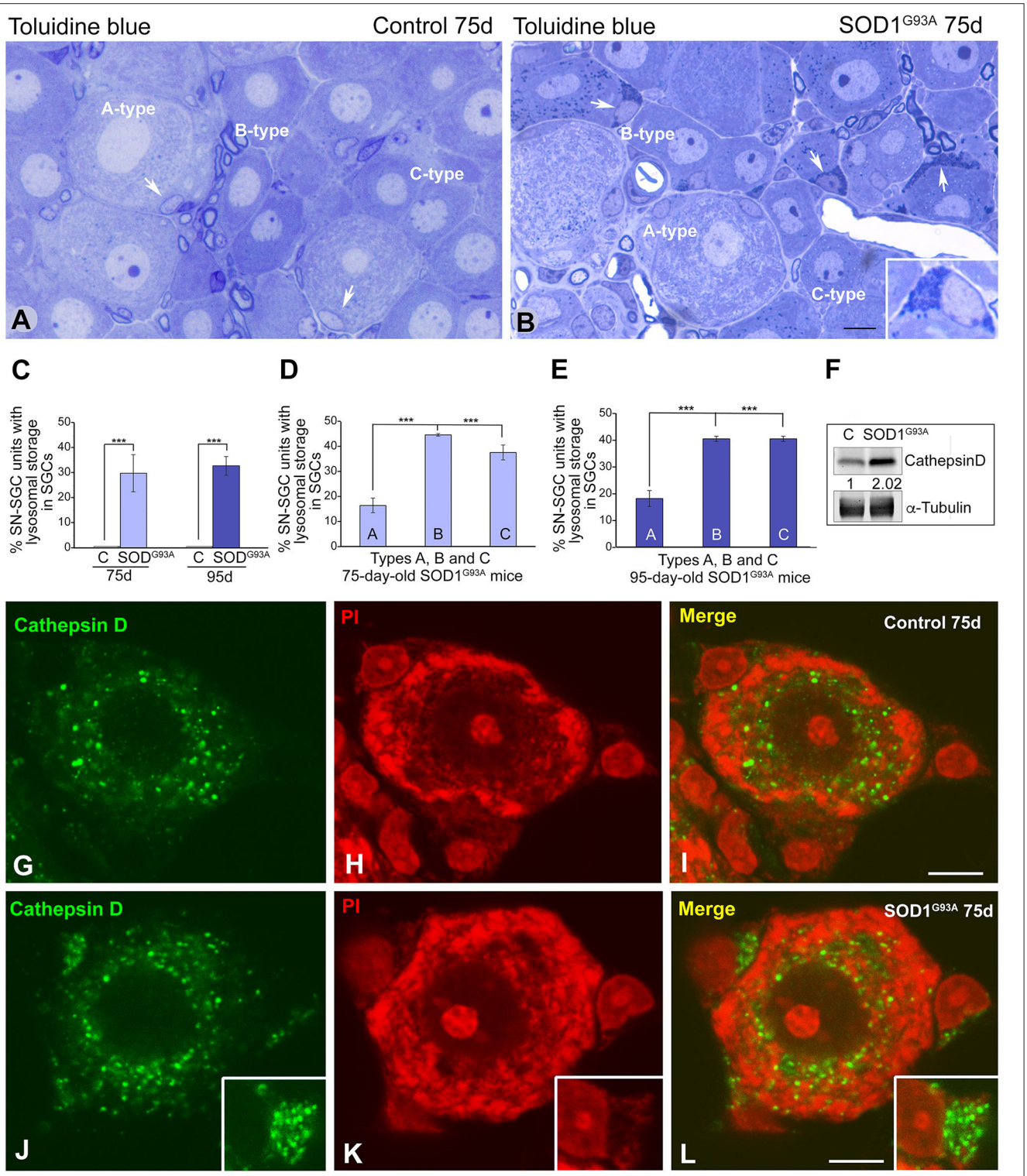


**FIGURE 4 |** SGCs from SOD1$^{G93}$ mice markedly increase the lysosomal compartment. **(A,B)** Semithin sections of DRG from control **(A)** and SOD1$^{G93}$ mice **(B)** stained with toluidine blue illustrating the organization of SN-SGC units. Note the accumulation of basophilic granules identified as lysosomes in some SGCs from SOD1$^{G93}$ mice (arrows and inset in **B**). **(C)** Quantitative analysis of SN-SGC units from control and SOD1$^{G93}$ mice carrying basophilic granules in the SGC cytoplasm at 75 and 95 days of age. **(D,E)** Quantitative analysis of the proportion of types **(A–C)** SN-SGC units from SOD1$^{G93}$ mice carrying basophilic granules in the SGC cytoplasm at 75 **(D)** and 95 **(E)** days of age. **(F)** Representative example of a western blot of cathepsin D expression in DRG from SOD1$^{G93}$ and wild-type mice.

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**FIGURE 4 |** Alpha-tubulin was used as loading control. Note the increased expression of cathepsin D in SOD1$^{G93}$ mice. **(G–L)** Immunostaining for cathepsin D counterstained with propidium iodide (PI) of SN-SGC units from control **(G–I)** and SOD1$^{G93}$ mouse **(J–L)**. Note the few immunostained lysosomes in control SGCs and their prominent storage in SGCs from the SOD1$^{G93}$ mouse (insets). Conversely, there are no appreciable differences in the distribution of lysosomes between the control and SOD1$^{G93}$ sensory neurons. Scale bar: **(A,B)** 10 μm; **(G–L)** 5 μm. Student *t*-test in **(C–E)** $p < 0.001$, $^{***}p < 0.001$.

SOD1$^{G93A}$ mice and significantly increased levels were found in the latter (**Figure 3B**) as evidenced by the results of comparative analysis of the optical density of the fluorescent BODIPY-C11 signal (**Figure 3C**). Moreover, the cytoplasmic distribution of the BODIPY-C11 signal in SGCs was not homogeneous, and some cytoplasmic domains exhibited higher fluorescence intensity (**Figures 3D,E**), which may reflect either the formation of SOD1 aggregates or the existence of clusters of membrane-bound organelles with high levels of lipid peroxidation.

The cellular stress response of ROS and free radical generation may induce the formation of lipid droplets that accumulate as neutral lipid in non-adipocyte cells (Liu et al., 2015). We used the lipophilic fluorescent probe BODIOY-493/503 to assess whether mutant SOD1$^{G93A}$ overexpression induced the formation of lipid droplets. Lipid droplets labeled with this probe were frequently detected in SGCs from the 75-day-old SOD1$^{G93A}$ mice and to a lesser extent in sensory neurons but not in samples from control mice (**Figure 3F**). The electron microscopy analysis confirmed the presence of lipid droplets with a variable electron density in SOD1$^{G93A}$ SN-SGC units, which appeared either as isolated lipid droplets surrounded by cytoplasmic organelles (**Figures 3G,H**, **6F**) or formed part of more complex lysosome-related structures (**Figure 3I**).

## Overexpression of Mutant SOD1$^{G93A}$ Induces a Lysosomal Storage in SGCs

We stained semithin sections of DRG with toluidine blue and performed an electron microscopy analysis to determine the morphological alterations induced by the overexpression of mutant SOD1$^{G93A}$ in SN-SGC units. In control SN-SGC units, toluidine blue staining revealed the typical distribution of large (type A), medium (type B), and small size (type C) SN-SGC units in the endoneural tissue microenvironment (**Figure 4A**). In SOD1$^{G93A}$ mice, the global structural organization of SN-SGC units was preserved at the presymptomatic stage (75 days of age). Intriguingly, numerous SGCs, particularly SGCs from small- and medium-sized units (types B and C), showed a substantial accumulation of basophilic granules, which were identified as lysosomes, in extensive areas of cytoplasm (**Figure 4B**). Interestingly, when it occurred, the expansion of the lysosomal compartment was frequently observed in several SGCs of the same SN-SGC unit. Abnormal lysosomal storage was rarely observed in SGCs from control DRG (**Figure 4A**). The proportion of SN-SGC units exhibiting lysosomal storage defect in glial cells, which was estimated using toluidine blue-stained semithin sections, was ∼30 and 33% in SOD1$^{G93A}$ mice at 75 and 95 days of age, respectively (**Figure 4C**). Specifically, this proportion was significantly higher in type B and C units than in larger type A units at both ages (**Figures 4D,E**).

The lysosomal nature of the basophilic granules was confirmed in dissociated SN-SGC units immunostained for the proteolytic enzyme cathepsin D, a lysosomal marker, and counterstained with propidium iodide. A few cathepsin D-positive lysosomes were observed in control SGCs, whereas large clusters of immunostained lysosomes were frequently observed in SGCs from SOD1$^{G93A}$ mice (**Figures 4G–L**). Lysosomes were preferentially distributed in the perinuclear cytoplasm in sensory neurons from in both control and SOD1$^{G93A}$ mice, but signs of aberrant lysosomal storage were not detected (**Figures 4G–L**). A western blot analysis confirmed the increase in levels of the cathepsin D protein in DRG lysates from SOD1$^{G93A}$ mice compared with wild-type animals (**Figure 4F**).

A low-magnification electron microscopy analysis revealed the morphological organization of SN-SGC units in the DRG from control and SOD1$^{G93A}$ mice at 75 days of age (**Supplementary Figures 1A,B**). This electron microscopy examination confirmed the presence of SGCs with a lysosomal storage defect (**Supplementary Figure 1A**). At a higher magnification, the ultrastructural features of SGCs from control DRG were characterized by a cell nucleus with a peripheral distribution of heterochromatin and a perinuclear cytoplasm enriched in mitochondria and Golgi complexes. The cytoplasm also exhibited some endoplasmic reticulum cisternae and isolated lysosomes (**Figures 5A,B**). Moreover, cell bodies and organelle-poor flat processes of SGCs completely wrapped neuronal perikaryal as previously reported (Pannese, 1981; Hanani, 2005) (**Figures 5A,B**). In contrast, at the presymptomatic stage, SGCs from the SOD1$^{G93A}$ mice frequently showed an unstructured perinuclear cytoplasm due to the extensive accumulation of densely packed electron-dense bodies identified as lysosome-related structures (**Figures 5C,D**). Although the lysosomal storage defect preferentially appeared in SGC bodies, numerous lysosomes were also distributed in some flattened processes (**Figure 5E**). The aberrant accumulation of electron-dense bodies appears to reflect disrupted lysosomal homeostasis (cacostasis), which leads to a non-inherited lysosomal storage pathology in these cells. Abnormal lysosomal storage was accompanied by reductions in both the number of Golgi complexes and protein synthesis machinery, particularly free polyribosomes and ER cisterns (**Figures 5C,D**).

The storage lysosomes displayed a variety of ultrastructural morphologies. They included small round primary lysosomes with a homogeneous texture and larger polymorphic secondary lysosomes that varied in size or morphology (**Figures 5C–E**). Some large and complex structures contained intralysosomal (i) electron-lucent areas; (ii) domains with a multilamellar (myelin-like) configuration composed of closely packed lamellae; and (iii) bodies or particles with a very high electron density (**Figures 5C,F,G**). Regarding the latter, the highly

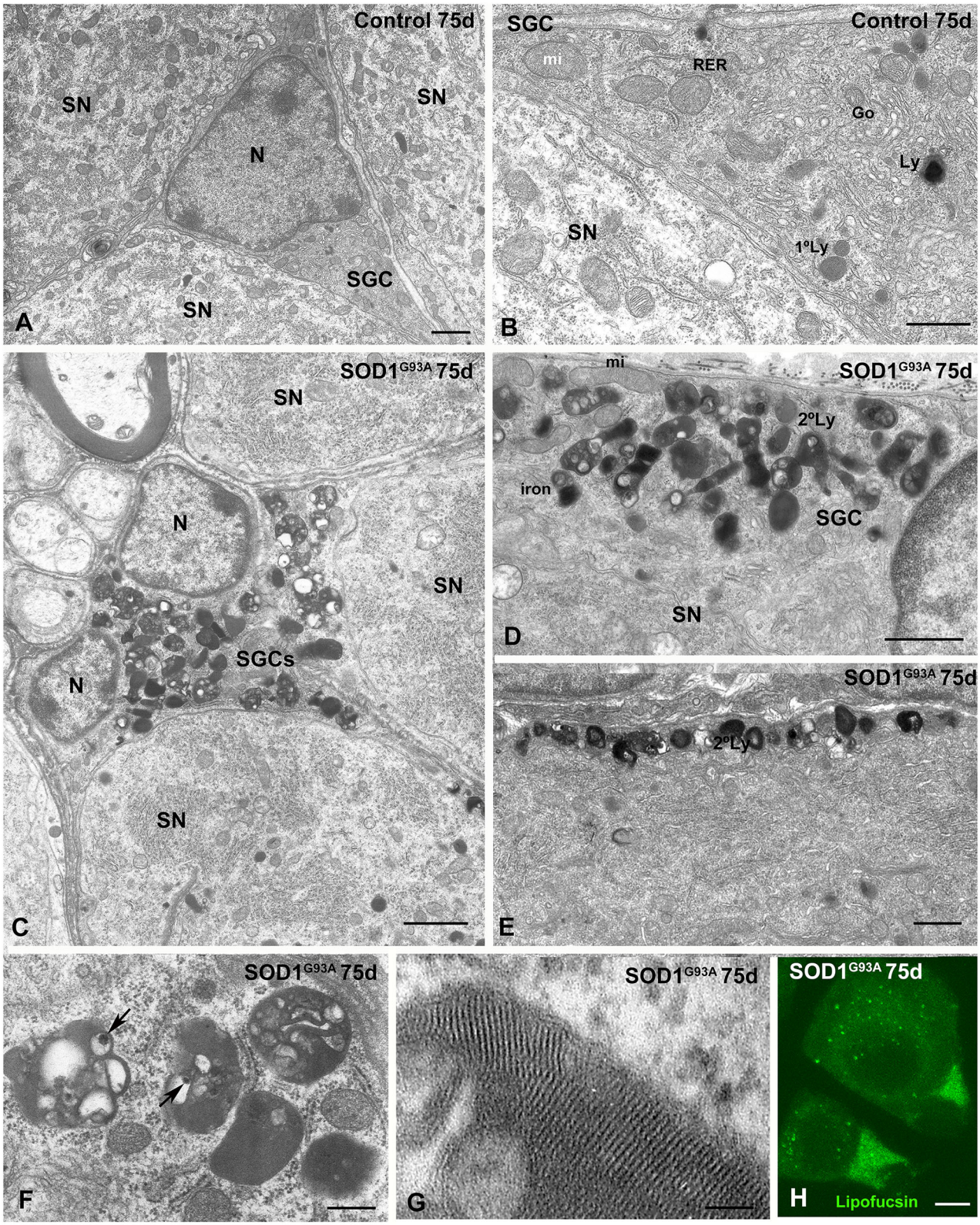


**FIGURE 5 |** Lysosomal storage disorder in SGCs from SOD1$^{G93}$ mouse at presymptomatic stage (75 days of age). **(A,B)** Ultrastructure of control SGSs surrounding sensory neurons (SN). Note in the **(B)** the characteristic organization of cytoplasmic organelles, including the Golgi complex (Go), endoplasmic reticulum,

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**FIGURE 5 |** mitochondria, and some scattered lysosomes (Ly). N: nucleus. **(C)** Panoramic vision of a SGC from a $SOD1^{G93A}$ mouse surrounding three sensory neurons (SN). Note the prominent storage of lysosome-related structures with an heterogenous morphology. **(D,E)** Detail of the focal accumulation of lysosomes at the marginal cytoplasm **(D)** and in a glial expansion **(E)** in SGCs from the $SOD1^{G93}$ mouse. Many secondary lysosomes include electron-lucent areas. **(F)** High magnification of characteristic secondary lysosomes and residual bodies (lipofuscin bodies) containing material of diverse nature including electron-lucent areas and very electron-dense particles (arrows). **(G)** Detail of the intra-lysosomal organizations of a lamellar myelin-like structure. **(H)** Lipofuscin autofluorescence of SN-SGC units from $SOD1^{G93}$ mouse. Note some lipofuscin granules in the cytoplasm of sensory neurons and the higher autofluorescent signal intensity in the cytoplasm of SGCs. Scale bar: **(A–E)** 1 μm; **(F)** 400 nm; **(G)** 50 nm; **(H)** 10 μm.

electron dense structures may represent focal deposits of iron or other metals derived from the intralysosomal oxidation of metalloproteases (**Figure 5F**). Moreover, some complex lysosome-related structures containing large electron-lucent areas may be residual bodies of lipofuscin containing indigestible oxidized lipids and proteins (**Figure 5F**). The presence of residual bodies of lipofuscin was confirmed by their strong autofluorescence signal detected using confocal microscopy (**Figure 5H**), a typical feature of lipofuscin granules (Luzio et al., 2007). Moreover, a diffuse cytoplasmic pool of lipofuscin was also observed (**Figure 5H**).

During the symptomatic stage (95 days of age), the cytoplasmic accumulation of components of the lysosomal system was increased, and some SGCs exhibited signs of advanced cell degeneration, including vacuolar degeneration of lysosomes and other organelles as well as cytoplasmic swelling (**Figures 6A–I**). Intralysosomal highly electron-dense bodies that were presumably enriched in metals, autophagosomes containing heterogeneous cellular structures and lipid droplets were frequently observed (**Figures 6C,E–G**). At this symptomatic stage, the SGC alterations were commonly accompanied by an increase in the size of lysosomal compartment and occasional mitochondrial vacuolization in sensory neurons of the SN-SGC units containing SGCs with a lysosomal storage defect (**Figure 6A**). Based on this finding, SGC degeneration may lead to the metabolic dysfunction of sensory neurons, which specifically affects lysosomal homeostasis.

## DISCUSSION

The present study strengthens the current conception of ALS as a predominant but not an exclusively motor circumscribed disease in which other neuronal and non-neuronal systems may be affected. In our study, the employed behavioral tests mainly evaluate protopathic manifestations, the stimuli of which are principally transmitted by type B and C sensory neurons. Thus, we particularly focused on these neurons and their associated SGCs in the present study.

Interestingly we observed the presence of subtle sensory manifestations even before of the appearance of motor symptomatology. Consistent with findings reported in a previous study by Vaughan et al. (2015) examining two strains of transgenic mice harboring $SOD1^{G93A}$ and $TARDBP^{A315T}$ mutations, the results from the present study suggest that the degeneration of other non-motor areas might precede and/or contribute to the MN damage in ALS. Therefore, at the spinal cord level, complex sensory-motor networks are altered in patients with ALS (Held et al., 2019). Therefore, some degree of involvement of the sensory system may easily contribute to MN damage through different mechanisms, including prion-like propagation (Prusiner, 2012). Previous studies have reported disorders at different levels of the sensory pathway including peripheral receptors, small intraepidermal sensory fibers, sensory neurons in the DRG, anterolateral and dorsal ascending spinal tracts and the sensory cortex (Guo et al., 2009; Vaughan et al., 2015; Rubio et al., 2016; Sassone et al., 2016). However, none of these studies had previously reported SGCs as potential disease targets. Based on the increasing importance of neuron-glia interactions in ALS, the neuron-SGC functional units of DRG have emerged as a pure system to assess not only the pathogenic events but also the chronology of the process due to the particularities of this "functional ecosystem."

Regarding SOD1 expression in SGCs, our results indicate that the overexpression of mutant $SOD1^{G93A}$ leads to its intracellular accumulation in SGCs of the DRG at presymptomatic motor stages. We propose that the abnormal cytoplasmic accumulation of mutant $SOD1^{G93A}$ in SGCs is potentially neurotoxic by itself and may contribute to the dysfunction of SN-SGC units and the subsequent primary sensory alterations reported here.

In addition to the motor cortex and spinal cord, the abnormal accumulation of mutant SOD1 has also been reported in other brain regions, including the temporal cortex, hippocampus, and cerebellum (Steinacker et al., 2014) as well as DRG neurons in individuals with ALS (Sábado et al., 2014). Importantly, to the best of our knowledge, this is the first study suggesting SGCs as a potential non-motor target in ALS. Several reports have provided evidence that the accumulation of mutant SOD1 in MNs from animal models of ALS leads to altered proteostasis (Riancho et al., 2015). In fact, in $SOD1^{G93A}$ mice, we observed disruptions in proteostasis accompanied by abnormal SOD1 accumulation, the aggregation of ubiquitylated proteins and alterations in the autophagy-lysosomal system in SGCs during the presymptomatic stage. Interestingly, the $SOD1^{G93A}$ mutation has been reported to destabilize and promote protein misfolding and its subsequent aggregation (Sibilla and Bertolotti, 2017). Furthermore mutant SOD1 displays an increased aggregation propensity compared with the wild-type protein (Prudencio et al., 2009). The aggregation of the mutant SOD1 protein may be increased by its ability to propagate its misfolded conformation by acting as a prion-like protein that escapes protein quality control and alters the native SOD1 folding process (Münch and Bertolotti, 2011; Prusiner, 2012; Ayers et al., 2014; Sibilla and Bertolotti, 2017).

Our results are consistent with the induction of oxidative stress in SGCs. Aberrant oxidative reactions catalyzed by

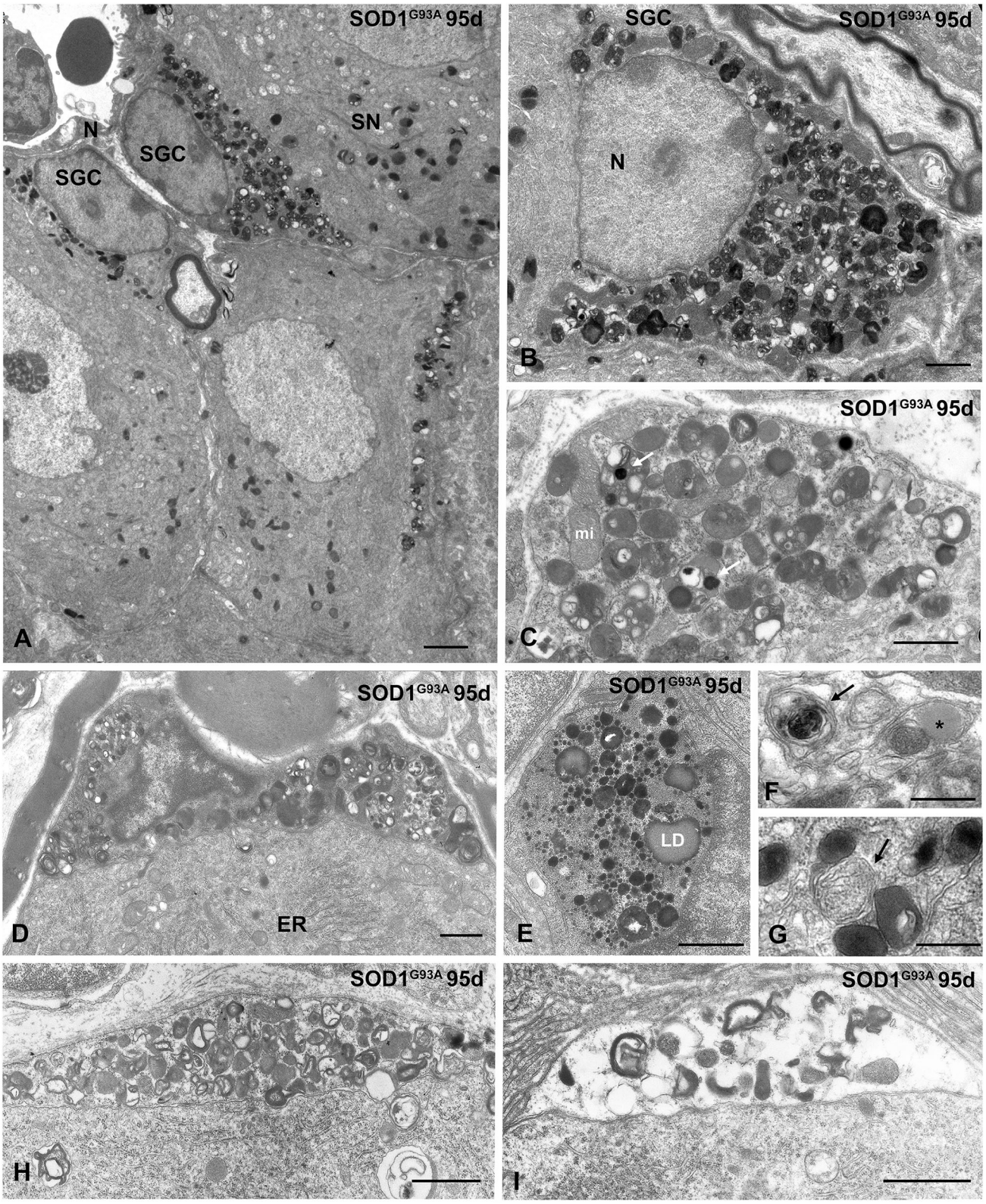


**FIGURE 6 |** Advanced lysosomal storage disorder in SGCs from SOD1$^{G93}$ mice at symptomatic stage (95 days of age). **(A)** Representative example of several SGSs from a SOD1$^{G93}$ mouse with lysosomal storage. One of the associated sensory neurons (SN) shows abundance of lysosomes and cytoplasmic vacuolation of

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**FIGURE 6 |** mitochondria. N: nucleus. **(B)** Massive storage of densely packed lysosomes and residual bodies in the cytoplasm of a SGC with severe disruption of the cytoplasmic organization. **(C)** Storage of lysosomes in a SGC. Note the presence of very electron-dense intralysosomal structures (white arrow) presumably of metal nature and some mitochondria (mi). **(D)** A SGC with high accumulation of lysosomes and prominent heterochromatinization at the nuclear periphery. Note the well-preserved cytoplasmic organization of the associated sensory neuron with a stack of rough endoplasmic reticulum cisterns (ER). **(E)** Large cytoplasmic area with high concentration of small rounded and homogenous primary lysosomes, larger and heterogenous secondary lysosomes, and lipid droplets (LD). **(F,G)** Detail of SGC cytoplasm showing autophagosomes (arrows) and a lipid droplet (asterisk in **F**). **(H,I)** Two progressive stages of vacuolar degeneration in SGCs. Note the complete disruption of glial cell cytoplasm in **(I)**. Scale bar: **(A)** 2 μm; **(B–E)**, **(H,I)** 1 μm; **(F,G)** 0.5 μm.

mutant SOD$^{G93A}$ have been proposed to contribute to increased oxidative stress and cellular toxicity in ALS (Andrus et al., 2002; Ilieva et al., 2007; Barber and Shaw, 2010; An et al., 2014). In this context, the results of our experiment using the BODIPY-C11 probe revealed increased lipid peroxidation in SGCs of the DRG from SOD1$^{G93A}$ mice. Moreover, lipid peroxidation generates several highly reactive oxidizing agents, including lipofuscin, which are capable of damaging macromolecules and cellular organelles (Höhn and Grune, 2013). Thus, we propose that SGCs of the DRG are important targets of oxidative stress in individuals with ALS.

Notably, lipid droplets composed of neutral lipids were observed in SGCs from the SOD1$^{G93A}$ mice, as evidenced by the BODIPY-493-503 probe and confirmed with electron microscopy. In addition to their essential role in energy storage, growing evidence also links lipid droplets to neuron-glia metabolic coupling (Pennetta and Welte, 2018). In this context, the high level of lipid peroxidation reported here might represent a protective detoxification mechanism for the oxidative stress in sensory neurons. This protective function is relevant to ALS because metabolic abnormalities in lipid metabolism and the lipidome are prevalent in the spinal cord of patients with ALS and SOD1 mice (Chaves-Filho et al., 2019). Moreover, recent evidence suggests a metabolic switch from glucose to lipids as the energy source in SOD1 mouse models of ALS (Schmitt et al., 2014). If this process also occurred in the DRG from the SOD1 mice, neurons would likely rely on SGCs to store excess neutral lipids in droplets as a neuroprotective mechanism by reducing lipotoxicity (Liu et al., 2015; Chaves-Filho et al., 2019). Interestingly, we detected some lipid droplets inside autolysosomes, suggesting a mechanism of lipophagy (Welte and Gould, 2017). Consistently, Rudnick et al. (2017) observed the activation of autophagy in MNs during the progression of ALS in SOD1$^{G93A}$ mice, and we often detected autophagosomes in SGCs from this mouse model of ALS.

The most prominent cellular change observed in SGCs from the SOD1$^{G93A}$ mouse model of ALS is the substantial accumulation of cathepsin D-positive lysosomes and residual bodies, including lipofuscin granules. This cellular response likely substantially affects SGC function and preferentially occurs in types B and C SN-SGC units, consistent with the tactile and thermal-related nociceptive dysfunctions observed in the sensory tests. To the best of our knowledge, our study provides the first observation of the involvement of a lysosomal storage disorder in ALS pathogenesis. Moreover, altered autophagy-lysosomal homeostasis appears to be a major subcellular pathway underlying the toxicity of mutant SOD1$^{G93A}$ toxicity in SGCs. Importantly, increased lysosomal storage in SGCs occurs at the presymptomatic stage (75 days of age) in the SOD1$^{G93A}$ mouse model of ALS, a time prior to the appearance of detectable cellular alterations in sensory neurons. This finding supports the hypothesis that SGCs from the DRG are also primary cellular targets of oxidative stress and proteostasis disturbances in ALS (**Supplementary Figure 2**). Moreover, the dysfunction of the autophagy-lysosomal system was noted in several SGCs of the same SN-SGC unit, suggesting that the prion-like nature of the mutant SOD1 protein may potentially contribute to the propagation of its toxicity through intercellular communication mechanisms.

We postulate that the pathogenic cascade that leads to excessive storage of lysosomes in SGC is triggered by oxidative stress induced by the abnormal accumulation and toxicity of the mutant SOD1$^{G93A}$ protein. Regarding the potential defects in the activity of lysosomal enzymes in SGCs, aberrant changes in glucosylceramide, galactolipids, and sphingomyelin levels have been reported in the spinal cord of patients with ALS and SOD1 mouse models of the disease (Dodge et al., 2015).

The lysosomal storage disorder in SGCs appears to reflect a severe disturbance of proteostasis that particularly affects cellular mechanisms of macromolecular degradation. In this context, dysfunction of both the ubiquitin proteasome and autophagy-lysosomal systems has been reported in the spinal cord of patients with ALS and SOD1$^{G93A}$ mice (Rudnick et al., 2017). Consistent with this observation, we observed an abundance of autophagosomes in SGCs from this mouse model of ALS.

## CONCLUSIONS

Our study strengthens the current widely accepted concept of ALS as a non-MN exclusively circumscribed disorder, supporting the sensory involvement noted in the SOD1 transgenic murine model of the disease. Specifically, we first highlighted SGCs as a new potential target for the disease (**Supplementary Figure 2**). The preferential lysosomal pathology observed in these cells meets the criteria for a lysosomal storage disorder induced by oxidative stress (Kielian, 2019; Marques and Saftig, 2019). Moreover, SGCs appear to have an essential function in maintaining sensory neurons homeostasis, thus reinforcing the crucial role of astroglial cells in ALS (Baker et al., 2015). Based on both the existence of sensory-motor networks in the spinal cord and the prion hypothesis, SGCs might be responsible for the sensory manifestations and potentiate or propagate motor neuron pathology. The inclusion of these "new guests" into ALS pathogenesis will provide new pathogenic perspectives that might result in the development novel therapeutic strategies.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by COMITE DE ÉTICA DE LA UNIVERSIDAD DE CANTABRIA.

## AUTHOR CONTRIBUTIONS

JR, ML, and MB designed and supervised the experiments and wrote the manuscript. MR-S, JR, OT, and MB performed the experiments and analyze the data. MR-S, JR, OT, MB, and ML read and editing the article. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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# Mitochondrial Dynamics and Bioenergetic Alteration During Inflammatory Activation of Astrocytes

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Mitochondria are essential cellular organelles that act as metabolic centers and signaling platforms and have been identified as an important subcellular target in a broad range of neuropathologies. Studies on the role of mitochondria in neurological disorders have primarily focused on neurons. However, dysfunctional mitochondria in glial cells, particularly astrocytes, have recently gained research attention due to their close involvement in neuroinflammation and metabolic and neurodegenerative disorders. Furthermore, alterations in mitochondrial energy metabolism in astrocytes have been reported to modulate cellular morphology and activity and induce the release of diverse proinflammatory mediators. Moreover, emerging evidence suggests that dysregulation of mitochondrial dynamics characterized by aberrant fission and fusion events in glial cells is closely associated with the inflammatory activation of glia. In this mini-review, we cover the recent advances in the molecular aspects of astrocytic mitochondrial dynamics and their metabolic changes under the pathological conditions of the central nervous system (CNS).

**Keywords: glia, astrocyte, mitochondria, fission, fusion, metabolism, neuroinflammation**

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## INTRODUCTION

Astrocytes are homeostatic cells of the central nervous system (CNS), comprising around 40% of the glial population (Pelvig et al., 2008), and exhibit remarkable morphological and functional heterogeneity. Besides providing metabolic and physical support to neurons, astrocytes regulate the blood-brain barrier (Posada-Duque et al., 2014), the extracellular balance of ions and neurotransmitters (Sofroniew and Vinters, 2010), synaptogenesis (Baldwin and Eroglu, 2017), and axon pathfinding, thus rendering these glial cells necessary for brain homeostasis. Studies have demonstrated that astrocytes receive and carry information to other neural cells in a coordinated effort in response to diverse CNS insults, including injury and infection, and initiate reparative mechanisms through the activation of immune defenses (Sofroniew, 2009; Bylicky et al., 2018).

Astrocyte-mediated neuroinflammation is associated with metabolic and degenerative pathologies such as Alzheimer's disease (AD; Fu and Jhamandas, 2014), Parkinson's disease (PD; Cabezas et al., 2014), multiple sclerosis (Brosnan and Raine, 2013), traumatic brain injury (Barreto et al., 2011), diabetes or obesity (Rahman et al., 2018), and aging (Zhang et al., 2017).

In response to CNS insults, such as injury and diseases, astrocytes undergo a morphological, biochemical, transcriptional, and functional transformation termed as astrogliosis or astrocyte reactivity, which leads to a spectrum of heterogeneous changes in a context-specific manner that vary depending on the etiology and severity of the CNS insults (Sofroniew, 2009; Anderson et al., 2014; Escartin et al., 2019). This inflammatory phenotypic change of astrocytes is characterized by higher expressions of glial fibrillary acidic protein (GFAP), aldehyde dehydrogenase one family member L1, and vimentin proteins, accompanied by their hypertrophic morphology and thick processes (Pekny et al., 1999; Pekny and Nilsson, 2005). Consistent with this, Escartin et al. (2019) have extensively discussed the common features and the core hallmarks of reactive astrocytes in their recent review and suggested that GFAP overexpression and morphological changes are the most common marker for reactive astrocytes. During this process, astrocytes regulate their metabolism to generate lactate, glutamate, and ketone bodies as energy substrates for neurons, as these nutrients are deprived in the lesioned brain (Auestad et al., 1991; Dienel, 2013). However, under specific circumstances, astrogliosis exerts harmful effects such as excessive inflammation through diverse secretory mediators and interference with axon growth and synapse sprouting (Sofroniew and Vinters, 2010).

In recent years, there has been an increasing research focus on glial mitochondrial dynamics and energy metabolism, particularly in the context of the phenotypic transitions of microglia and astrocytes (Motori et al., 2013; Nair et al., 2019). It has been reported that alterations in mitochondrial energy metabolism in these glial cells modulate the cellular morphology and activity. In agreement with this, the phenotypic changes in glia are driven by a mitochondrial metabolic shift; in particular, switching from oxidative phosphorylation to glycolysis is associated with the inflammatory activation of glia in diverse neuroinflammatory conditions (Jiang and Cadenas, 2014; Nair et al., 2019). A previous study reported that increased glycolysis fuels the energy-intensive processes required for the inflammatory activities of immune cells (Ganeshan and Chawla, 2014). Furthermore, alterations in mitochondrial morphology in glial cells, characterized by aberrant fission and fusion events, were found to be closely associated with neuroinflammation (Motori et al., 2013; Kim et al., 2019). In this mini-review, we summarize the recent advances in the molecular aspects of mitochondrial dynamics and bioenergetics in inflammatory astrocytes in the context of metabolic and neurodegenerative diseases.

## PATHWAYS LINKING MITOCHONDRIAL DYSFUNCTION TO INFLAMMATORY ACTIVATION OF ASTROCYTES

In diverse neuropathologies associated with excessive and acute/chronic neuroinflammation, including neurodegenerative diseases, traumatic brain injury, and stroke, astrocytes exhibit a highly reactive state (Sofroniew, 2009) and release a variety of pro-inflammatory and anti-inflammatory mediators, which have been implied to contribute to worsening or ameliorating the brain pathology (Bush et al., 1999; Okada et al., 2006). In recent years, multiple pathways, involving morphological and functional routes, have been identified that link mitochondrial dysfunction and inflammatory activation of astrocytes.

### Altered Mitochondrial Dynamics During Inflammatory Activation of Astrocytes

The mitochondrion of mammalian cells is tubular and is constantly maintained by fusion and fission reactions and through the exchange of different genes and proteins; these processes are essential for its appropriate functioning (Chang and Reynolds, 2006). These processes involve a group of proteins, such as dynamin-related protein (DRP1) and fission 1 protein mediating fission event, mitofusin 1 and 2 (MFN1 and 2), and optic atrophy 1 (OPA1) mediating fusion event (Liesa et al., 2009). An appropriate balance between fusion and fission reactions is essential for maintaining the mitochondrial architecture and distribution of mitochondrial components (Liesa et al., 2009). Breakdown of mitochondrial dynamics leads to mitochondrial damage, a condition that has been suggested to be associated with neuroinflammation, aging, and metabolic and neurodegenerative diseases (Detmer and Chan, 2007; Joshi et al., 2019; Liu et al., 2020).

Astrocytic mitochondria play a vital role in brain energy metabolism and immune modulation (Belanger et al., 2011; Joshi et al., 2019). Accumulating evidence demonstrates that the alteration in the metabolic signature of astrocytes reflects their response to neuroinflammation. In CNS pathologies associated with neuroinflammation, astrocytes alter their phenotypes into a reactive state, which results in mitochondrial alterations. A study conducted by Motori et al. (2013) provided insights into the astrocytic mitochondrial changes that occur in injured brain tissues. Upon inflammatory insults, astrocytes exhibit multiple forms of reactivity in different areas of the lesioned mouse brain, which affect the mitochondria in particular. The balance between mitochondrial fusion and fission events is altered in astrocytes during neuroinflammation and tissue injury, and this faulty regulation of the mitochondrial dynamics has been associated with astrogliosis. A highly damaged and proinflammatory brain environment leads to excessive fission of mitochondria in astrocytes, accompanied by an increase in the expression of phosphorylated-DRP1 ($Ser^{616}$), which also induces mitochondrial fragmentation. This phenomenon has been correlated with impaired mitophagy, a cargo-specific subset of autophagy (Sheng, 2014). It has been suggested that these alterations in astrocytic mitochondrial dynamics play a critical role in cellular aging and trigger neuroinflammation and neurodegenerative diseases. A previous study identified a correlation between the alterations in mitochondrial dynamics in astrocytes and the neuroinflammation in mice with chronic manganese-induced neurotoxicity (Sarkar et al., 2018). That study demonstrated that manganese treatment increased mitochondrial circularity and decreased MFN2 levels inducing an excessive mitochondrial fragmentation along with inflammatory activation of astrocytes characterized by an elevated GFAP expression. However, mito-apocynin, a

mitochondria-targeted antioxidant, significantly attenuated the manganese-induced expression of inflammatory genes and astrogliosis.

Increased mitochondrial fission and fragmentation have been implicated in neuroinflammation and pathogenesis of degenerative diseases. The role of mitochondrial fragmentation during the inflammatory activation of astrocytes has been reported by Joshi and group in the context of neurodegenerative diseases (Joshi et al., 2019). They demonstrated that excessive fragmented and damaged mitochondria released from microglia cause inflammatory activation of astrocytes, which has been suggested to potentiate inflammatory neurodegeneration in mouse models of AD, Huntington's disease (HD), and amyotrophic lateral sclerosis. In this study, the mitochondrial fragmentation in microglia was mediated by DRP1–FIS1 (a mitochondrial fission 1 receptor) pathway. While the extracellular damaged mitochondria were detrimental, a transfer of functional mitochondria was neuroprotective. The amounts of extracellular functional mitochondria were inversely proportional to the number of damaged mitochondria under pathological conditions. Also, the mitochondrial dysfunction in glial cells has been characterized by lower ATP levels, loss of mitochondrial inner membrane polarization, and increased mitochondrial reactive oxygen species (ROS) production, which has been correlated with astrocyte activation toward the A1 proinflammatory state. Like microglia, reactive astrocytes also display dysfunctional mitochondria and induce neuronal damage (Joshi et al., 2019). However, a selective heptapeptide inhibitor (P110) of excessive mitochondrial fission and fragmentation was found to suppress glial activation, neuroinflammation, and neurodegenerative phenotypes *via* inhibiting the binding of activated DRP1 to FIS1 without affecting physiological mitochondrial fission. They have shown that the increase in the inflammatory response of astrocytes is in part mediated by fragmented mitochondria derived from microglia; these findings were mostly demonstrated using *in vitro* culture models. Their study also demonstrated that activated astrocytes exhibit several fragmented mitochondria as well; however, the potential role of fragmented mitochondria released from astrocytes in inflammatory cascades has not been investigated. Future studies are required to better understand the crosstalk between microglia and astrocytes through fragmented mitochondria in neuroinflammation.

The development of status epilepticus causes alterations in mitochondrial functions compromising astrocytic viability. An animal study conducted by Ko et al. (2016) showed that widespread reactive astrogliosis with reduced length of astrocytic mitochondria is observed in the dentate gyrus, whereas elongated mitochondria are found in autophagic astrocytes in the cornu ammonis 1 (CA1) region of hippocampus under the status epilepticus condition. The region-specific alterations in mitochondrial dynamics in astrocytes correlate with DRP1 phosphorylation, which is regulated by mitochondrial cyclin-dependent kinase 5 (CDK5) that promotes mitochondrial fission. However, pharmacological inhibition of CDK5 (Olomoucine and roscovitine) and mitochondrial fission (Mdivi-1) was found to ameliorate astrogliosis and cellular apoptosis following status epilepticus (Ko et al., 2016; Hyun et al., 2017).

Chronic neuroinflammation is one of the hallmarks of PD pathophysiology (Lee et al., 2019). Clinical reports and animal studies have indicated that gliosis and an increase in the levels of inflammatory mediators are common features of PD pathogenesis (Wang et al., 2015). The persistent release of proinflammatory molecules, including cytokines and chemokines, by activated microglia and reactive astrocytes, results in the enhancement of dopaminergic neuronal degeneration in the substantia nigra. Focusing on astrocytes, pro-inflammatory astrogliosis has been observed in the brain of patients with PD, suggesting that reactive astrocytes are involved in the alteration of immune processes in PD pathophysiology (Yamada et al., 1992). It has been reported that an excess amount of misfolded α-synuclein in the brain of PD mouse model triggers astrocytes to alter into inflammatory phenotypes inducing widespread astrogliosis, microglial activation, increased expression levels of TNF-α and IL-6, and subsequent degeneration of dopaminergic neurons (Gu et al., 2010; Fellner et al., 2013). Another report indicated that the phosphatase and tensin homolog-induced putative kinase 1 (PINK1)/parkin pathway regulates mitochondrial dynamics and function of mammalian cells (Yu et al., 2011). Consistent with this, loss of function in PINK1/parkin due to mutations has been reported to induce mitochondrial damage and suggested to be involved in the early onset of PD (Exner et al., 2012). Remarkably, parkin dysfunction in astrocytes has been reported to impair mitochondrial function, contributing to the pathogenesis of PD (Ledesma et al., 2002). This finding suggests that parkin dysfunction might be involved in defective mitophagy in astrocytes and subsequent PD pathogenesis. Future study is required to investigate the role of mitophagy in astrocytes in neurodegenerative diseases. It has also been reported that parkin is involved in the astrocytic inflammatory response. In this regard, astrocytic activation by IL-1β induced a decrease in the level of parkin (Khasnavis and Pahan, 2014). Furthermore, another PD-related gene *DJ-1* has been demonstrated to alter mitochondrial dynamics in cultured astrocytes, resulting in a lower neuroprotective ability (Lev et al., 2013). Similarly, a decreased level of DRP1 is also found in the astrocytes of patients with PD (Hoekstra et al., 2015), implying an impairment of mitochondrial dynamics in the brain astrocytes of these patients. However, the mechanistic correlation between astrocytic mitochondrial dynamics and their inflammatory activation in PD pathophysiology remains largely unknown. Future studies are necessary to identify the mitochondrial pathways that regulate astrocytic phenotypes associated with neuroinflammation in PD.

## Mitochondrial Bioenergetic Perturbation During Inflammatory Activation of Astrocytes

Multiple lines of evidence suggest that glial (microglia and astrocytes) phenotypic transition is governed by a mitochondrial

**TABLE 1** | Experimental findings related to mitochondrial dynamics and bioenergetics in inflammatory astrocytes and neuropathologies.

| Disease/ neuropathologies | Model/stimuli | Mitochondrial dynamics and metabolic changes | Suggested mechanism | Remarks | References |
|---|---|---|---|---|---|
| Brain injury | Stab wound injury *in vivo*. LPS + IFN-γ stimulation *in vitro*. | Excessive mitochondrial fission, enhanced glycolysis, and reduced mitochondrial respiration. | Increased levels of p-DRP1 ($Ser^{616}$), mitochondrial ROS, and iNOS. | Impaired mitophagy and mitochondrial motility, multiple forms of astrocyte reactivity in different brain areas. | Motori et al. (2013) |
| Alzheimer disease | 5XFAD mice and cellular models. $_0A\beta_{42}$ or LPS or nigericin stimulation *in vitro*. | Pathological mitochondrial fragmentation, lower ATP levels, loss of normal inner mitochondrial membrane polarization, and increased mitochondrial ROS production. | DRP1-FIS1-mediated mitochondrial fission. | Astrocyte activation to A1 proinflammatory state and a release of proinflammatory cytokines such as TNF-α and IL-1β. | Joshi et al. (2019) |
| Huntington disease | R6/2 mice and cellular models. LPS or nigericin stimulation *in vitro*. | Pathological mitochondrial fragmentation, lower ATP levels, loss of normal inner mitochondrial membrane polarization, and increased mitochondrial ROS production. | DRP1-FIS1-mediated mitochondrial fission. | Astrocyte activation to A1 proinflammatory state and a release of proinflammatory cytokines such as TNF-α and IL-1β. | Joshi et al. (2019) |
| Amyotrophic lateral sclerosis | SOD1-G93A mice and cellular models. LPS or nigericin stimulation *in vitro*. | Pathological mitochondrial fragmentation, lower ATP levels, loss of normal inner mitochondrial membrane polarization, and increased mitochondrial ROS production. | DRP1-FIS1-mediated mitochondrial fission. | Astrocyte activation to A1 proinflammatory state and a release of proinflammatory cytokines such as TNF-α and IL-1β. | Joshi et al. (2019) |
| Parkinsonian syndrome | Chronic manganese-induced neurotoxicity. | Increased mitochondrial circularity and fragmentation, decrease in basal mitochondrial oxygen consumption, and reduced level of ATP. | Decreased level of MFN2. | Inflammatory activation of astrocytes characterized by an elevated GFAP expression and increased expression of proinflammatory factors such as TNF-α, IL-1β, IL-6, IL-12, and NOS2. | Sarkar et al. (2018) |
| Status epilepticus (SE) | Pilocarpine-induced SE in rat. | Increased mitochondrial fission and reduced fusion along with the reduced length of astrocytic mitochondria. | Increased expression of p-DRP1 ($Ser^{616}$) by mitochondrial cyclin-dependent kinase 5 and decreased levels of p-DRP1 ($Ser^{637}$) and OPA1. | Reactive astrogliosis. | Ko et al. (2016) and Hyun et al. (2017) |
| Aging | Aged rats (6–18 months old). IL-1β or TNF-α stimulation *in vitro*. | Higher mitochondrial respiration rate. | Age-dependent increase in $H_2O_2$ generation and NOX expression. | Functionality switch of astrocytes to a reactive state. | Jiang and Cadenas (2014) |
| Diabetes and obesity | Mouse models of STZ and HFD-induced diabetes and obesity. High glucose or palmitate stimulation *in vitro*. | Altered glycolytic metabolism is characterized by an increased level of lactate and extracellular acidification rate. | The metabolic shift from oxidative phosphorylation to glycolysis by upregulation of PDK2 and p-PDH ($S^{293}$ and $S^{300}$). | Increased levels of cytokines (TNF-α, IL-1β, and IL-6) and gliosis. | Rahman et al. (2020) |

metabolic shift particularly from oxidative phosphorylation to glycolysis (Jiang and Cadenas, 2014; Nair et al., 2019). Unlike microglia, the astrocytes are primarily glycolytic, demonstrating a lower oxidative metabolic rate (Walz and Mukerji, 1988). The phenotypic alterations observed in reactive astrocytes can be associated with mitochondrial metabolism; however, further studies are required to elucidate the role of mitochondrial bioenergetic distress in the inflammatory activation of astrocytes under different conditions.

A study conducted by Jiang and Cadenas (2014) demonstrated a metabolic–inflammatory axis in primary astrocytes. These cells exhibit increased responses with age to inflammatory cytokines such as IL-1β and TNF-α. Furthermore, exposure of astrocytes to IL-1β and TNF-α alters

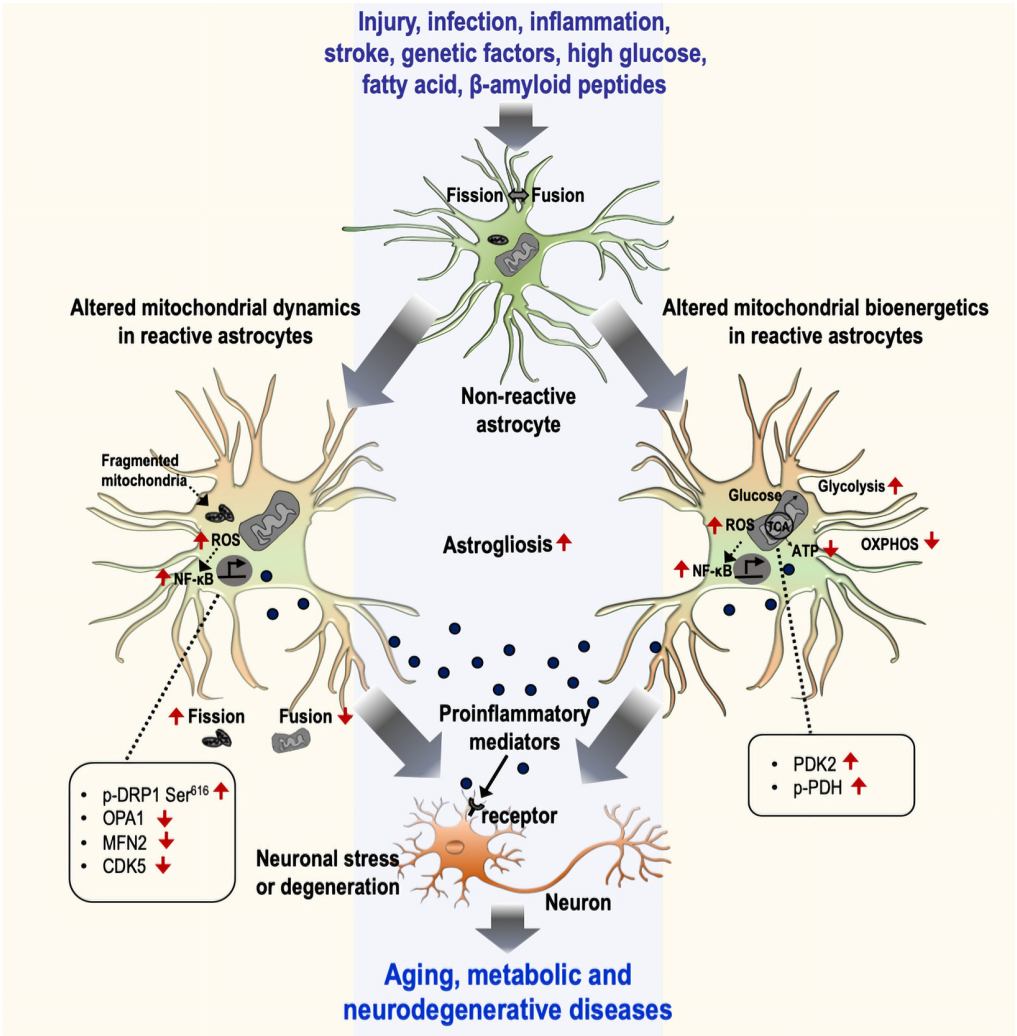


**FIGURE 1 |** Mitochondrial dynamics and bioenergetics in inflammatory astrocytes and neurological disorders. Astrocytes undergo a gradual activation process in response to various stimuli, including injury, infection, inflammation, stroke, genetic factors, high glucose, fatty acid, and β-amyloid peptides, that leads to the induction of inflammatory phenotypes through the alteration of mitochondrial dynamics (left) and bioenergetics (right). The alteration of mitochondrial dynamics is represented by an increase in fission and a decrease in fusion events characterized by changes in related proteins [p-DRP Ser$^{616}$, optic atrophy 1 (OPA1), MFN2, and CDK5], culminating in the increased number of fragmented mitochondria. Similarly, altered mitochondrial bioenergetics is represented by increased glycolysis and decreased mitochondrial oxidative phosphorylation. The change in the bioenergetic process is mediated by the regulation of mitochondrial proteins such as the upregulation of PDK2 and p-PDH. This phenomenon induces astrocytes toward their inflammatory activation through the excessive production of reactive oxygen species (ROS) and the subsequent activation of the NF-κB pathway. Reactive astrocytes release diverse pro-inflammatory mediators and induce inflammatory neuronal stress or toxicity associated with aging and metabolic and neurodegenerative diseases. OXPHOS, oxidative phosphorylation.

their mitochondrial bioenergetics, suggesting that the alterations in mitochondrial aerobic metabolism and inflammatory responses are a mutual process and support the functionality switch of astrocytes to a reactive state with age. This metabolic change is associated with an age-dependent increase in hydrogen peroxide generation and activation of NF-κB signaling. A previous study involving a seahorse analysis of bioenergetic status in manganese-treated astrocytes demonstrated an impaired basal mitochondrial oxygen consumption and adenosine triphosphate (ATP)-linked respiration rates, which have been correlated with manganese-induced astrocytic inflammatory phenotypes characterized by increased expression of proinflammatory factors such as TNF-α, IL-1β, IL-6, IL-12, and NOS2 (Sarkar et al., 2018). Also, a recent study showed that metabolic reprogramming of astrocytes governs hypothalamic inflammation and its sequelae in diabetes. In that study, Rahman et al. (2020) identified that pyruvate dehydrogenase (PDH) kinase (PDK)-2, a key regulator of the mitochondrial gatekeeping enzyme PDH, induces a metabolic shift from oxidative phosphorylation to glycolysis in astrocytes that contributes to neuroinflammatory responses characterized by increased levels of inflammatory cytokines (TNF-α, IL-1β, and IL-6) and gliosis; this further modulated the neuropeptidergic circuitry in the hypothalamus associated with altered feeding

behavior in diabetes. However, astrocyte-specific genetic ablation and pharmacological inhibition of PDK2 by AZD7545 and lactate dehydrogenase (a glycolytic enzyme) by oxamate or GSK2837808A were found to ameliorate the diabetes-induced hypothalamic inflammation and the subsequent metabolic syndrome, suggesting that PDK2 in hypothalamic astrocytes can be a potential therapeutic target for neuroinflammation and associated metabolic disorders.

## Triggers and Mediators of Mitochondrial Dynamics and Metabolic Changes in Inflammatory Astrocytes

Astrocytes undergo a gradual activation process in response to diverse stimuli, including injury, stroke, inflammation, neurotoxins, pathogens, over-nutrition, genetic factors, and β-amyloid peptides (Afridi et al., 2020). Natural byproducts, including ROS such as hydroxyl radicals ($OH^-$), superoxides ($O^{2-}$), and reactive nitrogen species such as Nitric oxide (NO) play vital roles in cell signaling, gene transcription, and microbial defense (Rizor et al., 2019). Several lines of evidence indicated the role of NO in regulating DRP1 activity and mitochondrial fission (Barsoum et al., 2006; Bossy et al., 2010). A study by Motori et al. (2013) has revealed that inflammation-induced NO production is required for DRP1 activation and subsequent mitochondrial fragmentation in astrocytes. These fragmented mitochondria in astrocytes exhibit increased ROS generation and compromised ATP production, which lead to the activation of the NF-κB pathway and the release of proinflammatory cytokines, initiating a toxic feedforward loop of chronic neuroinflammation toward neurotoxicity (Motori et al., 2013; Kaur et al., 2019). Furthermore, studies have reported that NO-induced activation of hypoxia-inducible factor 1-alpha and 5′ AMP-activated protein kinase signaling pathways enhance the expression of glycolytic genes in reactive astrocytes (Almeida et al., 2004; Brix et al., 2012). However, further research is required to correlate mitochondrial dysfunction and involvement of other inflammatory signaling pathways such as mitogen-activated protein kinase, c-Jun N-terminal kinases, and extracellular-signal-regulated kinase (Park et al., 2013, 2015) in reactive astrocytes.

Mitochondria are dynamic cellular organelles, and the balance between fusion and fission is firmly connected with their bioenergetics. Accumulating studies have linked mitochondrial dynamics to the balance between energy demand and nutrient supply, suggesting the changes in mitochondrial structure as a mechanism for bioenergetic adaptation to metabolic demands (Liesa and Shirihai, 2013). However, alterations in both mitochondrial dynamics and bioenergetic metabolism have been correlated and considered as prominent events in the pathogenesis of neuroinflammation and neurodegenerative diseases. In the context of inflammatory astrocytes, alterations in both mitochondrial dynamics and bioenergetics have been observed in several studies (Motori et al., 2013). However, further investigation is required to understand the molecular pathways underlying the coordination between mitochondrial dynamics and the energy metabolism in health, which are essential for the characterization of astrocytic inflammatory activation in metabolic and neurodegenerative diseases. In the light of limited knowledge, it has been suggested that the possible regulation of mitochondrial fusion by the energy state is brought about by the changes in the mitochondrial membrane potential-dependent cleavage of OPA1 (Ishihara et al., 2006). Moreover, fission can also be controlled by energy-dependent processes through the cAMP-dependent activation of DRP1 (Duvezin-Caubet et al., 2007). Proteomic analyses have demonstrated that posttranslational modifications of DRP1, OPA1, and MFN2 may interfere with the regulation of mitochondrial dynamics and the subsequent oxidative phosphorylation capacity in response to cellular energy state and nutrient availability (Benard et al., 2007). These findings may help future studies identify the molecular link between mitochondrial bioenergetics and dynamics in astrocyte function in health and diseases.

# CONCLUSIONS AND FUTURE PERSPECTIVE

The role of altered mitochondrial dynamics and bioenergetics in diverse neuropathologies is being excitedly investigated. Alteration of mitochondrial dynamics and bioenergetics in astrocytes has been implicated in neurometabolic and neurodegenerative conditions, including diabetes or obesity, aging, AD, HD, and PD (**Table 1**). Accumulating evidence suggests that dysregulation of mitochondrial fission and fusion events, defective mitophagy, and impaired bioenergetics are largely associated with the inflammatory activation of astrocytes. Consistent with this, altered mitochondrial morphology and function cause cellular stress through the activation of diverse signaling pathways, suggesting that mitochondrial dysfunction is associated with the changes in astrocytic inflammatory phenotypes (**Figure 1**). Although no selective drug or treatment strategies are targeting specifically astrocytic mitochondria at present, current and future studies may increase the feasibility of astrocyte-based therapeutic strategies targeting mitochondrial pathways to prevent and alleviate astrocyte-mediated neuroinflammation and subsequent neuropathologies.

# AUTHOR CONTRIBUTIONS

MR conducted the literature review, formulated, and wrote the manuscript. KS edited the manuscript and was involved in all aspects of manuscript preparation. All authors contributed to the article and approved the submitted version.

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frontiers in Aging Neuroscience

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# Microglial Hyperreactivity Evolved to Immunosuppression in the Hippocampus of a Mouse Model of Accelerated Aging and Alzheimer's Disease Traits

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Neuroinflammation is a risk factor for Alzheimer's disease (AD). We sought to study the glial derangement in AD using diverse experimental models and human brain tissue. Besides classical pro-inflammatory cytokines, we analyzed chitinase 3 like 1 (CHI3L1 or YKL40) and triggering receptor expressed on myeloid cells 2 (TREM2) that are increasingly being associated with astrogliosis and microgliosis in AD, respectively. The SAMP8 mouse model of accelerated aging and AD traits showed elevated pro-inflammatory cytokines and activated microglia phenotype. Furthermore, 6-month-old SAMP8 showed an exacerbated inflammatory response to peripheral lipopolysaccharide in the hippocampus and null responsiveness at the advanced age (for this strain) of 12 months. Gene expression of *TREM2* was increased in the hippocampus of transgenic 5XFAD mice and in the cingulate cortex of autosomal dominant AD patients, and to a lesser extent in aged SAMP8 mice and sporadic early-onset AD patients. However, gene expression of *CHI3L1* was increased in mice but not in human AD brain samples. The results support the relevance of microglia activation in the pathways leading to neurodegeneration and suggest diverse neuroinflammatory responses according to the AD process. Therefore, the SAMP8 mouse model with marked alterations in the dynamics of microglia activation and senescence may provide a complementary approach to transgenic mouse models for the study of the neuroinflammatory mechanisms underlying AD risk and progression.

**Keywords: neuroinflammation, SAMP8 mice, autosomal dominant Alzheimer's disease (ADAD), sporadic early-onset Alzheimer's disease (sEOAD), triggering receptor expressed on myeloid cells 2 (TREM2)**

## INTRODUCTION

Neuroinflammation and peripheral inflammatory conditions associated with aging or some pathological conditions are known risk factors for triggering sporadic Alzheimer's disease (AD) (Bolós et al., 2017; Tejera et al., 2019). Furthermore, lower peripheral inflammation is one of the markers associated with preserved memory at middle age (Corpas et al., 2019). Accordingly, experimental studies have shown neuroprotection by diverse anti-inflammatory agents against memory loss and AD-like pathology (Corpas et al., 2017; Ettcheto et al., 2017). Furthermore, some observational studies have shown the potential of non-steroidal anti-inflammatory drugs (NSAIDs) for prevention of AD (Wang et al., 2015a). However, a number of clinical trials of NSAIDs and other anti-inflammatory drugs reported to date have shown either their lack of efficacy against AD or serious side effects (Elmaleh et al., 2019). The recent failure of the NSAID naproxen to reduce the progression of presymptomatic AD (Meyer et al., 2019) has prompted the redesign of clinical studies (Hershey and Lipton, 2019) and efforts to broaden the pathways of study in the search for new inflammatory targets (Sharman et al., 2019). The relevance of neuroinflammation in the development of AD is further confirmed by the fact that inflammation-related proteins, including the astroglia-based YKL40 [also known as chitinase 3 like 1 (CHI3L1)] and the microglia-based triggering receptor expressed on myeloid cells 2 (TREM2), are currently considered to be potential AD biomarkers in CSF or blood (Molinuevo et al., 2018). The corresponding genes *CHI3L1* and *TREM2* are widely expressed in cerebral tissue, including the hippocampus and cortical areas sensitive to AD pathology (Guerreiro et al., 2013; Sanfilippo et al., 2019).

In experimental studies, mouse models of AD exhibit a diverse degree of neuroinflammation that generally correlates with the amyloid burden. For instance, the 5XFAD transgenic AD mouse model develops abundant amyloid plaque deposits surrounded by activated astrocyte and microglia (Oakley et al., 2006) in parallel with increased brain levels of pro-inflammatory cytokines (Griñán-Ferré et al., 2016b). However, the neuroinflammatory mechanism may differ between AD mouse models and humans (Hemonnot et al., 2019), which may have contributed to the failure of neuroinflammation targeted drugs in clinical trials of AD (Kodamullil et al., 2017). The senescence-accelerated mouse prone 8 (SAMP8) strain is a spontaneous mouse model of accelerated aging that has been proposed as a model of late-onset sporadic AD (Cheng et al., 2014; Liu et al., 2020). It exhibits neuroinflammation, memory loss and mild AD-like pathology (Álvarez-López et al., 2014; Griñán-Ferré et al., 2016a; Ito et al., 2020). At the systemic level, SAMP8 shows increased concentrations of circulating pro-inflammatory factors and increased activation of inflammatory pathways in several organs in comparison with the control strain senescence-resistant mouse 1 (SAMR1) (i.e., Miró et al., 2017; Fernández-García et al., 2019). Furthermore, we have previously demonstrated that cell cultures of SAMP8 embryonic or neonatal brain maintain the pathogenic dysfunctions found in the adult mouse brain (García-Matas et al., 2008; Díez-Vives et al., 2009; Cristòfol et al., 2012). Therefore, SAMP8 may be a good model in which to study the mechanisms underlying the risk of sporadic AD associated with age-related inflammation. Moreover, systemic inflammation may induce or aggravate neuroinflammation. In this regard, peripheral administration of lipopolysaccharide (LPS) from bacterial endotoxin is widely used to induce inflammatory responses in mice for modeling neurodegeneration processes (for a review see: Catorce and Gevorkian, 2016). LPS is the major component of the outer membrane leaflet of Gram-negative bacteria. A structural motif of LPS is recognized by the innate immune cells as one of the pathogen-associated molecular patterns (PAMPs) that stimulate Toll-like receptors (TLRs); LPS mainly stimulates TLR4. The LPS/TLR4 signaling pathway may trigger potent immune responses (Lu et al., 2008). In brief, LPS-activated peritoneal macrophages and dendritic cells of the innate immune system produce Interleukin 1 (IL1α and IL1β), a first-line mediator in the signaling cascade that would help to fight the infection; this includes vagal stimulation to induce sickness behavior, and further synthesis of IL1β in cells lying outside the blood–brain barrier that would reach brain target cells (mainly microglia, but also endothelial cells and macrophage-like cells among others) to induce prostaglandins and pro-inflammatory cytokines (Konsman et al., 2002). A comparable response of transient sickness behavior and microglial activation is also elicited in humans after a single peripheral administration of LPS (Schedlowski et al., 2014; Sandiego et al., 2015), thus giving value to the LPS challenge in experimental models. Besides changes in the classical pro-inflammatory cytokines and nitric oxide pathways, analysis of the proposed AD biomarkers CHI3L1 and TREM2 may help us to understand the mechanisms involved in neuroinflammatory changes.

We aimed to analyze the reactive gliosis of SAMP8 mouse brain and those of astrocytes and microglia *in vitro*, either in basal conditions or after an acute injury with LPS. Using SAMR1 and SAMP8 mice in adulthood and advanced age we aimed to discern age-related changes. Next, we analyzed *CHI3L1* and *TREM2* gene expression in several scenarios, including the transgenic mouse 5XFAD and post-mortem human samples with advanced AD pathology. A study of these genes in the hippocampus of SAMP8 and 5XFAD mice and in the posterior cingulate cortex of human patients with sporadic early-onset AD (sEOAD) and autosomal dominant AD (ADAD) may help reveal the neuroinflammatory mechanisms underlying AD risk and progression.

## MATERIALS AND METHODS

### Animals

The senescence-prone SAMP8 mouse strain and the control senescence-resistant SAMR1 strain were developed by selective breeding at Kyoto University (Takeda et al., 1994). SAMP8 mice show accelerated brain senescence with cognitive impairment, neuroinflammation, oxidative stress, traits of amyloid and tau pathologies, and epigenetic alterations (López-Ramos et al., 2012; Cosín-Tomás et al., 2014; Griñán-Ferré et al., 2018). Male SAMP8 and SAMR1 mice aged 6 and 12 months were used for this study. Six-month-old SAMP8 mice show the full pathological

phenotype, whereas 12-month-old mice are near the mean survival age and have been poorly analyzed (Porquet et al., 2013). Heterozygous transgenic AD mice of the strain 5XFAD (Oakley et al., 2006) and their wild type siblings (WT), 9-month-old males, were used for selected analysis. All mice were bred at the Animal House of the University of Barcelona (UB, Barcelona, Spain). First progenitors of the SAMP8/SAMR1 strains and 5XFAD were obtained from Harlan (Envigo, Barcelona, Spain) and from Jackson Laboratory (Bar Harbor, ME, United States), respectively. Animals were maintained under standard laboratory conditions of food and water *ad libitum*, 22 ± 2°C, and 12 h:12 h light-dark cycle. All experimental protocols and procedures were approved by the local Ethics Committee for Animal Experimentation (CEEA, UB; DAAM 7136 and DAAM 9323), in accordance with the Decree 214/97 of the Generalitat de Catalunya, Spanish legislation and the European Union Directive 2010/63/EU for animal experiments.

## Glial Cell Cultures

Astrocytes and microglia cultured from neonatal SAMP8 neocortex show senescent and pathological traits that might greatly contribute to pathological brain aging in these mice (García-Matas et al., 2008, 2015; Díez-Vives et al., 2009). Here we used mixed glial cultures enriched in astrocytes and almost pure microglia cultures to discern inflammation mechanisms at the cellular level.

Mixed glial cultures enriched in astrocytes were prepared from the cerebral cortices of 2-day-old SAMP8 and SAMR1 mice as previously described (Sola et al., 2011). Briefly, brains were dissected free of the meninges, diced into small cubes and dissociated by incubation with a 0.5% trypsin-EDTA solution (Gibco) for 25 min. Cells were seeded at $5 \times 10^4$ cells/cm$^2$ in multi-well plates or on glass coverslips in DMEM supplemented with 2.5 mM glutamine, 100 μg/mL gentamycin and 20% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% $CO_2$. The culture medium was changed every 3–4 days and FBS was progressively lowered to 10% during the first 2 weeks of culture. Experiments were routinely carried out at 21 days *in vitro*. Established mixed glia cultures of both SAMR1 and SAMP8 consisted of 85–90% astrocytes, 10–15% microglia, and 0.1–1% oligodendroglia.

Microglial cultures were obtained from mature mixed glial cultures prepared as described above, followed by mild trypsinization to detach an upper layer of mixed glia while maintaining a bottom layer of microglia attached to the plate (Saura et al., 2003). Cultures were used immediately. Established microglia cultures of both SAMR1 and SAMP8 consisted of >98% microglial cells.

## Lipopolysaccharide Treatment

*In vivo*, SAMP8 and SAMR1 mice were subjected to a pro-inflammatory injury by administration of LPS from *Escherichia coli* (serotype 026:B6; Sigma, Saint Louis, MO, United States). LPS was dissolved in sterile physiological saline solution to obtain 100 μg in 0.1 mL for intraperitoneal injection. Mice received a dose of 3 mg per kg body weight or the equivalent volume of vehicle. The experiment was terminated after 3 h of treatment with LPS or vehicle. The experimental groups were as follows: SAMR1-Control 6 months ($n$ = 10); SAMR1-Control 12 months ($n$ = 7); SAMP8-Control 6 months ($n$ = 6); SAMP8-Control 12 months ($n$ = 3); SAMR1-LPS 6 months ($n$ = 10); SAMR1-LPS 12 months ($n$ = 7); SAMP8-LPS 6 months ($n$ = 5); and SAMP8-LPS 12 months ($n$ = 4). SAMP8 mice have a reduced lifespan with median and maximum life expectancies of 10 and 16 months, respectively (Porquet et al., 2013). Therefore, older SAMP8 mice were visually inspected for overall health appearance prior to their inclusion in the study.

*In vitro*, pro-inflammatory injury was induced in glial cell cultures by adding LPS at the final concentration of 100 ng/mL in the culture medium. Interferon-γ (IFN; Sigma) at the final concentration of 0.1 ng/mL was simultaneously added to potentiate the effects of LPS (Straccia et al., 2011). Experiments were terminated after 24 h of exposure to LPS + IFN or vehicle (saline). Conditioned culture media and/or cells were immediately collected for further assays. All experiments were performed in cells from at least $n$ = 3 independent primary cultures.

## Sickness Behavior

Decreased motivation to engage in social exploratory behavior is used to assess sickness behavior induced by an infection (Dantzer, 2001). Sickness behavior shows the adaptive reorganization of an animal's priorities. SAMP8 and SAMR1 mice were subjected to the social behavior test immediately before administration of LPS or vehicle and again 3 h later. A juvenile conspecific male mouse was introduced into the test subject's home cage for a 5-min period. The social interaction between the subject and the juvenile intruder was video-recorded and the length of time spent engaged in social investigation was determined from the video records. Social behavior was determined as the amount of time that the experimental subject spent investigating (e.g., anogenital sniffing and trailing) the intruder (Fishkin and Winslow, 1997). The results were expressed as the ratio between the time devoted to social behavior after the treatment, and the respective baseline response.

## Mouse Blood and Brain Tissue Samples

After completion of the behavioral test, 6- and 12-month-old SAMP8 and SAMR1 mice were decapitated to collect trunk blood. Blood was allowed to clot at 4°C for 30 min and centrifuged to obtain serum. The cerebral cortex and hippocampus were immediately dissected on a cold plate. Samples of blood serum and cerebral tissues were stored at −80°C for further analysis. All samples were obtained 3 h after LPS or vehicle injection. Brain tissue of 9-month-old 5XFAD ($n$ = 4) and WT mice ($n$ = 4) was also obtained and stored at −80°C until analysis.

## Human Tissue Samples

Brain tissue samples of sEOAD, ADAD and neurologically healthy controls (NHC) were obtained from the Neurological Tissue Biobank of Hospital Clínic – IDIBAPS and the Neuropathology Institute of the Hospital Universitari de Bellvitge. All subjects or their legal representatives provided

written consent for the use of the brain samples and the study was approved by the Ethics Committee of the Hospital Clínic of Barcelona. All procedures were conducted in accordance with the 1964 Declaration of Helsinki and its later amendments. Postmortem brain tissue was evaluated following standardized pathological procedures and each patient's disease was classified according to international consensus criteria (Hyman et al., 2012; Montine et al., 2012). Following these criteria, AD patients included in the study were classified as A3, B3, and C3. All patients were previously screened for the APOE genotype and for mutations in the PSEN1, PSEN2, and APP genes (Antonell et al., 2013). Eight samples per group were used for this study. Characteristics of the subjects are displayed in **Table 1**. ADAD subjects bore PSEN1 mutations (L286P, V89L, M139T and E120G). The three groups were balanced for sex, postmortem delay and APOE genotype. ADAD and NHC groups were also matched by age, but sEOAD was significantly older than the other two groups [one-way ANOVA, $F(2,21) = 12.92$, $p < 0.001$; sEOAD vs. ADAD $p < 0.01$ and sEOAD vs. NHC, $p < 0.001$].

**TABLE 1 |** Characteristics of the human subjects included in the study.

| Subject | Sex | Age (y) | *APOE* genotype | PMD (h:min) | FAD mutation | Group |
|---|---|---|---|---|---|---|
| E1 | Male | 57 | 4/3 | 4:30 | – | sEOAD |
| E2 | Male | 61 | 3/3 | 19:15 | – | sEOAD |
| E3 | Female | 63 | 3/3 | 9:00 | – | sEOAD |
| E4 | Male | 60 | 3/3 | 5:00 | – | sEOAD |
| E5 | Female | 74 | 3/3 | 9:00 | – | sEOAD |
| E6 | Male | 69 | 3/3 | 3:30 | – | sEOAD |
| E7 | Male | 68 | 3/3 | 9:00 | – | sEOAD |
| E8 | Female | 65 | 3/3 | 16:00 | – | sEOAD |
| | | 65 ± 1.8 | | 9:24 ± 1:50 | | |
| P1 | Female | 56 | 3/3 | 5:00 | *PSEN1 (L286P)* | ADAD |
| P2 | Male | 54 | 3/3 | 7:30 | *PSEN1 (V89L)* | ADAD |
| P3 | Male | 64 | 3/3 | 14:45 | *PSEN1 (M139T)* | ADAD |
| P4 | Male | 57 | 3/2 | 9:30 | *PSEN1 (V89L)* | ADAD |
| P5 | Male | 44 | 3/3 | 5:30 | *PSEN1 (E120G)* | ADAD |
| P6 | Male | 53 | 3/3 | 5:15 | *PSEN1 (M139T)* | ADAD |
| P7 | Female | 48 | 4/3 | 16:25 | *PSEN1 (M139T)* | ADAD |
| P8 | Male | 57 | 3/3 | 15:15 | *PSEN1 (M139T)* | ADAD |
| | | 54 ± 2.0 | | 9:54 ± 1:36 | | |
| C1 | Female | 45 | 3/3 | 14:40 | – | NHC |
| C2 | Male | 46 | 3/2 | 9:35 | – | NHC |
| C3 | Male | 47 | 3/3 | 4:55 | – | NHC |
| C4 | Male | 49 | 3/3 | 7:35 | – | NHC |
| C5 | Male | 53 | 3/3 | 7:25 | – | NHC |
| C6 | Male | 58 | 4/3 | 4:00 | – | NHC |
| C7 | Male | 59 | 3/3 | 6:25 | – | NHC |
| C8 | Male | 50 | 3/3 | 12:00 | – | NHC |
| | | 51 ± 1.8 | | 8:18 ± 1:12 | | |

*For each group, mean ± SEM of age and PMD are shown. ADAD, autosomal dominant Alzheimer's disease; FAD, familial Alzheimer's disease; NHC, neurological healthy control; PMD, postmortem delay; sEOAD, sporadic early-onset Alzheimer's disease.*

## RNA Extraction and qPCR Analysis

Total RNA was isolated from mouse tissue samples using the mirVana$^{TM}$ miRNA Isolation Kit with phenol (Applied Biosystems, Foster City, CA, United States) in accordance with the manufacturer's instructions for obtaining total RNA, including small RNA. Isolation of total RNA from frozen brain tissue of the posterior cingulate area at the thalamus level was performed using an RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). RNA yield, purity, and quality were determined using a NanoDrop$^{TM}$ ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). RNAs with a 260/280 ratio of >1.9 were selected. Random-primed cDNA synthesis was performed using the High-Capacity cDNA Archive kit (Applied Biosystems). Gene expression was measured in a CFX96 Real-Time qPCR Detection System (Bio-Rad, Hercules, CA, United States), using specific TaqMan FAM-labeled probes (Applied Biosystems). The expression of the genes *Il1b*, *Il6* and *Tnf*, coding for the common pro-inflammatory cytokines IL1β, Interleukin 6 (IL6), and tumor necrosis factor α (TNFα), respectively, were analyzed in SAMP8 and SAMR1 cortex and hippocampus. The expression of the mouse genes *Chil1* and *Trem2* that code for CHI3L1 and TREM2, respectively, were analyzed in the hippocampus of SAMP8, SAMR1, 5XFAD, and WT mice. The human counterpart genes *CHI3L1* and *TREM2* were analyzed in human samples of the posterior cingulate area at the thalamus level. Mouse data were normalized to *TBP* gene expression and human data to *PGK1* and *B2M*. mRNA levels were expressed as fold change of the control group (6-month-old SAMR1 treated with vehicle, WT mice or NHC, as appropriate). A list of primers utilized is presented in **Table 2**.

## Morphology of Cultured Glial Cells

The activated phenotype of glial cells was confirmed by microscopic examination of stained cultures. Glial cells grown on glass coverslips were fixed with 4% paraformaldehyde and astrocytes and microglia were specifically stained using standard histological procedures. Astrocytes were immunostained with the primary antibody to Glial fibrillary acidic protein (GFAP) (1:500; Dako Z0334; Agilent, Santa Clara, CA, United States) followed by the fluorescent Alexa Fluor 546 species-specific conjugated secondary antibody (1:1000; Thermo Fisher Scientific, Waltham, MA, United States). Microglia were stained with lectin from *Bandeiraea simplicifolia* conjugated with fluorescein (1:400; Sigma, L2895). Coverslips were mounted upside down on glass slides with Mowiol 4-88 (Sigma 81381) media. Astrocyte cultures from SAMR1 and SAMP8 showed similar basal morphology and similar morphological transformation in response to LPS + IFN. Microglia proliferation and relative number of cells with morphological changes were analyzed by cell count in microphotographs using Cell F software (Olympus, Shinjuku, Tokyo, Japan).

**TABLE 2 |** Genes analyzed and TaqMan FAM-labeled probes for real-time qPCR.

| Function | Symbol | Full name | TaqMan Assay | NCBI RefSeq | Species |
|---|---|---|---|---|---|
| Pro-inflammatory cytokine | *Il1b* | Interleukin 1β | Mm00434228_m1 | NM_008361.3 | Mouse |
| | *Il6* | Interleukin 6 | Mm00446191_m1 | NM_031168.1 | Mouse |
| | *Tnf* | Tumor necrosis factor α | Mm00443258_m1 | NM_001278601.1 NM_013693.3 | Mouse |
| Unknown, astrogliosis | *Chil1* | Chitinase like protein 1 or YKL-40 | Mm00801477_m1 | NM_007695.3 | Mouse |
| | *CHI3L1* | Chitinase 3 like protein 1 or YKL-40 | Hs01072228_m1 | NM_001276.2 | Human |
| Immune response, microgliosis | *Trem2* | Triggering receptor expressed on myeloid cells 2 | Mm00451744_m1 | NM_031254.3 | Mouse |
| | *TREM2* | Triggering receptor expressed on myeloid cells 2 | Hs00219132_m1 | NM_001271821.1 NM_018965.3 | Human |
| Reference gene | *Tbp* | TATA-box binding protein | Mm00446971_m1 | NM_013684.3 | Mouse |
| | *B2M* | β-2-Microglobulin | Hs00187842_m1 | NM_004048.2 XM_005254549.3 | Human |
| | *PGK1* | Phosphoglycerate kinase 1 | Hs00943178_g1 | NM_000291.3 | Human |

## Nitrite Assay

Nitric oxide generation by activated glia in culture was measured by the colorimetric Griess reaction that detects nitrite ($NO_2^-$), a stable reaction product of nitric oxide and molecular oxygen. Briefly, 100 μL of conditioned medium were incubated with 100 μL of Griess reagent for 10 min at room temperature. The optical density was measured at 540 nm using a microplate reader (iEMS Reader MF; Labsystems, Vantaa, Finland). The nitrite μM concentration was determined from a sodium nitrite standard curve.

## Cytokine Determinations

The levels of the pro-inflammatory cytokines IL1β, IL6, and TNFα were determined in homogenates of SAMP8 and SAMR1 cerebral cortical tissue and in conditioned culture media. The protein concentration of tissue homogenates was determined using the Bradford assay. IL1β in the serum of the mice was also determined to identify the peripheral response to LPS. Cytokines were determined using commercial ELISA kits, following the manufacturer's instructions. Mouse IL1β Quantikine ELISA Kit (MLB00C) was purchased from R&D Systems (Minneapolis, MI, United States), Murine IL6 ELISA Set (861.020.005) from Diaclone (Besançon, France) and Mouse TNFα ELISA Ready-SET-Go (88-7324-22) from Thermo Fisher. Samples were measured at 450 nm using a plate reader (iEMS Reader MF; Labsystems, Vantaa, Finland). Data were expressed as pg/ml of blood serum or pg/mg of protein, as appropriate.

## Statistical Analysis

The results are expressed as mean ± SEM. The distribution of the data was checked with the Shapiro–Wilk test, and data were log-transformed into normality where required. Data from the SAMP8 and SAMR1 mice with two factors (strain and treatment in the *in vitro* experiments) or three factors (strain, treatment and age in the *in vivo* experiments) were analyzed by ANOVA to obtain the respective main effects and interaction effects. All ANOVA factors have two levels and therefore no further analysis was performed in the absence of statistically significant interaction. Experimental groups were compared using Fisher's least significant difference (LSD) test when there was an interaction between factors. Student's *t*-test was used for comparison between 5XFAD and WT mice. The human tissue results were analyzed by one-way ANOVA followed by LSD *post hoc* test. Statistical analyses were performed using IBM SPSS Statistics v22.

# RESULTS

## Inflammatory Phenotype of SAMP8 Mice

Lipopolysaccharide induced a reduction in social exploration indicative of sickness behavior as expected, showing the effectiveness of the treatment in 6- and 12-month-old SAMR1 and SAMP8 mice. The results 3 h after the injection are depicted in **Figure 1A**. SAMR1 and SAMP8 mice showed similar responses to LPS injection. Furthermore, both SAMR1 and SAMP8 showed lower social exploration with age [three-way ANOVA, main effect of treatment: $F(1,44) = 55.086$, $p < 0.001$; main effect of age: $F(1,44) = 4.323$, $p = 0.043$].

Protein levels of the first-line pro-inflammatory cytokine IL1β in the blood serum of these animals are shown in **Figure 1B**. There was a significant increase in response to LPS stimulus in all groups, without effect of strain or age [three-way ANOVA, main effect of treatment: $F(1,18) = 18,371$, $p < 0.001$].

Protein and mRNA levels of the pro-inflammatory cytokines IL6, IL1β, and TNFα for these mice 3 h after the injection of LPS or vehicle are shown in **Figure 1C**. Protein levels of IL1β, IL6, and TNFα generally increased in the cerebral cortex of SAMR1 and SAMP8 mice in response to LPS systemic injury as indicated by statistical significance of treatment in ANOVA [three-way ANOVA, main effect of treatment: $F(1,51) = 15.639$, $p < 0.001$ for IL1β; $F(1,42) = 15,475$, $p < 0.001$ for IL6; and $F(1,48) = 5.179$, $p = 0.027$ for TNFα]. However, the results of 6-month SAMR1 protein levels and 12-month SAMP8 mRNA levels showed no tendency to increase those of TNFα after treatment with LPS. Furthermore, TNFα levels generally decreased with age [three-way ANOVA, main effect of age: $F(1,48) = 7.165$, $p = 0.010$ for TNFα]. A visible trend for lower IL1β and IL6 levels in the control treatment in 12-month-old mice compared to 6-month-old mice for both strains did reach significance when vehicle-injected mice were analyzed separately from LPS groups (not indicated in the figure) [two-way ANOVA, main effect of age: $F(1,24) = 7.723$, $p = 0.010$ for IL1β; $F(1,22) = 10.257$, $p = 0.004$ for IL6].

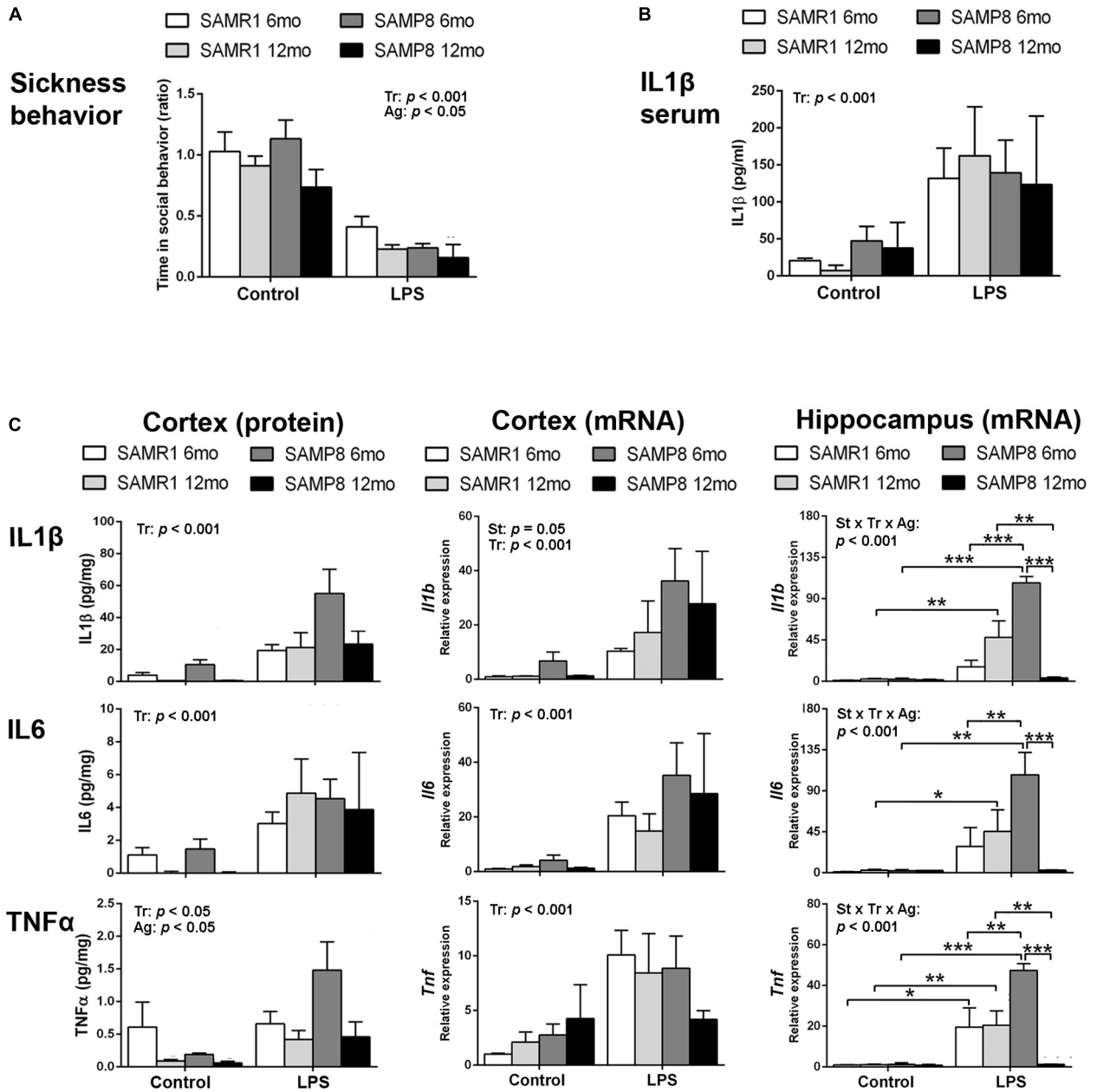


**FIGURE 1 |** Hippocampus of SAMP8 mice revealed an exacerbated acute inflammatory response to lipopolysaccharide (LPS) at young age and null responsiveness at old age. Sickness behavior 3 h after 3 mg/kg i.p. of LPS showed the effect of treatment and a lowering effect of age in the response of both SAMR1 and SAMP8 mice **(A)**. Blood serum levels of Interleukin 1β (IL1β) were increased in all groups of mice treated with LPS for 3 h regardless of strain and age **(B)**. Cerebral cortical levels of protein and mRNA of pro-inflammatory cytokines Interleukin 1β (IL1β/Il1b, protein/gene), Interleukin 6 (IL6/Il6, protein/gene) and Tumor necrosis factor α (TNFα/Tnf, protein/gene) were generally elevated by LPS in all mice, with lower protein levels of TNFα in all aged mice and higher mRNA levels of Il1b in SAMP8 mice; mRNA levels of all cytokines in the hippocampus showed exacerbated levels in 6-month-old SAMP8 compared to SAMR1 mice and low levels of cytokines indicative of null responsiveness to a pro-inflammatory injury in 12 month-old SAMP8 mice 3 h after LPS injection **(C)**. *P*-values for two-way ANOVA analysis are indicated at the top area of the graph: St, strain main effect; Tr, treatment main effect; Ag, age main effect; and St × Tr × Ag, interaction effect. *P*-values for Fisher's LSD *post hoc* tests are indicated as: $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the corresponding SAMR1 group, the corresponding control treatment group, or the corresponding younger mice group, as indicated. *N* = 3–10 mice/group.

LPS induced increased mRNA levels of *Il1b*, *Il6,* and *Tnf* in the cerebral cortex of both strains of mice [three-way ANOVA, main effect of treatment: $F(1,30) = 40.574$, $p < 0.001$ for *Il1b*, $F(1,33) = 28.382$, $p < 0.001$ for *Il6*, and $F(1,30) = 19.163$, $p < 0.001$ for *Tnf*]. Furthermore, SAMP8 showed higher levels of *Il1b* mRNA [main effect of strain: $F(1,30) = 4.159$, $p = 0.050$ for *Il1b*].

In the hippocampus, LPS also induced an increase in the mRNA of the three cytokines [three-way ANOVA, main effect of

treatment: $F(1,23) = 27.187$, $p < 0.001$ for *Il1b*, $F(1,22) = 30.821$, $p < 0.001$ for *Il6*, $F(1,23) = 34.536$, $p < 0.001$ for *Tnf*]. However, the levels attained were very high in the 6-month-old SAMP8 mice compared to SAMR1 and to the corresponding levels in SAMP8 cerebral cortical tissue. Furthermore, 12-month-old SAMP8 mice showed significantly lower mRNA levels of the three cytokines than in 6-month-old SAMP8 mice after LPS injection [three-way ANOVA, main effect of age: $F(1,23) = 4.839$, $p = 0.038$ for *Il1b* and $F(1,23) = 10.082$, $p = 0.004$ for *Tnf*; effect of treatment × strain × age interaction: $F(1,23) = 17.257$, $p < 0.001$ for *Il1b*, $F(1,22) = 4.326$, $p = 0.049$ for *Il6*, $F(1,23) = 10.542$, $p = 0.004$ for *Tnf*].

Overall, SAMP8 mice were similarly responsive than SAMR1 mice against LPS injection at the peripheral level. However, there was a differential response in the brain tissue. Specifically, SAMP8 mice showed a general increase of *Il1b* expression in the cerebral cortex and, most noticeably, an extreme activation of the gene expression of *Il1b*, *Il6* and *Tnf* induced by LPS in the hippocampus at young age followed by null responsiveness to infection-like stimulus at old age. Therefore, there was an exacerbated immune response in young SAMP8 and immunosuppression in aged SAMP8 hippocampi.

## Inflammatory Phenotype of SAMP8 Glial Cultures

Phenotypic images of astrocytes and microglia in mixed glial cultures from SAMR1 and SAMP8 mice are shown in **Figure 2A**. SAMP8 astrocytes were visualized by immunostaining of the GFAP marker in mixed glial cultures highly enriched in

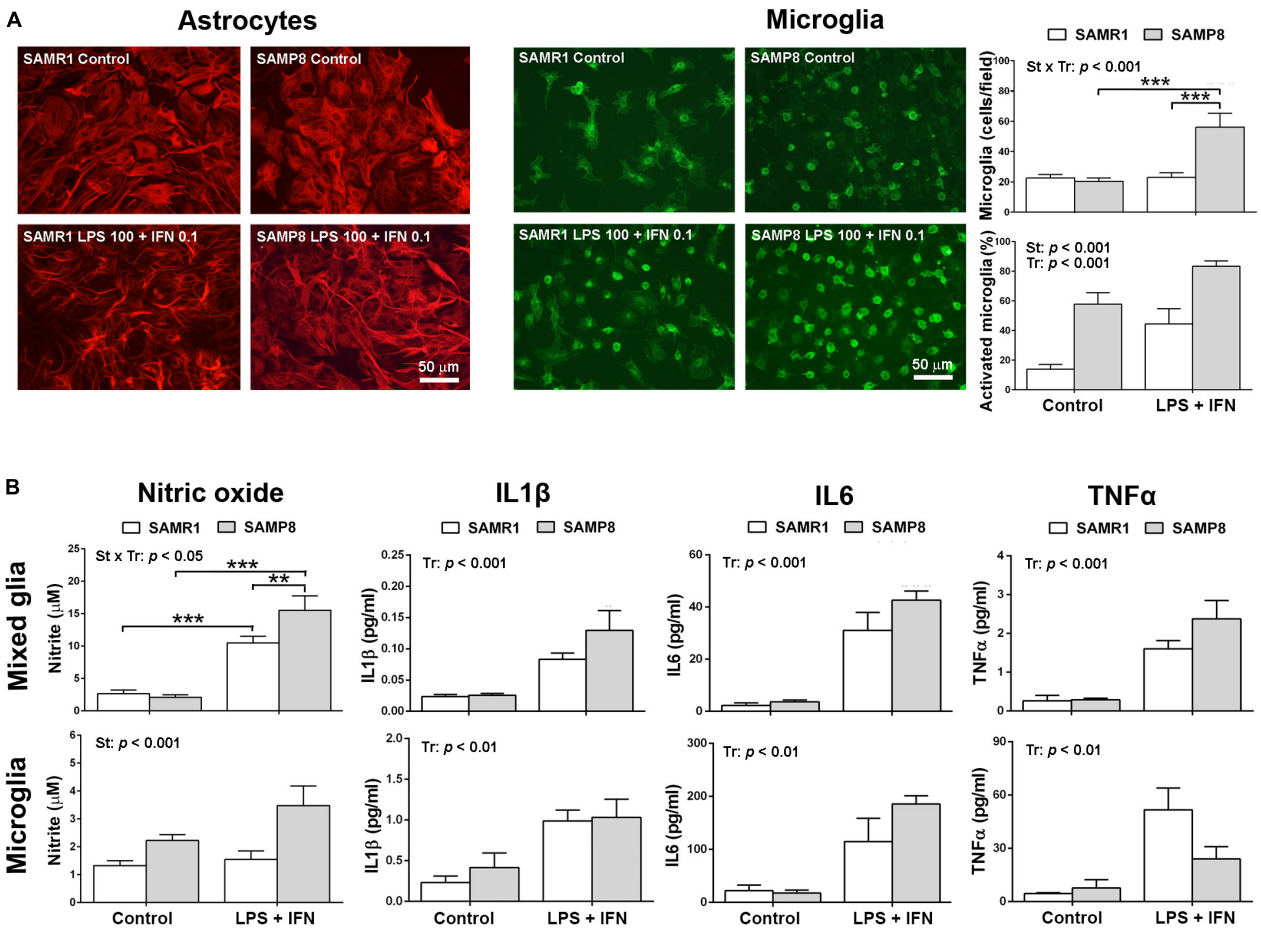


**FIGURE 2 |** Mixed glial cultures of SAMP8 senescent mice had a pro-inflammatory phenotype mainly driven by microglia. Representative images of astrocytes stained with glial fibrillary acidic protein (GFAP) and microglia cultures stained with lectin in mixed glial cultures in control conditions or submitted to a 24-h treatment with lipopolysaccharide (LPS) 100 ng/ml + interferon γ (IFN) 0.1 ng/ml; histograms of the average cell counts of microglia per microscopic field and the percentage of microglia with reactive phenotype shown by globular morphology, as indicated **(A)**. Histograms of the nitric oxide generation, and the levels of pro-inflammatory cytokines Interleukin 1β (IL1β), Interleukin 6 (IL6) and Tumor necrosis factor α (TNFα) released into the culture media of mixed cultures and microglia cultures, in control conditions or treated with LPS 100 ng/ml + IFN 0.1 ng/ml, as indicated **(B)**. *P*-values for two-way ANOVA analysis are indicated at the top area of the graph: St, strain main effect; Tr, treatment main effect; and St × Tr, interaction effect. *P*-values for Fisher's LSD *post hoc* tests between the groups indicated in the graph are as follows: $^{**}p < 0.01$, $^{***}p < 0.001$. $N = 6$–10 (microglia micrographs) from 3 independent cultures per group for analysis of microglia number and phenotype in mixed glial cultures, $N = 18$–24 (mixed glia)/15–26 (microglia) from 4 to 6 independent cultures per group for nitrite determination and $N = 4$–8 (mixed glia)/3–6 (microglia) independent cultures per group for cytokine analysis. Scale bar = 50 µm.

these cells. In control conditions, SAMP8 astrocytes had a similar morphology to SAMR1 astrocytes. Both astrocyte strains acquired a typical activated morphology with filiform processes after pro-inflammatory injury induced by LPS + IFN treatment. However, SAMP8 microglia stained with lectin showed a globular morphology indicative of the activated state in control conditions similar to that of SAMR1 microglia after LPS + IFN injury. Pro-inflammatory treatment also induced proliferation in SAMP8 microglia, but not in SAMR1 microglia, as confirmed by cell count (upper histogram, **Figure 2A**) [two-way ANOVA, main effect of strain: $F(1,29) = 11.42$, $p = 0.0021$, main effect of treatment: $F(1,29) = 15.26$, $p = 0.0005$, and effect of strain × treatment: $F(1,29) = 14.72$, $p = 0.0006$]. Furthermore, the morphological transformation appeared more intense in SAMP8 microglia treated with LPS + IFN than in the SAMR1 counterparts. Cell counts revealed a higher percentage of activated microglia in SAMP8 under both control and pro-inflammatory conditions (lower histogram, **Figure 2A**) [two-way ANOVA, main effect of strain: $F(1,29) = 39.19$, $p < 0.0001$, main effect of treatment: (1, 29) = 17.97, $p = 0.0002$].

Pro-inflammatory physiological changes in SAMR1 and SAMP8 cultures were evaluated by the levels of nitric oxide and cytokine release. The results are shown in **Figure 2B**. Mixed glial cultures increased nitric oxide generation following treatment with LPS + IFN, as determined by nitrite accumulation in the culture media. This response was higher in SAMP8 cultures than SAMR1 cultures [two-way ANOVA, main effect of treatment: $F(1,74) = 89.93$, $p < 0.0001$; main effect of strain: $F(1,74) = 3.992$, $p = 0.0494$; effect of strain × treatment interaction: $F(1,74) = 6.255$, $p = 0.0146$]. SAMP8 pure microglia cultures also showed higher nitric oxide generation than SAMR1 microglia, although the effect of treatment or strain × treatment interaction did not reach significance [two-way ANOVA, main effect of strain: $F(1,75) = 12.61$, $p = 0.0007$]. However, there was a tendency for a difference between nitric oxide levels after LPS + IFN injury in SAMR1 and SAMP8 microglia that was significant by a direct Student's *t*–test comparison (not indicated in the figure) [$t(44) = 2.706$, $p = 0.010$].

As a whole, glial cell cultures obtained from newborn brain of SAMP8 mice showed higher responsiveness to a pro-inflammatory LPS + IFN injury than those of SAMR1 mice. This effect was primarily driven by microglia, as demonstrated by nitric oxide generation in control and stimulated conditions in pure microglia cultures. Furthermore, the morphological analysis confirmed a state of basal activation, as shown by a higher number of cells with globular morphology, and a hyperreactivity to LPS + IFN, as shown by an increase in the total number and in the percentage of activated SAMP8 microglia.

## Alzheimer's Disease Neuroinflammatory Markers

Phenotypic gene expression of *Chil1* and *Trem2* in SAMR1 and SAMP8 hippocampus 3 h after acute LPS injury or control conditions is shown in **Figure 3A**. Both *Chil1* and *Trem2* mRNA increased with age, although the effect was almost null in the vehicle-injected SAMR1 mice [three-way ANOVA, main effect of age: $F(1,25) = 7.602$, $p = 0.011$ for *Chil1*, and $F(1,24) = 7.174$, $p = 0.013$ for *Trem2*]. Meanwhile, LPS treatment induced *Chil1* expression in 12-month-old SAMR1 mice but not in the younger counterparts, whereas LPS-treated SAMP8 mice at both ages showed similar expression than control 12-month-old SAMP8 [three-way ANOVA, effect of treatment × strain × age interaction: $F(1,25) = 4.386$, $p = 0.047$ for *Chil1*]. The variability of the *Trem2* data did not allow obtaining further ANOVA significant results in addition to the main effect of age; however, there was a tendency for a difference between 12-month-old SAMR1 in control conditions and LPS treatment that was significant by a direct Student's *t*–test comparison (not indicated in the figure) [$t(11) = 2.669$, $p = 0.0218$].

The AD mouse model 5XFAD showed greater hippocampus expression of both *Chil1* and *Trem2* than WT mouse siblings, as shown in **Figure 3B** [$t(6) = 7.39$, $p < 0.001$, for *Chil1* and $t(6) = 7.39$, $p < 0.001$ for *Trem2*].

The mRNA levels of the corresponding human genes *CHI3L1* and *TREM2* in posterior cingulate tissue samples are shown in **Figure 3C**. No significant differences were obtained between the NHC and AD groups for *CHI3L1*. However, TREM2 mRNA levels were higher in the AD groups than the NHC group. This increase was of borderline significance for sEOAD but highly significant for ADAD. Furthermore, ADAD showed higher *TREM2* expression than sEOAD [one-way ANOVA, $F(2,21) = 9.839$, $p = 0.001$].

Here we found a differential expression in the respective markers for astrogliosis (*Chil1*/*CHI3L1*) and microgliosis (*Trem2*/*TREM2*) in the several brain tissues analyzed from mouse models and AD patients. SAMP8 hippocampus showed increased expression of *Chil1* at old age, although the increase of *Trem2* did not reach statistical significance compared to SAMR1. sEOAD patients showed also a borderline significance in the increase of *TREM2*. However, ADAD patients and 5XFAD mice showed a distinct increased expression of *Trem2*/*TREM2*. 5XFAD mice also showed high levels of *Chil1* expression.

# DISCUSSION

## SAMP8 Mice Showed Dysregulation of the Neuroimmune Response to an Acute Stimulus in the Hippocampus

The SAMP8 mouse model of accelerated brain aging and sporadic AD traits showed a higher neuroinflammatory profile compared to SAMR1, as expected from previous reports (Tha et al., 2000; Álvarez-López et al., 2014; Griñán-Ferré et al., 2016a; Rancán et al., 2017). However, we found a major dysregulation of the neuroimmune response to an acute stimulus in the SAMP8 hippocampus that ranged from hyperresponsiveness at young age to null responsiveness at older age. It is known that the main pro-inflammatory cytokines IL1β, IL6, and TNFα are transiently overexpressed in various regions of mouse brain after an LPS injury (Layé et al., 1994; Catorce and Gevorkian, 2016). Accordingly, both SAMP8 and SAMR1 mice were responsive to LPS by showing an increase in the protein

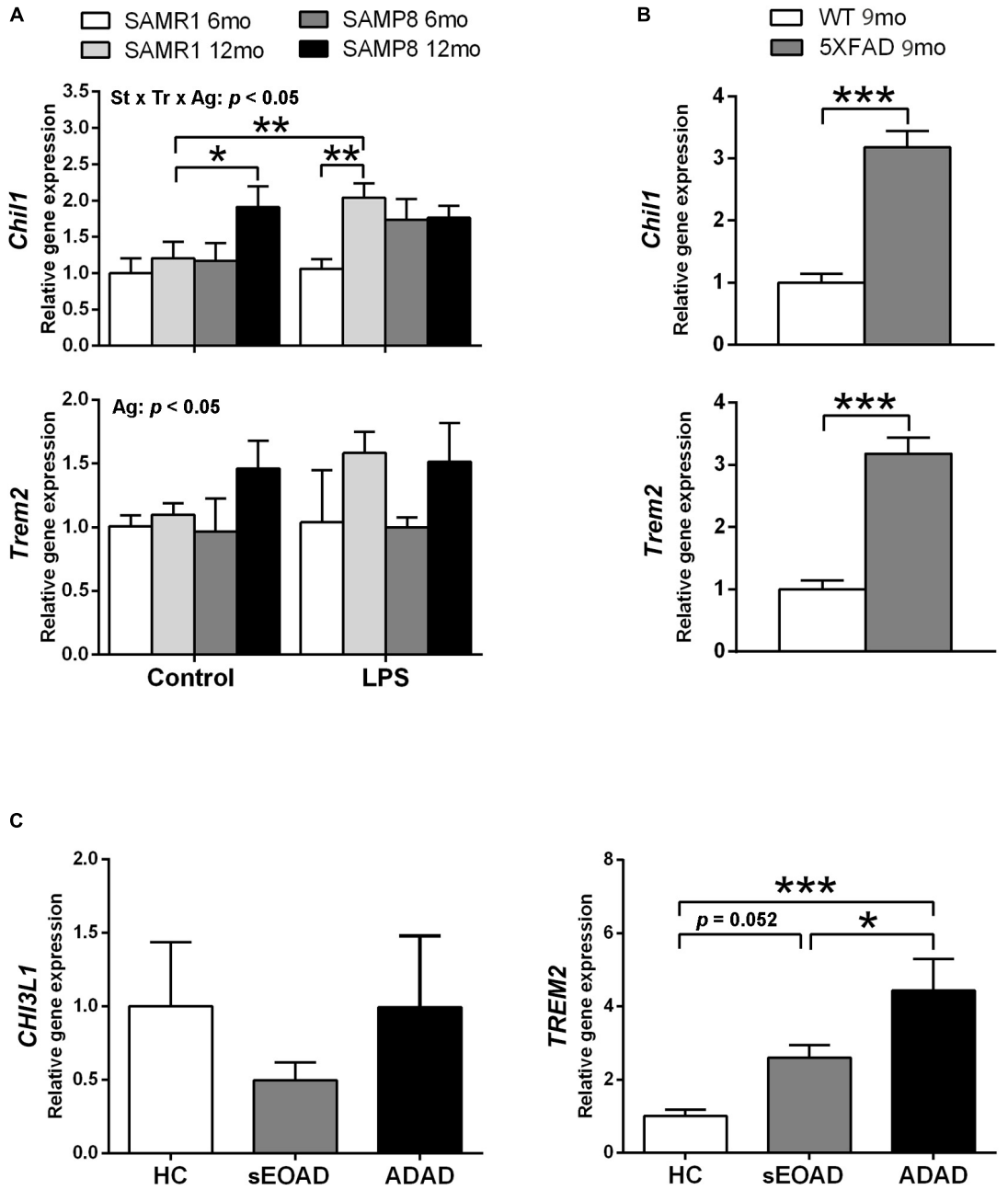


**FIGURE 3 |** Gene expression of Alzheimer's disease (AD)-associated markers YKL40, also known as Chitinase 3-Like 1 (CHI3L1/Chil1, human/mouse gene), and Triggering receptor expressed on myeloid cells (TREM2/Trem2, human/mouse gene) showed differential patterns in the hippocampus of SAMP8 mice in comparison with AD samples. Trem2 expression showed a general increase with age in SAMP8 and SAMR1 mice, whereas Chil1 showed a greater increase with age in SAMP8 under control treatment and in SAMR1 3 h after 3 mg/kg i.p. of lipopolysaccharide (LPS) **(A)**. Both Chil1 and Trem2 genes were highly expressed in the hippocampus of the AD mouse model 5XFAD **(B)**. The respective human homologous gene CHI3L1 showed no changes of expression in the posterior cingulate cortex of early onset AD (sEOAD) or autosomal dominant AD (ADAD) as compared to neurologically healthy controls (NHC), whereas TREM2 showed increased expression in AD **(C)**. *P*-values for three-way ANOVA analysis are indicated at the top area of the graph (Ag, age main effect; and St × Tr × Ag, strain × treatment × age interaction effect) and *p*-values for Fisher's LSD *post hoc* tests between the groups indicated in the graph are indicated as follows: $^{*}p < 0.05$ and $^{**}p < 0.01$, $N = 3$–7, in **(A)**. *P*-values for *t*-test are indicated as: $^{***}p < 0.001$, $N = 4$, in **(B)**. *P*-values for LSD tests after one-way ANOVA are indicated as: $^{*}p < 0.05$ and $^{***}p < 0.001$, $N = 8$, in **(C)**.

and mRNA levels of these cytokines in the cerebral cortex. A similar response to LPS was also found at the peripheral level. Interestingly, the hippocampus analysis showed a much higher inflammatory response in 6-month-old SAMP8 mice than in the SAMR1 mice of the same age. Furthermore, 12-month-old SAMP8 hippocampus showed no responsiveness to

LPS challenge, whereas SAMR1 mice conserved a response of increased cytokine gene expression. Two previous studies performed in young mice reported either no strain differences after a lower dose of 20 μg/kg body weight LPS (Tha et al., 2000) or higher protein levels of the NLRP3 inflammasome in whole brain tissue and IL6 in blood serum of SAMP8 than SAMR1 after 0.33 mg/kg body weight LPS (Ito et al., 2020). Therefore, both young age and a strong LPS challenge may be required to cause an exacerbated generation of pro-inflammatory mediators in SAMP8. Likewise, an insufficient resolution response to age-related inflammation has been reported in SAMP8 hippocampus (Wang et al., 2015b). In fact the hippocampus is highly vulnerable to deterioration by neurotoxic activation of glial cells (Ojo et al., 2015). Accordingly, genome-wide association studies have shown that immune response and related pathways are highly activated in AD brain, suggesting that an increasingly deregulated response with age and infections may result in progressive neurodegeneration and AD (Liu and Yu, 2019). We speculate that chronic neuroinflammation in the SAMP8 hippocampus causes a deregulation of the local neuroimmune response as shown by hyperreactivity to an infectious-like stimulus in young adulthood age and immunosuppression in old age; these processes would contribute to memory loss and neurodegeneration.

## SAMP8 Microglia From Neonatal Mice Showed a Pro-inflammatory State *in vitro*

The *in vitro* analysis showed a higher contribution of microglia than astrocytes in the SAMP8 neuroinflammatory processes. SAMP8 microglia generally showed the amoeboid morphology that is associated with an activated state (Aldana, 2019). However, SAMP8 astrocytes did not show overt reactive phenotype in control conditions, although they have shown some functional deficiencies *in vitro* (García-Matas et al., 2008, 2015). Experiments in cell cultures have shown that both microglia and astrocytes are able to synthesize IL1β, IL6 and TNFα (Lam et al., 2017; Rodgers et al., 2020). Here there was a similar response to LPS + IFN injury in the release of pro-inflammatory cytokines in mixed glial cultures enriched in astrocytes and in microglia cultures. However, we found higher nitric oxide production in LPS-challenged SAMP8 mixed glia and more crucially, in SAMP8 pure microglia regardless of treatment conditions. These effects were paralleled by increased proliferation and morphological changes of SAMP8 microglia. Excessive nitric oxide generated by microglia through sustained expression of inducible nitric oxide synthase (iNOS) is believed to contribute to age-associated neurodegeneration (Yuste et al., 2015). Accordingly, higher nitric oxide content (Wang et al., 2013) and iNOS expression (Griñán-Ferré et al., 2016a) have been reported in SAMP8 brain tissue than in SAMR1. Neuroinflammatory priming has also been reported in microglia isolated from the brains of adult SAMP8 as compared to SAMR1 mice (Ito et al., 2020). Exacerbated inflammatory responses in neurodegenerative disorders are mainly attributed to microglia, in a proposed vicious cycle of sustained microglial activation, neuroinflammation and neurodegeneration (Aldana, 2019; Ahmad et al., 2019). Nevertheless, there is a current consent on the complex dynamics and heterogeneity of microglia activation beyond the initially proposed pro-inflammatory M1 (classical activation) and immunosuppressive M2 (alternative activation) states (Ransohoff, 2016; Song and Suk, 2017; García-Revilla et al., 2019). Microglia polarization can also evolve to alternative activated phenotypes involved in repair functions (Colton et al., 2006; Masuda et al., 2019). Specifically, a subset of microglia, the "disease-associated microglia" (DAM), may be able to sense neuronal damage signals from AD and other conditions and promote phagocytosis, barrier formation and activation of protective pathways (Deczkowska et al., 2018). Furthermore, the presence of microglia with a senescent phenotype in the hippocampus of 12-month-old SAMP8 would be consistent with the senescent dystrophic microglia that have been described in the human aging brain (Streit et al., 2004). Senescent microglia no longer perform their functions of immune surveillance and response or other active neuronal supportive functions including activity-dependent remodeling of synaptic connections (Fields et al., 2014).

## Gene Expression of the Astrogliosis Marker CHI3L1 Was Increased in SAMP8 and 5XFAD Mice but Not in Alzheimer's Disease Brains

In the brain, *CHI3L1* is expressed mainly by astrocytes and it increases in regions of neuroinflammation in AD and tauopathies (Querol-Vilaseca et al., 2017), and also in other neuroinflammatory conditions (Bonneh-Barkay et al., 2010). We found increased expression of *Chil1* gene in the hippocampus of 12-month-old SAMP8 and 9 month-old 5XFAD mice, in agreement with the progression of age-related astrogliosis (Girard et al., 2014; Corpas et al., 2017) and the advanced stage of neurodegeneration in both mouse models. Noticeably, *Chil1* expression increased in 12-month-old hippocampus of control SAMR1 mice after LPS challenge. Therefore, the level of neuroinflammation needed to upregulate *Chil1* may require the interaction of advancing age and systemic infection in these mice. Also, increased expression of *Chil1* has been shown in other AD transgenic mouse models at advancing age (Colton et al., 2006; Xiao et al., 2016). In human brain, increased expression of the *CHI3L1* gene has been reported in postmortem tissue samples of sporadic AD patients in their 70s and 80s (Colton et al., 2006; Llorens et al., 2017) and further potentiated by systemic infection (Rakic et al., 2018). However, we found no significant changes in expression in the mRNA of the younger AD cohorts tested despite its severe pathology. Intriguingly, it may be required an older age and/or the presence of infection for detecting increased expression of *CHI3L1* despite the increased protein levels in the CSF of AD patients (Schindler et al., 2019; Antonell et al., 2020).

The function of CHI3L1 protein remains unknown. *In vitro*, soluble inflammatory mediators released by macrophages or microglia have been reported to induce *CHI3L1* transcription

and morphological changes in astrocytes (Bonneh-Barkay et al., 2012). The results of an *in vivo* study of traumatic brain injury have suggested that CHI3L1 is protective (Wiley et al., 2015), whereas the results from a study with chronic brain infusion of amyloid beta suggested otherwise (Choi et al., 2018). It is possible that expression of *CHI3L1* with advancing neurodegeneration is deleterious, as shown for instance by its inverse correlation with survival in amyotrophic lateral sclerosis (Sanfilippo et al., 2019).

## Gene Expression of the Microgliosis Marker TREM2 Was Highly Increased in Mice and Alzheimer's Brain With Familial Disease Type

Upregulation of the *Trem2* gene in SAMP8 hippocampus was detected in aged mice, consistent with a previously reported age-related increase in whole brain protein levels in these mice (Jiang et al., 2014b). Remarkably, the gene upregulation was driven by aging and was statistically unrelated to peripheral challenge with LPS in both SAMP8 and SAMR1. Furthermore, middle-aged 5XFAD mice with advanced AD-like pathology showed robust upregulation of *Trem2* in agreement with previous results reported in this and other transgenic AD mouse models (Jiang et al., 2014a; Brendel et al., 2017; Hüttenrauch et al., 2018). Our results in human AD brain showed increased *TREM2* mRNA in the posterior cingulate brain area of AD patients, although the increase of the sEOAD group did not reach significance compared to NHC. We cannot rule out a contribution of age factor on the small increase of *TREM2* mRNA levels in sEOAD patients, who were slightly older than NHC and ADAD groups. However, previous authors have also shown a trend to increase the overall *TREM2* expression levels in sporadic AD cases in comparison to controls (Roussos et al., 2015; Del-Aguila et al., 2019). Remarkably, we found a differential increase between sEOAD and ADAD patients, namely, gene-driven AD increased *TREM2* expression levels by almost twice as much as sporadic AD, a similar fold difference as in the transgenic 5XFAD mouse model compared to aged SAMP8 mice. Therefore, the increased expression of *TREM2* in ADAD brain could indicate a differential microglia response depending on the AD etiology. What is more, the differential level of expression of *TREM2* does not seem driven by the degree of pathology, since sEOAD and ADAD had a similar high level of neuropathological changes. Gene expression of *TREM2* has not been previously analyzed in ADAD brain, to our knowledge. However, higher gene expression of *TREM2* in the superior temporal gyrus has been reported in AD carriers of a missense mutation of this gene (Roussos et al., 2015). Also, the analysis of the differential expression of gene transcripts for the three TREM2 isoforms (Zhong and Chen, 2019) by RNA-Seq has shown an association between AD cases and higher levels of the shortest transcript that lacks the transmembrane domain, in the parietal lobe of sporadic AD brain and *TREM2* mutation carriers (Del-Aguila et al., 2019). Unfortunately, our qPCR analysis did not discern the different *TREM2* transcripts.

The transmembrane glycoprotein TREM2 is a crucial component of a receptor-signaling complex that regulates the immune response and phagocytic activity of microglia, macrophages, dendritic cells and osteoclasts (Sudom et al., 2018). Furthermore, it is involved in neuroprotection mediated by the DAM phenotype (Deczkowska et al., 2018). *TREM2* gene variants with loss-of-function effects are a known risk factor for late-onset AD (Sudom et al., 2018). However, higher levels of TREM2 protein have been reported in the middle temporal cortices of late-onset AD patients in their 80s than in age-matched control tissue, and this increase was correlated with that of phosphorylated tau and apoptotic markers (Lue et al., 2015). This is consistent with the proposed deleterious effect of TREM2 at advanced stages of AD when it would aggravate neuroinflammation (Karanfilian et al., 2020). Animal studies have shown that brain levels of soluble TREM2 increase during aging in parallel with amyloidosis and microglia activation (Brendel et al., 2017). At middle age, *Trem2* studies of gene modulation showed a protective effect in transgenic AD mice (Jiang et al., 2014a) and SAMP8 mice (Jiang et al., 2014b). TREM2 is emerging as a critical factor in the regulation of complex microglia activation states (García-Revilla et al., 2019).

## Conclusion

Pathological changes of microglia activation in the postmortem AD brain have been described in the pioneering studies of the McGeer's group in the late 1980s and 1990s (McGeer et al., 1987). Later studies overwhelmingly suggest that reactive microglia play an important role in the neuroinflammatory processes of AD. Age-related microglia changes showing a moderately activated phenotype were subsequently described in the normal human brain (Sheng et al., 1998). There is also current agreement on the AD risk induced by low-grade chronic neuroinflammation in the aging brain.

Here we confirmed the presence of increased expression of *TREM2* in AD microglia and unveiled a differential expression between sporadic and gene-driven AD, despite a similarly severe pathology in human (sEOAD and ADAD) and similarly severe cognitive loss and neuroinflammation in mouse models (SAMP8 and 5XFAD). Unexpectedly, we found no changes in the gene expression of the astrocytic protein CHI3L1 (also known as YKL-40) in the human brain of our cohorts of sEOAD or ADAD, while it is known to increase in sporadic AD at older ages. However, the mouse counterpart Chil1 did increase its gene expression. Furthermore, we found that the SAMP8 mice show early microglia-exacerbated reactivity to an infectious-like stimulus *in vitro* and *in vivo*, followed by immunosuppression at old age in the hippocampus. Therefore, SAMP8 mice open a new scenario in the relationship between the progressive impairment of the innate immune system and the expression of AD-related gliosis markers. However, microglia phenotypic changes and derangement of their innate immune function driven by age-related neuroinflammation and AD pathology are complex and warrant further study using diverse experimental systems, including genetic and non-genetic AD models and human samples.

## DATA AVAILABILITY STATEMENT

The original contributions generated for this study are included in the article results, further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Hospital Clínic of Barcelona, Barcelona, Spain. Written informed consent was not provided because brain samples were obtained from the Neurological Tissue Biobank of Hospital Clínic - IDIBAPS and the Neuropathology Institute of the Hospital Universitari de Bellvitge. Tissue donations follow the legal procedures for their use in research studies. The animal study was reviewed and approved by the Ethics Committee for Animal Experimentation (CEAA) of the University of Barcelona, Spain.

## AUTHOR CONTRIBUTIONS

RCr, PK, CSo, JM, RS-V, AA, AL, and CSa contributed to the conception and design of the study. AL and CSa jointly supervised the study. PM-M, RCo, EG-L, MC-T, RCr, and CSa did the experimental analysis and data processing. RCo and CSa wrote the manuscript draft. All authors read and approved the final manuscript.

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frontiers in Aging Neuroscience

REVIEW
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# Failure of Glial Cell-Line Derived Neurotrophic Factor (GDNF) in Clinical Trials Orchestrated By Reduced NR4A2 (NURR1) Transcription Factor in Parkinson's Disease. A Systematic Review

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Parkinson's disease (PD) is one of the most common neurodegenerative maladies with unforeseen complex pathologies. While this neurodegenerative disorder's neuropathology is reasonably well known, its etiology remains a mystery, making it challenging to aim therapy. Glial cell-line derived neurotrophic factor (GDNF) remains an auspicious therapeutic molecule for treating PD. Neurotrophic factor derived from glial cell lines is effective in rodents and nonhuman primates, but clinical findings have been equivocal. Laborious exertions have been made over the past few decades to improve and assess GDNF in treating PD (clinical studies). Definitive clinical trials have, however, failed to demonstrate a survival advantage. Consequently, there seemed to be a doubt as to whether GDNF has merit in the potential treatment of PD. The purpose of this cutting edge review is to speculate as to why the clinical trials have failed to meet the primary endpoint. We introduce a hypothesis, "Failure of GDNF in clinical trials succumbed by nuclear receptor-related factor 1 (Nurr1) shortfall." We demonstrate how Nurr1 binds to GDNF to induce dopaminergic neuron synthesis. Due to its undisputable neuro-protection aptitude, we display Nurr1 (also called Nr4a2) as a promising therapeutic target for PD.

**Keywords: Parkinson's disease, glial cell line-derived neurotrophic factor (GDNF), nuclear receptor related factor one (Nurr1)-also called Nr4a2, dopamine neurons, transcription factor**

**Abbreviations:** GDNF, Glial cell-line derived neurotrophic factor; Nurr1, Nuclear receptor related factor one; DA, Dopamine; PD, Parkinson's disease; AAV, Adeno-associated virus; TH, Tyrosine hydroxylase; DAT, Dopamine transporter; VMAT, Vesicular monoamine transporter; TSS, Transcription start site; ScRNA, seq-Single-cell RNA sequencing; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PITX3, Pituitary homeobox 3; GFL, GDNF family ligands; CoREST, Co repressor element 1 silencing transcription factor; AADC, Aromatic L-amino acid decarboxylase.

## INTRODUCTION

In recent years, substantial work has been carried out in the area of neurotrophic factors (Ferreira et al., 2018; Skaper, 2018; Stoker and Barker, 2020). The discovery of neurotrophins and the family of glial cell line-derived neurotrophic factor has provided significant insight into the growth, plasticity, neuroprotection, and repair (Bar et al., 1998; Patel and Gill, 2007; Yasuhara et al., 2007; Allen et al., 2013). Glial cell-line derived neurotrophic factor (GDNF) is a potent neurotrophic factor located at p12–p13.1 on chromosome five and comprises two (I and II) promoters and five exons (Baecker et al., 1999). Promoter I is located on exon IV upstream. Promoter II is positioned upstream of exon I and contains two enhancers, two silencers, and numerous binding sites for the transcription factors (Baecker et al., 1999; Airavaara et al., 2011). GDNF is involved in the growth, survival, maintenance of mesencephalic dopamine (DA) neurons as well as regeneration of adult dopamine neurons after damage (Lin et al., 1993; Lapchak, 1996; Lapchak et al., 1997b; Ibáñez and Andressoo, 2017). Just as Isaac Newton once wrote, "If I have seen more, it is by standing on the shoulders of giants;" The milestone that we have achieved so far (in GDNF studies) is due to the outstanding work of the fore legends. In-depth studies on neurodegenerative diseases, especially Parkinson's disease (PD), have been conducted to date. In toxin-induced models of PD, the GDNF is worthwhile, but clinical results have been disappointing. This review article objectively analyses how GDNF began as a hypothesis and extensively examine its trajectory in clinical trials. We also extrapolate the bias of GDNF in neuroprotection and later give our hypothesis as to why this protein failed to meet the primary endpoint in clinical trials. In support of the idea, we stress the need to switch to/or inject nuclear receptor-related factor 1 (Nurr1; agonist or AAV-Nurr1 vector) before starting GDNF therapy.

## GDNF MILESTONE STARTED AS A HYPOTHESIS

In 1981, a theory entitled "A coalescing hypothesis for the origin of amyotrophic lateral sclerosis, Parkinsonism, and Alzheimer" was proposed by Appel (1981). This meant that both of these conditions were due to the absence of a "hormone" or growth factor that would normally be secreted and retrograde transferred by the target tissue of damaged neurons after being picked up by the presynaptic terminal. He continued by explaining that, Neurotrophic failure in PD can be characterized by striatal cells' inability to provide the requisite neurotrophic dopamine hormone with substantia nigra cells subsequently damaged. The hippocampus and cortical cells' failure to supply the relevant cholinergic neurotrophic hormone can cause Alzheimer's disease defects. The central nervous system tissue culture provides a clear system for assessing these neurotrophic hormones that might allow testing the theories.

## GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR TRAJECTORY IN CLINICAL TRIALS

### It All Began in 1993, a Journey Coupled With Hope and Despair

Dr. Leu-Fen Lin and Dr. Frank Collins, both research scientists at a small pharmaceutical firm named Synergen, isolated a protein called neurotrophic factor derived from glial cells in 1991. Two years later, they shared their discovery in this journal (Lin et al., 1993). In the normal functioning of the brain, glial cells are essential. The first observations of the neuroprotective properties of GDNF in animal models of PD started to be documented 1 year after the original Lin, and Collins GDNF study (Hoffer et al., 1994; Beck et al., 1995; Tomac et al., 1995), and the first report of positive effects in nonhuman primate models of PD was published 1 year later, and rigorous studies followed afterward (Bowenkamp et al., 1995; Gash et al., 1995, 1996, 2005; Sauer et al., 1995; Zhang et al., 1997; Rosenblad et al., 2000; Ai et al., 2003). Amgen (a biotechnology company) immediately began setting up clinical trials to determine whether the neuroprotective properties of GDNF would translate into humans, sensing that they were on to a potentially blockbuster cure for Parkinson's. Sadly, since it does not infiltrate through the blood-brain barrier, a protective membrane protecting the brain, GDNF cannot be given orally (Grondin et al., 2003; Tosi et al., 2020). Amgen researchers hoped that, through inserting a tube into the ventricular system and then injecting GDNF, it would penetrate and circulate the brain and eventually reach the dopamine neurons. Findings of this attempt indicated that this was not an ideal therapeutic process. The ventricular route of administration was deficient because the GDNF was unable to penetrate so far into the brain and had a minimal effect as a result. Worse still, since the cerebrospinal fluid provides connections to the spinal cord and other central nervous system areas, various side effects, including hyponatremia, fatigue, vomiting, and paresthesia (prickly sensation in peripheral nerves), have been recorded in the 38 research participants who received GDNF (Nutt et al., 2003). The side effects caused Amgen to end the study.

Putamen was considered significant in searching out to another brain structure for GDNF infusion (Cothros et al., 2019). Dopamine neurons in the brain reside in a region called substantia nigra, but they project their fibers (or axons) to many other locations, including putamen, and this is where much of their dopamine is supplied. Comprehending that putamen is where many of the dopamine neurons' fibers can be located, the investigators in Bristol hoped that by pervading GDNF to the vicinity, they would promote dopamine neurons to generate further fibers. They recruited five people with advanced PD and inserted tiny tubes into the putamen, which would allow the GDNF to be injected into that area. In this study, five participants were treated with GDNF for 1 year. The study results showed that GDNF therapy resulted in a 39% increase in OFF-medication motor abilities (according to the Unified Parkinson's Disease Rating System, UPDRS), a

61% improvement in how participants viewed their capacity to perform everyday activities. A 64% drop in drug-induced dyskinesia (and not detected off-medication), no significant health side effects (none of the problems mentioned in the first study). Notably, the researchers performed brain imaging tests of these subjects and recorded a 28% rise in striatum dopamine storage after 18 months (Gill et al., 2003).

## Two Years Follow-Up Results

As a 2-year follow-up analysis indicated, the same five patients reported a 57% and 63% increase in their OFF-medication motor and everyday life sub-scores (Unified Parkinson's Disease Rating Scale) behaviors after 2 years of therapy, the impact of GDNF treatment tended to have long-lasting effects in these persons (Patel et al., 2005). And a case study of one trial patient was also released by the researchers, indicating that the beneficial effects of GDNF were already affecting 3 years after the treatment had stopped being administered (Patel et al., 2013). A post-mortem examination of one participant's brain showed the regeneration of putamen dopamine fibers (Love et al., 2005). In 2007, "Unilateral intraputamenal glial cell line-derived neurotrophic factor in patients with PD: reaction to 1 year of care and 1 year of withdrawal" was needed for independent replication of the research (Slevin et al., 2007). Ten individuals with Parkinson's were unilaterally implanted with a tube to administer GDNF to the putamen in this phase I research. This means that GDNF was being treated on only one side of the brain. However, after 12 months of therapy, the OFF- and ON-medication states' participants increased by 42% and 38% compared to the overall UPDRS rating. The patients were excluded from the GDNF (the sponsor intentionally halted therapy) after 12 months of treatment but were evaluated for a further 12 months. By 9–12 months after quitting GDNF injection, GDNF therapy's effects were entirely lost (UPDRS ratings had returned to baseline levels). More notably, seven out of 10 participants reportedly developed GDNF antibodies (Slevin et al., 2007).

## Was the Open-Label vs. Double-Blind Considered?

The major concern with the GDNF phase 1 clinical trial was that it was an open-label trial. Patients in the study and the doctors carrying out the study all knew who was receiving the drug. The research was not blind, which opened the door to a significant placebo effect possibility. A double-blind clinical trial for GDNF with 34 participants was launched by Amgen in conjunction with the phase I study. Both the researchers and the volunteers were double-blind and did not know who had GDNF or a control treatment. The technique used a very different pump (compared to the one used in the Bristol study) to inject the GDNF into the brain, and some speculated that this might have led to the result of this study. In June 2004, Amgen declared that its clinical trial investigating the effectiveness of GDNF in the treatment of advanced PD had demonstrated a biological benefit but had not shown any clinical change (compared to placebo treatment) after 6 months of use. This is the press release[1]. The research was stopped by Amgen later that year (in September). Two explanations, they cited: (1) pre-clinical results from nonhuman primates who were treated for 6 months in the highest dose category (followed by a 3-month washout period) revealed a substantial depletion of neurons in the cerebellum (Chebrolu et al., 2006; Luz et al., 2016). (2) In 18 of the 34 participants in the study (four of whom had acquired neutralizing activity) were identified (Tatarewicz et al., 2007). In 2006, the analysis findings were published (Lang et al., 2006a).

The latest Bristol study findings emerged in 2019. In the form of a randomized placebo-controlled, single-center trial sponsored by the UK National Health Service (and funded by Parkinson's UK and The Cure Parkinson's Trust), this new double-blind investigation of directly administered GDNF took place. Notably, the main difference from what had happened before with this trial was the use of a fresh delivery device designed to provide the putamen with excellent coverage. This study's results were published in February 2019 (Whone et al., 2019b), and it revealed that the study did not reach its prescribed primary endpoint. At the end of this double-blind study, all patients were allowed to enroll in an open-label extension study using the same GDNF dose regimen and intermittent infusion parameters as in the initial double-blind study. This open-label extension trial, which also lasted 40 weeks, began before the double-blind parent investigation results were known. The main objective was to obtain longer-term safety data and to collect further exploratory data on the clinical effects of GDNF over a more extended period of repeated tissue exposure (Whone et al., 2019a). All 41 participants in the parent study were enrolled in the extension study, and all were included in the evaluations. Again no significant differences have occurred.

## Was GDNF Gene Therapy a Solution?

In prior clinical trials, GDNF was injected into the brain in the form of protein. The idea came that why should it not be used in the form of a gene; the endpoint would probably have been promising. As most neurotrophic factors are labile agents that do not successfully pass the blood-brain barrier readily, viral vectors have a possible means of transmitting GDNF to degenerating dopaminergic neurons (Piguet et al., 2017; Axelsen and Woldbye, 2018; Niethammer et al., 2018). Previous experiments using adenoviral and adeno-associated viral vectors (AAV) have shown that transmission of the GDNF gene to the nigrostriatal system before a 6-OHDA lesion protects against dopaminergic neuron death in rats (McBride et al., 2003; Mandel et al., 1997; Bensadoun et al., 2000). When all these GDNF clinical trials were going on, other members of the GDNF neurotrophic factor family were being studied in Parkinson's models especially Neurturin in the context of gene therapy. The first phase of the clinical trial was reported[2] (trial registration number NCT00252850 by CERE-120 company)

[1]https://www.amgen.com/media/news-releases/2004/06/amgens-phase-2-study-of-gdnf-for-advanced-parkinsons-disease-fails-to-meet-primary-endpoint-six-months-of-treatment-showed-biological-effect-but-no-clinical-improvement/
[2]https://clinicaltrials.gov/

and the findings showed that the procedure was safe and well-accepted (Marks et al., 2008), so the company launched a Phase II clinical trial (NCT00400634), and the results were released in 2010 (Marks et al., 2010). In this study, 58 patients from nine sites in the USA participated in the trial between December 2006 and November 2008. In patients treated with AAV2-neurturin, there was no substantial change in the primary endpoint [difference −0.31 relative to control individuals (SE 2.63), 95% CI −5.58–4.97; $p$ = 0.91; Marks et al., 2010; Bartus et al., 2013]. In 13 out of 38 patients infected with AAV2-neurturin and four out of 20 control subjects, severe adverse effects occurred. Three patients developed tumors in the AAV2-neurturin group and two in the sham surgery group. In 2015, a longer-term follow-up series of findings was released, with 51 patients involved in the study. No substantial variation was observed in the primary endpoint classes or in the majority of secondary endpoints. Two participants encountered cerebral hemorrhages with intermittent signs. AAV2-neurturin was not linked to any potentially relevant adverse events. Interpretation: the transmission of AAV2-neurturin bilaterally in PD to the putamen and substantia nigra was not superior to sham surgery. The treatment was well accepted, and there were no AAV2-neurturin-related clinically relevant adverse events (Warren Olanow et al., 2015). As a result of this conundrum, substantial reviews and, of course, that enunciate diverse views (Matcham et al., 2007; Kirkeby and Barker, 2019; Paul and Sullivan, 2019; Manfredsson et al., 2020) and editorials (Lang et al., 2006b; Penn et al., 2006) have been unveiled.

## GDNF IS BIASED IN NEUROPROTECTION

### Lentiviral Nigral GDNF Transmission Does Not Inhibit Neurodegeneration in a Parkinson's Disease Familial Rat Model

The evolution of genetic animal models of PD has been made possible by the discovery of mutations in the α-synuclein gene in rare autosomal dominant variants of hereditary PD (Brundin et al., 2017; Koprich et al., 2017). In rats expressing α-synuclein, a selective and progressive loss of nigral dopaminergic neurons associated with dopaminergic striatum denervation has been reported (Ip et al., 2017). The appearance of abundant α-synuclein-positive inclusions and extensive neuritic pathology is correlated with neuronal degeneration, thus causing a progressive and selective loss of dopamine neurons (Giasson et al., 2002). This recapitulates the major characteristics of PD. Lo Bianco et al. (2004) injected Lenti-GDNF in substantia nigra 2 weeks before nigral administration of lenti-A30P. Although robust expression of GDNF was observed in the entire nigrostriatal pathway due to retrograde or anterograde transport, in the lentiviral-based genetic rat model of PD, lenti-GDNF did not prevent dopaminergic neurodegeneration induced by α-synuclein. These findings suggest that GDNF treatment cannot modulate the cellular toxicity associated with abnormal folded protein accumulation (α-synuclein; Lo Bianco et al., 2004; Decressac et al., 2011).

### Nurr1 Mediated Down-Regulation Interferes With GDNF Signaling in Nigral Dopamine Neurons

Nurr1 is strongly expressed in the developing and adult ventral midbrain and is necessary for the acquisition and preservation of the dopaminergic phenotype in nigrostriatal neurons (Zetterstrom et al., 1997; Le et al., 1999b; Jankovic et al., 2005). In the absence of Nurr1, the dopaminergic neuronal markers, tyrosine hydroxylase (TH) and dopamine transporter (DAT), as well as the receptor tyrosine kinase signaling fail to demonstrate the formation of ventral midbrain neurons (Eells et al., 2001; Hermanson et al., 2003; Luo, 2012; Hegarty et al., 2013). In addition to the expression of the DA synthesis and release machinery components, down-regulation of Nurr1, caused by α-synuclein, affected the nigral DA neurons' ability to respond to GDNF *via* Ret expression regulation (Decressac et al., 2012a). Over-expression of Nurr1 in the infected cells essentially reverses the blockade of the GDNF response, and increased expression of Nurr1 can provide proximity defense of nigral DA neurons against α-synuclein toxicity, even in the absence of exogenously administered GDNF (Decressac et al., 2012a).

### Nigrostriatal GDNF Overexpression Induces a Robust Weight Loss in Both Animal Models and Clinical Trials

While GDNF has gained attention as a protein that may cure PD, a progressive neurological condition, owing to its impact on nigrostriatal DA neurons, there is a biologically significant side effect of GDNF that needs to be reported. Bodyweight loss was a regularly reported side effect along these lines while administering exogenous GDNF intracerebroventricularly to rodents, rhesus macaques, or humans (Lapchak et al., 1997a; Nutt et al., 2003; Su et al., 2009; Whone et al., 2019a,b). The leading cause of weight loss is elusive for GDNF-treated patients. Some studies indicate that GDNF administered intranigrally may gain access to ventricular spaces and distribute to hypothalamic nuclei, where neurotransmission changes influence food intake and may eventually contribute to weight loss (Hudson et al., 1995). A similar effect reported by Lapchak, a study which showed that GDNF spreads through the third ventricle and the hypothalamus from the lateral ventricle to the fourth, and this would suggest that GDNF alters the hypothalamic neurotransmission needed for feeding behavior (Lapchak and Hefti, 1992; Lapchak and Araujo, 1994). The weight loss phenomenon in GDNF treated animals has also been explored in obesity cases (Boston, 2004; Tümer et al., 2006; Manfredsson et al., 2009; Mwangi et al., 2014). Recently, Tümer et al. (2006) demonstrated that recombinant adeno-associated virus (rAAV)-mediated hypothalamic overexpression of GDNF induced substantial weight loss in elderly rats and decreased the trajectory of expected weight gain in young rats, indicating that circuits outside the basal ganglia could be involved in the capacity of GDNF to cause weight loss (Tümer et al., 2006). To foster weight loss in mice and nonhuman primates, GDF15 binds to the GDNF family receptor alpha-like (GFRAL), a distant relative of receptors for a particular class of TGF-β

superfamily ligands (Mullican et al., 2017; Saarma and Goldman, 2017). Therefore, the weight loss in PD treated with GDNF may have been due to alteration of the hypothalamic circuitry, resulting in a reduction in food consumption, thus increasing energy expenditure.

## WHY HAS GDNF FAILED IN CLINICAL TRIALS?

### Inadequate Transcription Factor NR4A2 (Nurr1) That Should Bind to GDNF (A New Hypothesis)?

While GDNF has achieved significant results in rodents (Beck et al., 1995; Kozlowski et al., 2000; Rosenblad et al., 2000) and nonhuman primates (Gash et al., 1995, 1996, 2005; Zhang et al., 1997; Ai et al., 2003), it has not given therapeutic benefits in PD patients in clinical trials. Why has it failed? To answer this question, we narrate various hypotheses, henceforth, offer our thoughts. One hypothesis postulates that the GDNF pathway is disrupted due to alpha-synuclein pathology in SNpc neurons of PD patients (Decressac et al., 2011) and that PD patients do not respond to GDNF therapy for this cause. Although this hypothesis is backed by laboratory evidence (Decressac et al., 2012a,b), further experiments are warranted, in particular by studying the brains of PD patients, to show impairments in the GDNF pathway in α-synuclein related cases. Another hypothesis indicates that drug delivery procedures were suboptimal, resulting in restricted dissemination in the brain parenchyma of neurotrophic factors that prevented the likelihood of a therapeutic advantage being observed (Bartus et al., 2015). This hypothesis appears to be confirmed by the study of catheters and infusion protocols used in the GDNF trials, laboratory studies evaluating GDNF delivery protocols in primates (Patel et al., 2005; Salvatore et al., 2006; Bartus et al., 2011), and brains of patients involved in the Neurturin clinical trial (Bartus et al., 2015), but still inconclusive findings obtained in subsequent studies, even after a high dose of GDNF and modification in infusion catheters. Another hypothesis is that nigrostriatal degeneration may have been too advanced for the patients to respond to growth factor therapy in patients selected for clinical trials. Two sets of evidence support this hypothesis. The first is from the Neurturin experiment, in which patients with <5 years of the disease reported better motor recovery (Marks et al., 2010). The second comes from a report by Kordower et al. (2013). Brain study in PD patients found that SNpc cells and putamen dopaminergic terminals appear to be lost during the first 4–7 years of diagnosis. This indicates that the rescue of the nigrostriatal system mediated by GDNF relies on the degree of degeneration (Quintino et al., 2019). To rescue the nigrostriatal system and motor deficits, GDNF had to be present in the striatum such that a significant amount of damaged SNpc neurons remain to respond to therapy.

Another hypothesis is that GDNF failure in clinical trials may have been triggered by an inadequate transcription factor NR4A2 (Nurr1) to bind to GDNF to evoke and defend dopamine neurons. Below, we critically review how the Nurr1 (Nr4a2) transcription factor is essential for GDNF to promote dopaminergic neuron protection.

## NURR 1 AT A GLANCE

NURR1 belongs to the family of transcription factors activated by ligands called nuclear receptors (Zetterstrom et al., 1997; Chu et al., 2002). NURR1 lacks a hydrophobic pocket for ligand binding, unlike most other nuclear receptors, and may therefore act as a nuclear receptor-independent of the ligand (Wang et al., 2003). NURR1 was first seen to be correlated with DA neuron activity by its essential role in the formation of midbrain DA neurons (Jankovic et al., 2005). Nurr1 is expressed early in post-mitotic cells in the ventral midbrain (from embryonic day 10.5 in mice) when they begin to express DA neuron features (Riddle and Pollock, 2003). Heterozygous Nurr1-deficient (Nurr1+/−) mouse DA neurons tend to be more vulnerable to toxic stress, including conditions believed to affect the survival of the DA neuron, such as susceptibility to toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA; Eells et al., 2002; Tan et al., 2003; Pan et al., 2008). Besides, in aged Nurr1+/– mice, progressive nigrostriatal dysfunction, a PD feature, has been observed (Jiang et al., 2005). By regulating transcription of the dopaminergic genes TH, DT, vesicular monoamine transporter (VMAT), and RET receptor tyrosine kinase, Nurr1 overexpression guides *in vitro* differentiation of mesodiencephalic dopaminergic neurons (mdDAs; Skerrett et al., 2014).

### Nurr1 Binds to GDNF to Stimulate the Synthesis of Dopaminergic Neuron Genes

We obtained the genomic structure of the human gene GDNF from Gene Bank's (Ensemble: ENSG00000168621, RefSeq: NM 199231). This promoter is strongly conserved between rats, mice, and humans, and it was earlier identified by Lamberti and Vicini (2014). We were able to classify various GDNF-binding transcription factors using the sequence retrieval tool database[3]. As seen in **Figures 1A,B**, NR4A42 (Nurr1) was tracked from −2,000 bp to +100 bp and found several binding sites. Then http://jaspar.genereg.net/ on GDNF binding, we obtained the NR4A2 (Nurr1) predicted sequence and relative score (**Figure 1C**). This demonstrates that there is a close interaction or crosstalk between GDNF and Nurr1 (NR4A2); thus, GDNF activity may be hampered when NR4A2 is physiologically affected. **Figure 2** illustrates how NR4A2 (Nurr1) binds to the GDNF promoter to evoke dopaminergic neurons synthesis.

### Nurr1 Regulates Tyrosine Receptor (RET) Expression in Dopamine Neurons of Adult Rat Midbrain (A Key Receptor for GDNF)

Tyrosine receptor is a member of the superfamily of receptor tyrosine kinase and the receptor complex signaling component

[3]https://epd.epfl.ch/index.php

A

GGCGCATTAACCCCGCGCCTCCATGCTCCTCCGGTCGGACCGCCAGCCATTTCGCACGCA
CCCGGGAGCTGGCACCGGCGGGGGGCTTGAGGGGGGAAGCTCGCGTTCCCCAGGTCCTAG
CTGCCAAAGTACCTTTCTGGGCTCATTTTGCATGGCCTGGTGCAGTTTTCCTGTGTCTGC
ACATCGCGACCCAGAACCTAGCTTTTTCCCGAGTTTGCAAACCAGCCCGCGAGGCAAGAG
GCGCTCGGTGCTGCAGGTCACTAGGAGTTTCTAGTCCCTACACCCTCTCGAGCCCAACAG
CTGCATAGCGAACAAACAGACGCTCAGGAGATGTAAAATGCATTAGTCTCTGCAGAGGTG
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CTAACCCACCCCCAGCTGCGTGGCCCTCCTTTGTAGGGGTGTGAGGATTGAGAAGCCAAA
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TTAGGTGGGAAGAATGAGGTGTGGGCGGCAGGCTGGGTGAGTGGTGCCCCCGAGCCTGCC
CTCGACTAGCCAGAAGCCCGGTTGGGACCCGAGGCAGGGGAATGCGCTTGATTTTATTTC
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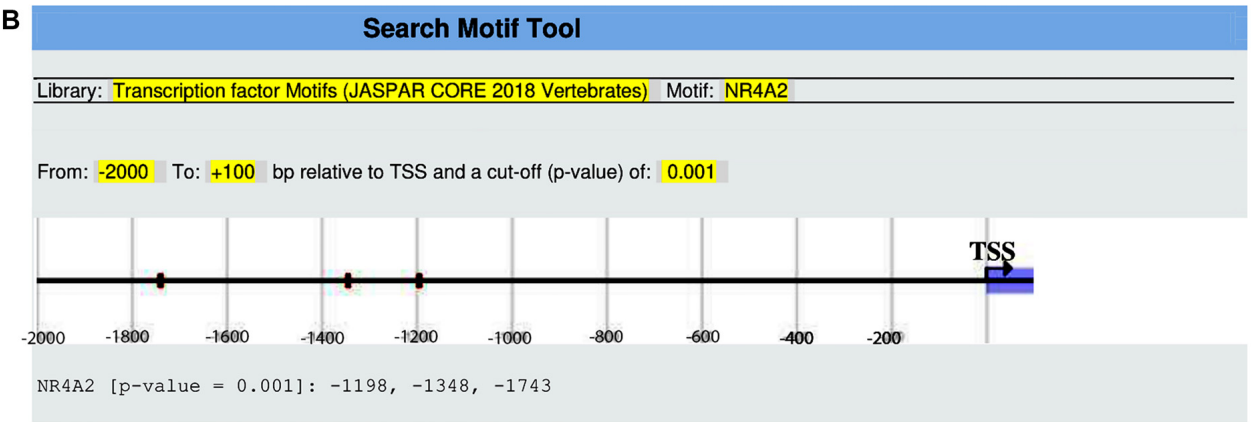
CGGGACAAGAAGGGGAGGTCTCTGGTTGGGGTGGAGGACGAAGGGTGGGAACTACCCGAT
TGCCCCCCAGGAATGGGGGATGTTGCGCACCAGTAGAGGGGACTGGACAGGAATCGTGGT
GGTGGGGGTGGGGGGTTAACTGGAGGGGACAGCAGCCCTGCTTGAAACTCTGACCCCTAA
GACCGTGTGGTGGAGAAGGGCAGCTGCAACCTGAACCAGGAGTGCGAGCTGCTCCTGGGG
CGCGCTGAGGAGGGAGAAGCGAACTGGGGACTTGCAAGGAGGGCAGGAGTGCCCGAGGAG
CCGCTGGCCTGCAGCGGTGCCGGAGGAGGGCGGTGACGAGGTTGGAGAGGGGCGCAGGGA
CCCGCAGGGAGCCCAGGCTTAACGTGCATTCTGCGGTTCTCTCCCCCACCTCCCGCCTGC
CCGCGCAGGTGCCGCCGCCGGACGGGACTTTAAGATGAAGTTATGGGATGTCGTGGCTGT
CTGCCTGGTGCTGCTCCACACCGCGTCCGCCTTCCCGCTGCCCGCCGGTAAGAGGCCTCC
CGAGGCGCCCGCCGAAGACCGCTCCCTCGGCCGCCGCCGCGCGCCCTTCGCGCTGAGCAG

**FIGURE 1** | Continued

**B**




**C**

| Matrix ID | Name | Score | Relative score | Start | End | Strand | Predicted sequence |
|---|---|---|---|---|---|---|---|
| MA0160.1 | NR4A2 | 9.42687 | 0.922285154 | 194 | 201 | + | CAGGTCAC |
| MA0160.1 | NR4A2 | 9.42687 | 0.922285154 | 589 | 596 | + | TAGGTCAC |
| MA0160.1 | NR4A2 | 6.88484 | 0.832967399 | 740 | 747 | − | AGGGCCAC |
| MA0160.1 | NR4A2 | 6.47078 | 0.818418852 | 1636 | 1643 | + | AAGGGCAG |
| MA0160.1 | NR4A2 | 6.44476 | 0.81750445 | 1455 | 1462 | + | GAGGTCTC |
| MA0160.1 | NR4A2 | 6.34173 | 0.813884592 | 1204 | 1211 | + | CAGGCCAA |
| MA0160.1 | NR4A2 | 5.99175 | 0.801587325 | 1610 | 1617 | − | GGGGTCAG |

**FIGURE 1 |** Glial cell-line derived neurotrophic factor (GDNF) promoter from −2,000 bp to +100 bp. **(A)** Red and yellow colors indicate regions with high nuclear receptor-related factor 1 (Nurr1; NR4A2) transcription factor binding affinity. **(B)** Binding regions, base pairs relative to the transcription start site (TSS) at a cut-off (*p*-value) of 0.001 is indicated. **(C)** The predicted sequence and relative score of NR4A2 binding to GDNF retrieved from http://jaspar.genereg.net/.

for glial cell line-derived neurotrophic factor family ligands (Golden et al., 1998; Trupp et al., 1999; Takahashi, 2001). The Ret canonical GDNF receptor, a tyrosine kinase receptor that signals by sarcoma protein (Src)/rat sarcoma (Ras)/mitogen-activated protein kinase (MAPK), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt, nuclear factor-κ beta (NF-κB), is activated by GDNF (Airaksinen and Saarma, 2002; Paratcha and Ledda, 2008; Kramer and Liss, 2015). In a study done by Drinkut et al. (2016), they observed that even significant overexpression of GDNF in the striatum does not have a neuroprotective or regenerative effect in the absence of Ret in dopaminergic neurons. Therefore, the canonical GDNF receptor Ret appears to be mandatory for mediating the beneficial survival and axonal re-sprouting effect of GDNF (Kowsky et al., 2007; Kramer et al., 2007). Galleguillos et al. (2010) unilaterally knocked-down Nurr1 expression in the substantia nigra (SN) of adult rats using adeno-associated vector to confirm whether Nurr1 controls RET expression during adulthood. A 57.3% drop in Nurr1 mRNA in the SN followed by reduced extracellular DA levels in the striatum was seen in Nurr1 knockdown animals. RET mRNA and protein decreased by 76.9% and 47%, respectively, in the injected SN. This proves that NR4A2 (Nurr1) is paramount for GDNF to mediate its neuroprotective effect *via* the tyrosine receptor.

## Single-Cell Transcriptomics Identifies Nr4a2 (Nurr1) as an Enriched Gene in a Model of Parkinson's Disease Treated With Stem Cell-Derived Graft

Single-cell genomics and transcriptomics of single cells have become strong techniques for the genome-wide analysis of single-cell biology. In its transcriptome, epigenome, and local microenvironment, every single-cell in an organism is unique. Because of random fluctuations in the mechanisms driving and regulating transcription and translation, even genetically identical cells exhibit stochastic gene expression (Maleszka et al., 2014; Dey et al., 2015; Pichon et al., 2018). The underlying heterogeneity within cells is a fundamental property of cellular systems for homeostasis and growth (Huang, 2009). Transcriptome spatiotemporal and cell type-specific analyses, the total of all RNA transcripts in a cell or organ, can provide a better understanding of the role of genes in the development and function of the brain and their potential

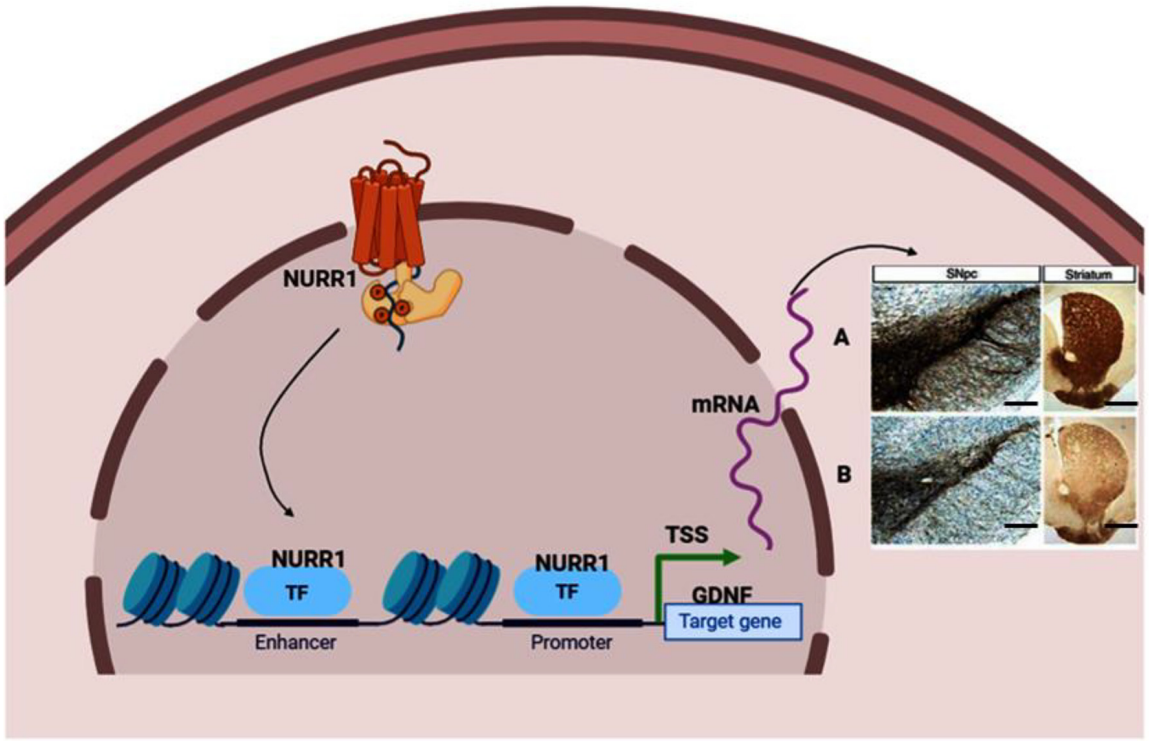



**FIGURE 2 |** Neuroprotective effect stimulated by transcription factor Nurr1 binding to GDNF promoter. **(A)** Nurr1 binds to GDNF promoter to stimulate the synthesis of dopaminergic neurons marked by tyrosine hydroxylase (TH), dopamine transporter (DAT), and vesicular monoamine transporter (VMAT) neurons in the substantia nigra and fibers in the striatum. **(B)** Inadequate Nurr1 leads to reduced dopamine (DA) neurons and fibers in the substantia nigra and striatum, respectively. Panels **(A,B)** indicate Immunohistochemistry stain (IHC) of TH positive neurons (In the substantia nigra compacta) and fibers (In the striatum). Magnification: 100×, Scale bar: 100 μm. TSS means Transcription start site; TF, Transcription factor. This figure was created with web-based software (www.biorender.com).

contribution to brain disorders (Keil et al., 2018; Anaparthy et al., 2019). In many brain regions, Single-cell RNA sequencing (scRNA-seq) has allowed researchers to identify various cell subpopulations, pinpoint gene signatures and novel cell markers (Cuevas-Diaz Duran et al., 2017; Ofengeim et al., 2017). Cell replacement has been a long-standing and realistic objective for the treatment of PD (Kim et al., 2020; Parmar et al., 2020). To uncover the previously unknown cellular diversity in a clinically relevant cell replacement PD model, scRNA-seq was used in the experiment by Tiklová et al. (2020b). Human embryonic stem cells (hESCs) were transplanted into the striatum of adult rats that had 6-hydroxydopamine (6-OHDA) unilaterally lesioned. These stem cells gave rise to neuron-rich grafts with innervation extending from the graft core to the dorsolateral striatum and prefrontal cortex. The grafts also contained the expected component of DA neurons as detected 6 months after transplantation by the expression of TH. Furthermore, in animals transplanted with human embryonic stem cell-derived Ventral Midbrain progenitors, paw use and rotational asymmetry induced by 6-OHDA lesions were corrected, confirming functional maturation after transplantation. Gene enrichment had also confirmed that several highly expressed genes were reported, including Nr4a2. Astrocytes, oligodendrocytes, leptomeningeal vascular cells, and neurons were checked for enrichment (Tiklová et al., 2020a; **Figure 3**). This is the additional evidence that NR4A2 is beneficial and may stand alone as a standard treatment for neurodegenerative disorders, including PD.

## The Lentiviral-Mediated NR4A2 (Nurr1) Genetic Engineering Mesenchymal Stem Cells Protect Dopaminergic Neurons in a Rat Model of Parkinson's Disease

Nuclear receptor-related factor 1 (Nurr1) plays a vital role in the growth and maturation of mesencephalic dopamine (DA) neurons (Arenas, 2005; Jankovic et al., 2005; Decressac et al., 2013). It also plays a defensive role in DA neurons' survival and by inhibiting the activation of microglia and astrocytes (Saijo et al., 2009; Jakaria et al., 2019). Reduced expression of Nurr1 upsurge the susceptibility of MPTP-induced damage of mesencephalic dopamine neurons (Le et al., 1999a). Numerous studies, such as age-based declines in Nurr1 immunoreactivity in human substance nigra (Chu et al., 2002), Nurr1 in PD and related diseases (Chu et al., 2006), decreased NURR1 gene expression in PD3 patients (Le et al., 2008; Liu et al., 2012), decreased NURR1 and Pituitary homeobox 3 (PITX3) gene expression in PD Chinese patients

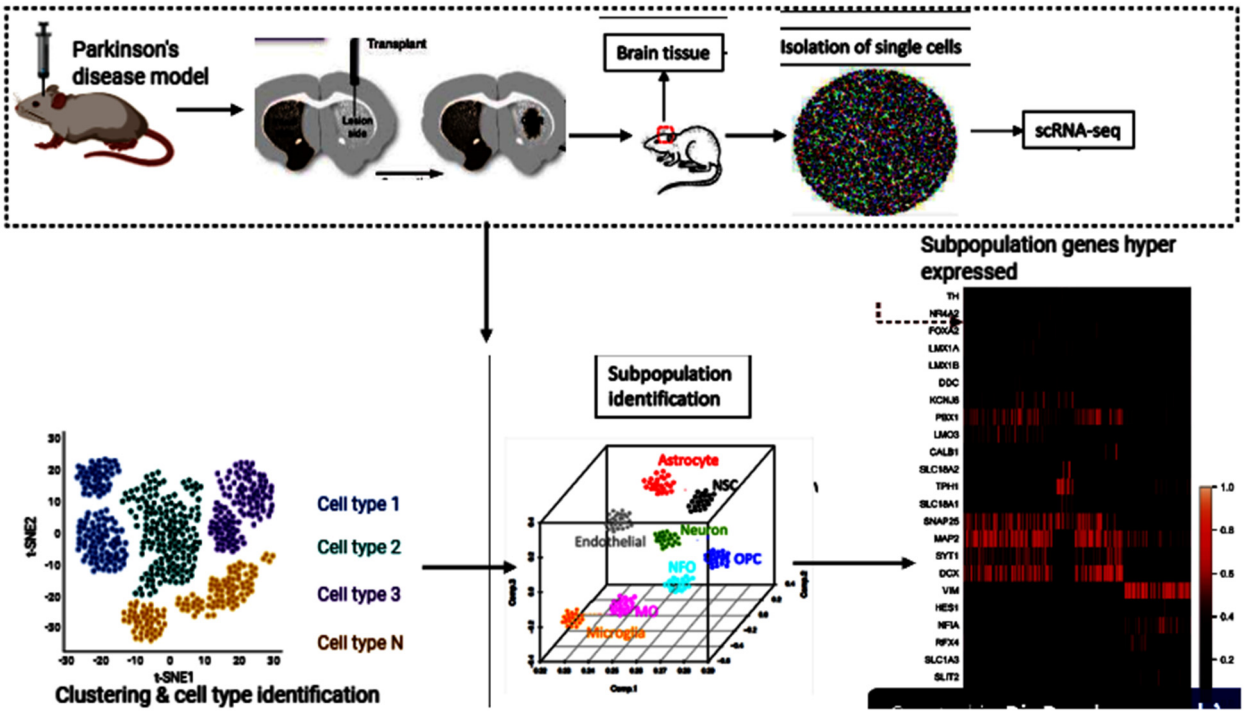



**FIGURE 3 |** Human embryonic stem cells (hESCs) therapy and single-cell RNA sequencing (scRNA-seq) downstream analyses. This figure was created with web-based software (www.biorender.com).

(Liu et al., 2012), decreased Nurr1 mRNA in peripheral blood lymphocytes in PD patients (Montarolo et al., 2016; Li et al., 2018; Yang et al., 2019), are erudite evidence that PD is related to the NR4A2 (Nurr1) shortfall. In the Wang et al.'s (2018) study investigating the therapeutic effects of transplantation of Nurr1 gene-modified mesenchymal bone marrow stem cells (MSCs) into 6-hydroxydopamine (6-OHDA)-induced PD rat models, MSCs was transduced with Nurr1 gene-expressing lentivirus and then transplanted into PD rats intrastriatally. Results revealed that Nurr1 gene-modified MSCs overexpress and *in vitro* secrete Nurr1 protein and also thrive in the brain and migrate. Nurr1 gene-modified MSCs significantly enhanced the pathological activity of PD rats 4 weeks after transplantation and increased the number of TH-positive cells in the substantia nigra (SN) and TH-positive striatum fibers, inhibited glial cell activation and decreased the expression of inflammatory factors in the SN (Wang et al., 2018). Taken together, these results indicate that intrastriatal Nurr1 gene-modified MSCs induced lentiviral vector transplantation has a substantial therapeutic effect for PD rats that could be potentially replicated in humans. **Figure 4** describes the Nurr1-based therapies in PD.

## Nurr1 Regulates Dopamine Synthesis and Storage in MN9D Dopamine Cells

The unraveling of dopamine as a neurotransmitter for the brain, its degradation in patients with PD, has been significant revolutionary events in the development of efficient therapy for patients with this condition (Antony et al., 2013). Another compelling solution to symptomatic PD treatment is to entirely rebuild the dopamine production machinery by introducing genes responsible for dopamine synthesis (Drinkut et al., 2012; Aly and Waszczak, 2015; Tenenbaum and Humbert-Claude, 2017). Nurr1 plays an essential role in dopaminergic neuron growth and transcriptional control of AADC, TH, DAT, and VMAT2 (Jankovic et al., 2005). Castro and colleagues showed in their previous research that Nurr1 causes cell cycle arrest and mature morphology (Castro et al., 2001). Hermanson and fellows have shown in another study that Nurr1 increases DA content and aromatic L-amino acid decarboxylase (AADC) and VMAT2 expression in MN9D cells (Hermanson et al., 2003). In addition, AADC and VMAT2 are deregulated in midbrain DA cells of Nurr1 knockout embryos. These together findings provide evidence of an instructive role for Nurr1 in regulating DA synthesis and storage. Therefore a decrease in the level of Nurr1 may impede the impact of GDNF or other GDNF family ligands (GFL) members.

## Nurr1 Pathway for Neuroprotection

Saijo et al. (2009) provided evidence for an unanticipated pathway by which Nurr1 mediates neuroprotection in their eloquently published article. These authors demonstrate that in microglia and astrocytes, mouse Nurr1 works to inhibit the development of inflammatory mediators that cause dopaminergic neurons to die (Saijo et al., 2009). NR4A2

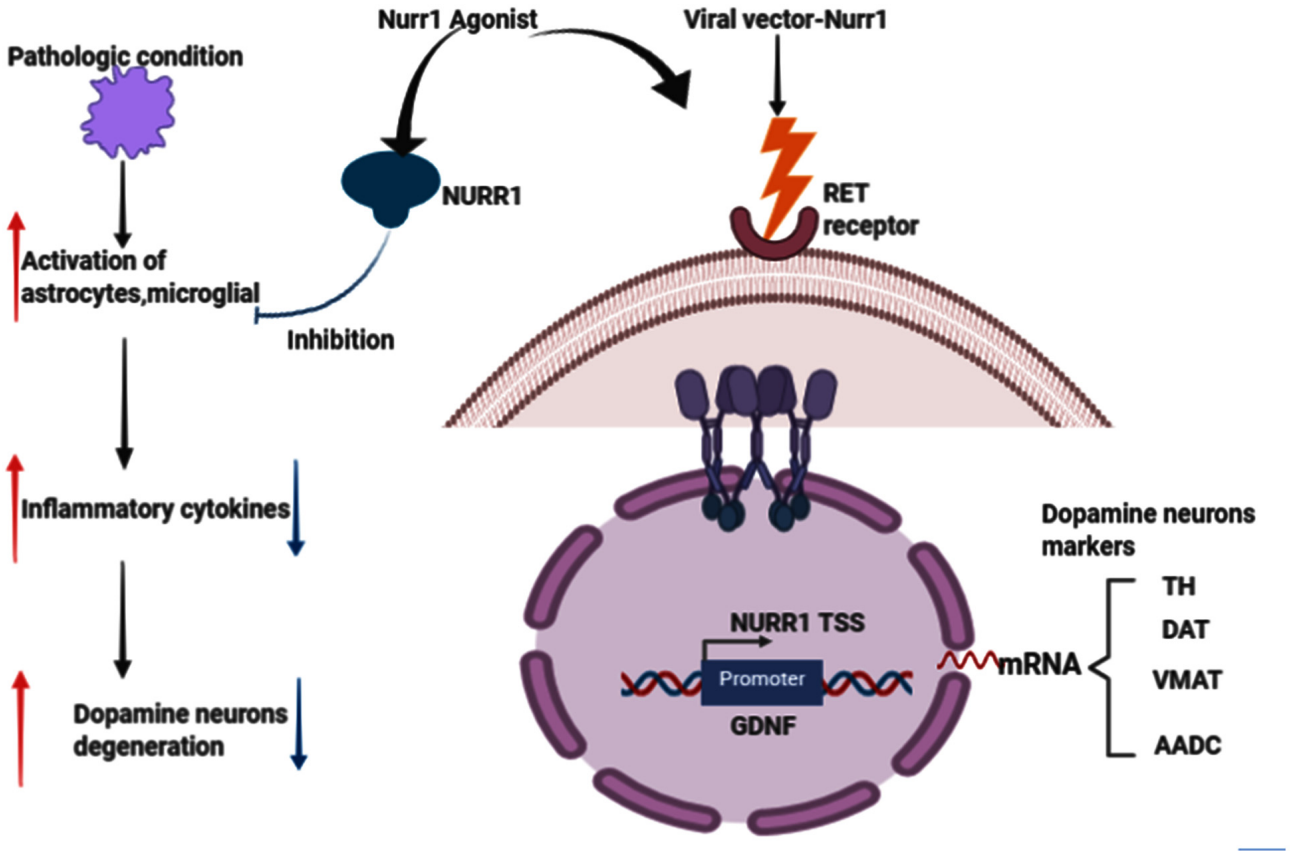



**FIGURE 4 |** Effects of Nurr1-based therapies in Parkinson's disease (PD). Nurr1 agonist or Viral vector delivery promotes the synthesis of several genes involved in neurotransmission and dopamine metabolism, including TH, DAT, VMAT, and Aromatic L-amino acid decarboxylase (AADC). Nurr1 treatment also inhibits astrocytes and microglial activation that would eventually cause dopamine neurons degeneration, thus PD. This figure was created with web-based software (www.biorender.com).

(Nurr1) does not have ligand-binding cavities; instead, it is an immediate-early gene whose expression is caused by several causes, including cyclic AMP, growth factors, hormones, and inflammatory signals. Saijo et al. (2009) discovered a previously unrecognized Nurr1 feature, the suppression of inflammatory gene expression. This suppression defends against the adverse effects of neuroinflammation and indicates new possible pathways that link the role of Nurr1 with PD. By delivering lipopolysaccharide (LPS) to the substantia nigra of mice using stereotaxic injections (a method that uses the coordinate system to target various regions of the brain accurately), the investigators laid the impetus for their discovery. This therapy causes local inflammation and leads to the destruction of tyrosine hydroxylase (TH+) expressing neurons. They proved that Nurr1 expression is caused by inflammation and that local Nurr1 knockdown (by injection of lentiviral vectors expressing short-hairpin RNAs) increases TH+ neuron death. Interestingly, rather than the neurons themselves, the main targets for the neuroprotective effects of Nurr1 tend to be the surrounding microglia and astrocytic cells (Qian et al., 2020). This is implicated in in-vitro studies of microglia and astrocytes in the release of neurotoxic factors causing neuronal death (Polazzi and Monti, 2010). Saijo and colleagues summarize their findings by reiterating that, by docking to NF-κB-p65 in a signal-dependent way on target inflammatory gene promoters, Nurr1 exerts anti-inflammatory effects. Inflammatory signals facilitate the expression of inflammatory genes through the activation of NF-κB signaling and the recruitment to inflammatory promoters of coactivator complexes such as p300/CBP by the NF-κB subunit p65 in conjunction with Co repressor element 1 silencing transcription factor (CoREST) complex (**Figure 5**).

## CONCLUDING REMARKS

GDNF is a potential therapeutic molecule for the treatment of PD. Clinical trials testing GDNF have failed despite an excellent profile in laboratory settings. It is essential to examine why this molecule's translation from the bench to bedside could not reach the primary endpoint. In the development and survival of dopamine-producing nerve cells in the brain, the nuclear receptor-related protein 1 or Nurr1 (also known as Nr4a2) plays an important role. This protein can regulate dopaminergic neurons' synthesis by binding to neurotrophic factors such

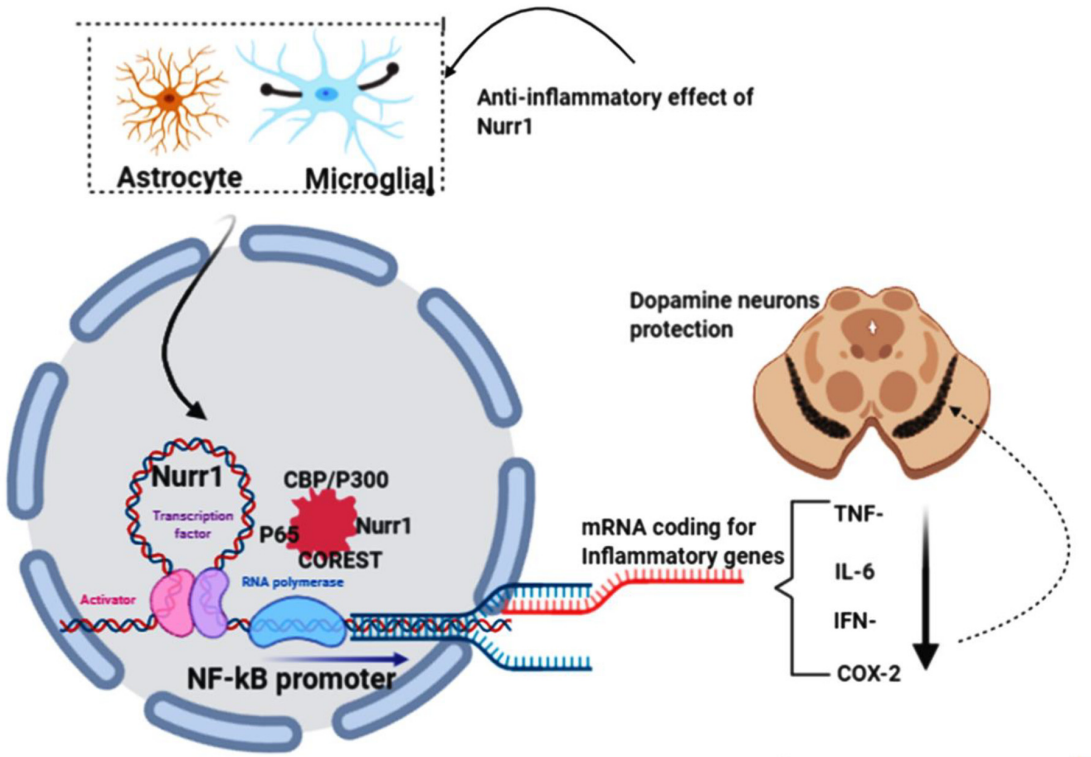



**FIGURE 5 |** NR4A2 (Nurr1) protects dopamine neurons by impeding neuro-inflammations. In pathological conditions, activated astrocytes and microglial induce the transcription of inflammatory genes known to prompt dopamine neurons' death in substantia nigra, thus PD. Nurr1, which is highly expressed in astrocytes and microglial, protects dopaminergic neurons by suppressing the transcription of inflammatory genes by binding to the NF-kB promoter in conjunction with coactivator complexes such as p300/CBP. This figure was created with web-based software (www.biorender.com).

as glial cell line-derived neurotrophic factor. Previous studies have shown that in Parkinson's patients, Nurr1 is involved in the loss of dopaminergic neurons. Pre-clinical data have shown that raising Nurr1 levels can decrease inflammation and increase neuron survival while declining protein contributes to motor symptoms similar to those seen in PD in rodents and nonhuman primates. Recent evidence from *in vitro* and *in vivo* studies has shown that Nurr1-activating compounds and Nurr1 gene therapy can increase DA neurotransmission and protect DA neurons from environmental toxin-induced cell damage or neuroinflammation mediated by microglia. The pharmacological effects of Nurr1-based PD therapies are: (1) to increase the expression of DA-related genes; (2) to protect or restore DA neurons from neurotoxins; and (3) to prevent the activation of microglia and to suppress neuroinflammation. These robust features make Nurr1 an appealing target for PD treatment. Since the GDNF clinical trials have not yet achieved the desired outcome and based on our speculated hypothesis, "Failure of GDNF in clinical trials succumbed by Nr4a2 (Nurr1) shortfall," we suggest that the clinical response to GDNF therapy may improve if the nigral or putamen injection is replaced or combined with either the pharmacological Nurr1 agonists or the AAV–NURR1 vector injection.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

PK conceived the idea and drafted this work while KK, AA, IN, YD, WB, WL and DG participated in writing and critically revised this article. All authors contributed to the article and approved the submitted version.

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frontiers in Aging Neuroscience

REVIEW
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# The Emerging Role of the Interplay Among Astrocytes, Microglia, and Neurons in the Hippocampus in Health and Disease

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For over a century, neurons have been considered the basic functional units of the brain while glia only elements of support. Activation of glia has been long regarded detrimental for survival of neurons but more it appears that this is not the case in all circumstances. In this review, we report and discuss the recent literature on the alterations of astrocytes and microglia during inflammaging, the low-grade, slow, chronic inflammatory response that characterizes normal brain aging, and in acute inflammation. Becoming reactive, astrocytes and microglia undergo transcriptional, functional, and morphological changes that transform them into cells with different properties and functions, such as A1 and A2 astrocytes, and M1 and M2 microglia. This classification of microglia and astrocytes in two different, all-or-none states seems too simplistic, and does not correspond to the diverse variety of phenotypes so far found in the brain. Different interactions occur among the many cell populations of the central nervous system in health and disease conditions. Such interactions give rise to networks of morphological and functional reciprocal reliance and dependency. Alterations affecting one cell population reverberate to the others, favoring or dysregulating their activities. In the last part of this review, we present the modifications of the interplay between neurons and glia in rat models of brain aging and acute inflammation, focusing on the differences between CA1 and CA3 areas of the hippocampus, one of the brain regions most susceptible to different insults. With triple labeling fluorescent immunohistochemistry and confocal microscopy (TIC), it is possible to evaluate and compare quantitatively the morphological and functional alterations of the components of the neuron-astrocyte-microglia triad. In the contiguous and interconnected regions of rat hippocampus, CA1 and CA3 Stratum Radiatum, astrocytes and microglia show a different, finely regulated, and region-specific reactivity, demonstrating that glia responses vary in a significant manner from area to area. It will be of great interest to verify whether these differential reactivities of glia explain the diverse vulnerability of the hippocampal areas to aging or to different damaging insults, and particularly the higher sensitivity of CA1 pyramidal neurons to inflammatory stimuli.

**Keywords: inflammaging, acute inflammation, CA1, CA3, triads, neurodegeneration, confocal microscopy**

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## INTRODUCTION

"Hitherto, gentlemen, in considering the nervous system, I have only spoken of the really nervous parts of it. But if we would study the nervous system, it is extremely important to have a knowledge of that substance which lies between the proper nervous parts, holds them together and gives the whole its form in a greater or lesser degree" quoted from a lecture given by Rudolf Virchow (Charitè Hospital, Berlin, April 3rd, 1858).

For over a century, the brain was considered a network of neurons that communicate to each other in a vacuum filled by glia cells, solely with scaffolding and trophic roles. In the 1990s the scenario changed and astrocytes, microglia as well as oligodendrocytes started to be considered real partners of neurons and major players in the physiological and pathological conditions of the Central Nervous System (CNS). In the last few years, the neuron-centric view of the brain has changed toward a new perspective. Indeed, in the CNS glia subtypes, such as astrocytes, resident microglia, perivascular microglia, and oligodendrocytes outnumber neurons. Much effort is now devoted to understanding the cellular interplay and the molecular mechanisms that underlie the cellular responses that can bring to CNS repair after injury or to neurodegeneration.

The networks of cell intercommunication change during life of an organism, especially during aging or disease, and these modifications can have great consequences in the brain, and especially in the hippocampus. Indeed, it is becoming more and more evident that proper interplay among the main cells of the brain, neurons, astrocytes, and microglia is fundamental for the physiological and functional organization of the central nervous system. Recruitment and activation of astrocytes and microglia in a complex spatial and temporal pattern require well-organized intercommunication between neurons and glia as well as among glial cells.

While for a long time glia activation has been considered detrimental for survival of neurons, more recently intercommunication among astrocytes, microglia and neurons appears fundamental during brain development, for synaptogenesis, for maintenance of healthy synapses and for brain maturation (Pfrieger, 2009; Heneka et al., 2010). These interactions change not only in health and disease, but, in similar disease conditions, they are different in different regions of the brain, and can prevent, modulate or even control, but also exacerbate, the mechanisms of neurodegeneration. Activated astrocytes and microglia undergo a set of transcriptional changes that cause morphological and functional modifications, transforming them into cells with different functions and properties. Understanding in more details how and why glia engage in phenotypic switches and how all this can influence surrounding glia cells and neurons is of the utmost importance.

In this review, we will discuss, in the framework of the published literature, some findings from our laboratory that describe the involvement of the neurons, astrocytes, and microglia triad in rodent models of normal brain aging and acute brain inflammation. We used the method of the triple labeling fluorescent immunohistochemistry coupled to confocal microscopy (TIC) to evaluate and compare the morpho-functional alterations of the components of the neuron-astrocyte-microglia triad, focusing on the differences between CA1 and CA3 areas of the hippocampus in different experimental models of brain pathologies.

This understudied area of research has an emerging importance, and will undoubtedly teach us much about brain functions in physiological and pathological conditions.

## DIFFERENT RESPONSES OF ASTROCYTES AND MICROGLIA IN THE HIPPOCAMPUS DURING AGING OR ACUTE INFLAMMATION

As the expectancy of lifespan of the population in developed countries increases, age-dependent cognitive impairment represents a major challenge both for preclinical and clinical research. Impairment of cognitive functions together with a variety of neurobiological modifications characterizes brain aging. Inflammaging, the low-grade, slow, chronic upregulation of pro-inflammatory responses, represents the progressive neurobiological modification that occurs in the aging brain (Franceschi et al., 2007; Deleidi et al., 2015). Inflammaging hits the majority of the CNS, and particularly the hippocampus, a brain region involved in memory encoding, which during normal aging displays numerous electrophysiological, structural and morphological changes. These inflammaging-induced alterations may be the cause of memory loss typical of advanced age and of some neurodegenerative disorders. The CA1 and CA3 areas of the hippocampus cooperate in the mechanisms of short term memory encoding. The comparison between these two hippocampal areas is of fundamental importance, since they have important, although diverse, roles in memory encoding and since they undergo significant structural and morpho-functional modifications in AD and ischemia (Bartsch and Wulff, 2015). This comparison is also important because it can be the basis of the higher sensitivity of pyramidal neurons in CA1 to neurodegenerative injuries in both experimental animal models and in patients (Mueller et al., 2010; Small et al., 2011; Bartsch et al., 2015).

It is now becoming clear that the lack of microglial and/or astrocyte support seems to be responsible for neuronal degeneration rather than inflammation *per se*. Recent studies

**Abbreviations:** AD, Alzheimer's Disease; AIF, Apoptosis Inducing Factor; ALS, Amyotrophic Lateral Sclerosis; APJs, Astrocytes branches; CNS, Central Nervous System; Cx43, Connexin43; CytC, Cytochrome C; GFAP, Glial Fibrillary Acidic Protein; GM-CSF, Granulocyte Macrophage-Colony Stimulating Factors; IBA1, Ionized calcium Binding Adaptor molecule; IFN, Interferon; IL, Interleukine; JAK, Janus kinase; LPS, Lipopolysaccharide; LRP-1, Lipoprotein receptor-related protein; M-CSF, Macrophage-Colony Stimulating Factors; MAPK, Mitogen Activated Protein Kinase; MHC, Major Histocompatibility Complex; MMP, Matrix metalproteinases; MPJs, Microglia projections; MRI, Magnetic Resonance Imaging; MPM, Multiphoton Microscopy; NF-Kb, Nuclear Factor-kappa B; PAI-1, Plasminogen activator inhibitor type 1; PET, Positron Emission Tomography; SPECT, Positron Emission Computed Tomography; SR, Str. Radiatum; TGF-β, Transforming Growth Factor-beta; TIC, Triple labeling fluorescent Immunohistochemistry coupled to Confocal microscopy; TLR4, Toll-like receptor 4; TNF, Tumor Necrosis Factor.

have demonstrated that at least two types of astrocytes and two types of microglia exist in the brain, with strikingly different properties. A1 and A2 astrocytes, and M1 and M2 microglia. A2 and M2 are non-reactive cells that seem to possess beneficial, neuroprotective properties. On the contrary, it appears that A1 and M1 are activated cells, with harmful properties for neurons. Indeed, A2 astrocytes promote outgrowth and survival of neurons, synaptogenesis, as well as phagocytosis, while A1 reactive astrocytes are neuroinflammatory, upregulate many genes increasing the expression of proinflammatory cytokines and other factors harmful for synapses (Liddelow and Barres, 2017). The M2 state of microglia is non-inflammatory and increases the secretion of a plethora of anti-inflammatory cytokines among which TNF-ß, IL-10, IL-4, and IL-13 (Allen and Barres, 2009). M1 microglia, activated by an acute insult, release proinflammatory cytokines among which TNFα, IL-6, IL-1, and IL-18 (Allen and Barres, 2009).

Nevertheless, according to Liddelow and Barres (2017) this quite recent classification of microglia and astrocytes in two different, all-or-none states appears rather too simplistic, and does not correspond to the variety of different phenotypes found in the CNS (De Biase et al., 2017; Keren-Shaul et al., 2017). A more recent hypothesis postulates that glia, as neurons, exist physiologically as heterogeneous, mixed populations, which may differ from a morphological and functional point of view. Consequently, there may exist *n* numbers of possible activation states of astrocytes and microglia, which are not related merely on the type of insult and the disease progression, but also on the cell type and on the CNS area in which cells are located (Zhang and Barres, 2010; Khakh and Sofroniew, 2015; Ben Haim and Rowitch, 2016; Liddelow and Barres, 2017; Khakh and Deneen, 2019; Pestana et al., 2020).

Even just from a morphological point of view, in the aged rat hippocampus many types of microglia can be identified:

- branched microglia at rest (or quiescent microglia): CNS adult form, characterized by long and branched apophyses and a small cell body;
- non-phagocytic reactive microglia: intermediate stage between the branching and the phagocytic form with pleomorphic bi-or tri-polar cell body, or as spindle or rod-shaped cells
- phagocytic microglia: mainly of amoeboid shape and large dimensions. It is situated in brain areas affected by necrosis or inflammation; it phagocytes foreign materials and exposes immune-molecules for the activation of T-lymphocytes. Interacts also with astrocytes and neurons to quickly re-establish tissue homeostasis (**Figure 1**).

Therefore, activation profile of astrocytes and microglia can be imagined as a continuum rather than an all-or-none phenomenon. It is intriguing to determine whether astrocytes and microglia located in different brain areas react to a similar stimulus/insult with the same functional modifications (Martín-López et al., 2013; Bribian et al., 2018), or whether they react in a different way to a similar insult. In the first case, it is possible to envisage that they are controlled by intrinsic cues, independent from the environment; in the second one, it is possible that different signals derived from the environment diversify the cellular responses (Martín-López et al., 2013; Bribian et al., 2018). Thus, it appears that astrocyte and microglia activation expresses in an apparent continuum of intensities, and these effects are different in different areas of the brain. For instance, it has been demonstrated that astrocytes and microglia reactivities differ not only in aging and acute inflammation, but also between CA1 and CA3 hippocampus (Cerbai et al., 2012; Lana et al., 2016).

Furthermore, data from our and others' laboratories highlight the importance of the astrocyte-microglia dialogue in maintaining the homeostatic conditions that allow to maintain brain health. While adaptive astrogliosis has been shown to have beneficial effects for neurons, suppression of astrocytes activation may increase neuronal vulnerability, exacerbate the progression of pathological conditions and alter tissue regeneration (Sofroniew, 2009; Burda and Sofroniew, 2014; Pekny et al., 2014). Indeed, some data demonstrate that hypertrophy of astrocytes is not only a negative phenomenon, but in some cases may reflect adaptive plasticity of these cells, as demonstrated in aged rodents in which an enriched environment increases the morphological complexity of astrocytes (Rodríguez et al., 2013; Sampedro-Piquero et al., 2014).

The communication dialogue between astrocytes and microglia is a two-way street, mediated *via* their respective secretion molecules (Jha et al., 2019). Astrocytes release substances for the activation and proliferation of microglia, such as GM-CSF (Granulocyte-Macrophage Colony Stimulating Factors) and M-CSF (Macrophage Colony Stimulating Factors) (Watkins et al., 2007). Microglia communicate with astrocytes through the release of growth factors and cytokines, including IL-1, which regulates the proliferation of astrocytes (Streit and Xue, 2013). It is still considered valid that microglia cells are fundamental in establishing and maintaining inflammatory responses that may cause neurodegeneration (Glass et al., 2010). Nevertheless, it is now becoming clearer and clearer that they also actively maintain their protective role during normal aging (Faulkner et al., 2004; Myer et al., 2006; Hanisch and Kettenmann, 2007; Li et al., 2008) by disposing of dying cells (Block et al., 2007).

## Microglia in Acute Inflammation and Aging

Microglia cells have a defined territory and patrol the brain parenchyma by constantly moving their processes (Morsch et al., 2015) to detect and eliminate apoptotic neurons or neuronal debris or by phagocytosis or phagoptosis (Koenigsknecht-Talboo and Landreth, 2005). It has been shown that microglia cells have highly mobile, ramified processes that allow a continuous and dynamic patrolling of the healthy brain tissue. In this way, microglia have an active role in the surveillance of brain parenchyma to eliminate damaged neurons (Davalos et al., 2005; Nimmerjahn et al., 2005; Morsch et al., 2015). Activation of microglia is quick and leads to morphological, immunophenotypic and functional changes that stimulate the migration of microglia to the brain area affected by damage. The activated microglia, after having fulfilled its function of

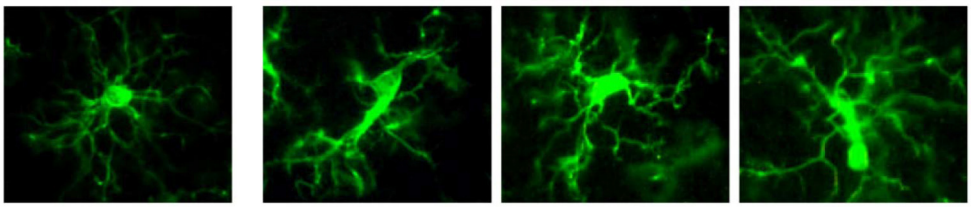


**FIGURE 1 |** Representation of the different stages of microglia activation, marked with the anti IBA1 antibody [from Cerbai et al. (2012)].

phagocytosis, is also able to regress rapidly to the quiescent form (Morsch et al., 2015).

It is becoming clear that activation of microglia is a highly regulated process. For instance, the transcripts of upregulated genes in microglia cells during aging-related chronic low-grade inflammation (inflammaging) differ fundamentally from those that are activated during acute inflammation, such as that induced by LPS infusion in young mice (Holtman et al., 2015). In acute inflammation, upregulated genes highly increase the expression of NF-κB signaling factors, whereas in inflammaging the signaling pathways related to phagosome, lysosome, or antigen presentation are upregulated, contributing to the phenotype of senescent microglia. In inflammaging, microglia display other specific morphological features in the hippocampus of aged rats (Cerbai et al., 2012) and in rodents express proinflammatory markers, such as MHC-II, IL-1β, IL-6, and TNFα, and contain lipofuscin granules [for references see Wolf et al. (2017)]. Aside from the differences in protein expression profile, in inflammaging both total and reactive microglia cells decrease in CA1 rat hippocampus (Cerbai et al., 2012), and their motility decreases in the retina of aged mice (Damani et al., 2011). Indeed, while in young animals microglia cells rapidly stretch their ramifications and increase their motility after administration of ATP, microglia cells during aging are much less mobile (Damani et al., 2011). In addition, microglia scavenging activity is different during aging and acute inflammation (Wolf et al., 2017; Lana et al., 2019) and the phagocytic activity and clearance capacity of microglia decreases with age, inversely correlating with Aβ plaque deposition in a mouse model of AD (Krabbe et al., 2013).

Activation of microglia, long considered only as a negative process that causes accumulation of neurotoxic phagocytes, is now considered a reversible, multi-staged process of generation of diverse types of reactive microglia cells that have protective capacity, at least in rodents (Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009; Kettenmann et al., 2013). Microglia ramifications are sensors that extend chemotactically toward injured cells in the "find-me" step of neuron phagocytosis (Hanisch and Kettenmann, 2007). Therefore, aging may impair and weaken the neuroprotective activity of microglia. Indeed, in rodents decreased migration of microglia may decrease its phagocytic efficacy, and favor the accumulation of degenerating neurons and proinflammatory neuronal toxic debris (Tian et al., 2012) a characteristic typical of CNS aging (Cerbai et al., 2012). Nevertheless, amplified, exaggerated, or chronic microglial activation can cause robust pathological changes and neurobehavioral complications, as in chronic inflammatory diseases (Glass et al., 2010; Norden and Godbout, 2013).

The endotoxin LPS, expressed on the wall of Gram-negative bacteria, behaves as an immunostimulant that activates microglia. The rapid activation of microglia after a damage, such as that caused by inoculation of LPS, is associated with the rapid activation of NF-kB, which does not require *de novo* protein expression. Indeed, the active form of NF-kB translocates from the cytoplasm to the nucleus, thus justifying the quick response of microglia to acute damage signals mediated by factors released by neurons, by pathogens or by immune cells. Inflammatory cytokines such as IL-6 and IL-1, both activators of NF-kB, recruit microglia (Gehrmann et al., 1995) while Interferon-γ and IL-4, released by T cells, stimulate the expression of MHC II on microglia, accelerating its proliferation. Matrix metalproteinases (MMPs), released from apoptotic cells, stimulate microglial activation. On the contrary, TGF-β1 and IL-10 negatively modulate microglia, reducing the expression of MHC II. Activated microglia rapidly express high levels of MHC II and several types of immunoglobulin family receptors, complement receptors, cytokines, chemokines (IFN-γ, IFN-β, IFN-α, IL-1, IL-6, IL-10, and IL-12) and receptors for mannose. Therefore, the cells acquire the ability to recognize and bind various antigens and present them to T lymphocytes (Rock et al., 2004).

Cytoskeleton remodeling of microglia can be monitored using immunostaining with antibodies for IBA1, a microglial marker of $Ca^{2+}$-dependent actin polymerization. During aging, IBA1 immunostaining in microglia cells is significantly lower than in LPS rats, and microglia cells of aged rats are characterized by extremely limited branchings, which on the contrary are very ramified in LPS rats (Lana et al., 2019).

Microglia, with morphological characteristics of phagocytic cells, are significantly more numerous in acute inflammatory conditions than in inflammaging (Cerbai et al., 2012), mirroring a defense reaction of the adult CNS to the robust acute inflammatory stimulus. In acute inflammation in the rat, microglia survey the brain parenchyma, possibly to prevent spreading of proinflammatory, damaging molecules from apoptotic neurons and debris (Cerbai et al., 2012; Lana et al.,

2016). This reactivity may be aimed at restoring normal physiological conditions, thus confirming the important role of microglia in the disposal of injured neurons in acute inflammation in the rat (Lana et al., 2019). The low-grade, chronic age-related processes of inflammaging may cause or be the cause of defective microglia patrolling of the parenchyma, and decreased targeting and phagocytosis of damaged neurons. Therefore, the efficiency of microglia in removal of damaged neurons from the nervous tissue may be lower during aging than in acute inflammatory conditions.

## Astrocytes in Acute Inflammation and Aging

Understanding the multiple, contrasting roles of astrocytes in neuropathological mechanisms is of great interest to unravel the pathogenesis of many neurodegenerative disorders. Astrocytosis (Nichols et al., 1993; Morgan et al., 1997, 1999), defined as significant increase of GFAP expression, is present in the absence of astrocytes proliferation in the hippocampus of aged rats (Cerbai et al., 2012). Increased GFAP, possibly caused by increased transcription of the soluble fraction of GFAP in response to oxidative stress (Sohal and Weindruch, 1996; Morgan et al., 1997, 1999; Wu et al., 2005; Middeldorp and Hol, 2011; Clarke et al., 2018), in rodents is a feature of reactive/activated astrocytes (Zamanian et al., 2012; Burda and Sofroniew, 2014; Liddelow et al., 2017). These findings suggest that astrocytes become more reactive during aging, although not more numerous. Inflammaging causes loss of function in astrocytes, as well as in other cell types, reducing their ability to maintain a physiological, healthy environment (Palmer and Ousman, 2018). Inflammaging can negatively alter the mutual interactions of astrocytes with surrounding cells, being the cause and/or effect of the inflammatory state characteristic of aging. Healthy astrocytes are indispensable for synaptogenesis, for maintenance and maturation of healthy synapses (Pfrieger, 2009; Heneka et al., 2010), and contribute importantly to memory associated processes (Verkhratsky et al., 2011). In line with these housekeeping effects of astrocytes, in aged rats the density of astrocytes is lower in both CA1 and CA3 than in the corresponding areas of young rats (Cerbai et al., 2012; Lana et al., 2016). This finding is confirmed in the frontal and temporal white matter of aged subjects (Chen et al., 2016) and in the hippocampus of aged mice (Long et al., 1998; Mouton et al., 2002) or aged Brown Norway rats (Bhatnagari et al., 1997). Furthermore, the astrocytes in CA1 and CA3 of aged rats (Cerbai et al., 2012; Lana et al., 2016, 2019) show a different morphology and have shorter, highly fragmented branches (**Figure 2A2**, open arrows) compared to young rats astrocytes (**Figure 2A1**).

In healthy conditions the branches of astrocytes form a continuous functional syncytium (Kiyoshi and Zhou, 2019) that extends throughout the CA1 hippocampus (**Figure 2A1**), while during aging the syncytium is interrupted. These morphological modifications closely resemble the phenomenon of clasmatodendrosis, first evidenced by Alzheimer and successively named by Cajal (Penfield, 1928; Perez-Nievas and Serrano-Pozo, 2018; Tachibana et al., 2019). Clasmatodendrotic astrocytes, irreversibly injured by processes that cause energy failure and acidosis (Friede and van Houten, 1961), show morphofunctional modifications such as vacuolization and swelling of the cytoplasm as well as beading and disintegration of the branches (Tomimoto et al., 1997; Hulse et al., 2001).

Clasmatodendrosis, found in ischemic cardiovascular and Binswanger's diseases, characterized by dysfunction of the blood brain barrier (Tomimoto et al., 1997), as well as in Alzheimer's Disease, may represent a response of astrocytes to energy failure and mitochondrial inhibition (Friede and van Houten, 1961; Kraig and Chesler, 1990; Hulse et al., 2001). Indeed, some studies show that aged astrocytes are characterized by metabolic remodeling (Yin et al., 2014), and the oxidative metabolism in astrocytes increases with age, thus limiting their capability to supply neurons with metabolic substrates (Jiang and Cadenas, 2014). Typical hallmarks of brain aging are the impairment of energy metabolism and redox homeostasis, which also contribute to age-related neuronal degeneration and cognitive decline (Biessels and Kappelle, 2005; Boveris and Navarro, 2008). These modifications are even more evident at early stages of neurodegenerative disorders (Yin et al., 2014). Clasmatodendrosis is also caused *in vitro* by mild acidosis (Hulse et al., 2001), a microenvironmental condition present in the aging brain (Ross et al., 2010), in ischemia (Sahlas et al., 2002), and in Aβ-deposition (Su and Chang, 2001). Furthermore, in patients with severe head trauma, clasmatodendrosis is correlated to the presence of contusions or oedemas, and with significant decrease of survival (Sakai et al., 2013). The morphological modifications of astrocytes brought about by clasmatodendrosis have functional consequences. The shrunken arborization of astrocytes branches is indicative of the altered functions that astrocytes acquire during aging (Middeldorp and Hol, 2011), a condition in which astrocytes lose their housekeeping functions of scaffolding and trophic support for neurons and acquire a role in the disposal of neuronal debris (Cerbai et al., 2012; Lana et al., 2016). Clasmatodendrotic astrocytes lose their spatial orientation, thus resulting in reduced astrocytic syncytium and decreased coverage of synapses, and this modification can be a key factor in decreasing synaptic connectivity and neuronal homeostasis (Rodríguez et al., 2009; Verkhratsky et al., 2010). Clasmatodendrotic astrocytes have decreased endfeet coverage of brain vessels (Chen et al., 2016), compromising the blood brain barrier and the neurovascular unit, possibly contributing to vascular modifications observed during aging and at the early stages of AD (Bell and Zlokovic, 2009).

## Interplay of Microglia and Astrocytes in Aging and Acute Inflammation

As reported above, microglia cells survey the brain parenchyma to find and eliminate neuronal debris or apoptotic neurons by phagocytosis (Koenigsknecht-Talboo and Landreth, 2005). Focusing on the possible mutual mechanical interactions of microglia and astrocytes in CA1 of aged rats, evidences show that microglia are irregularly distributed and have reduced cytoplasmic projections (Cerbai et al., 2012; Wong, 2013; Deleidi

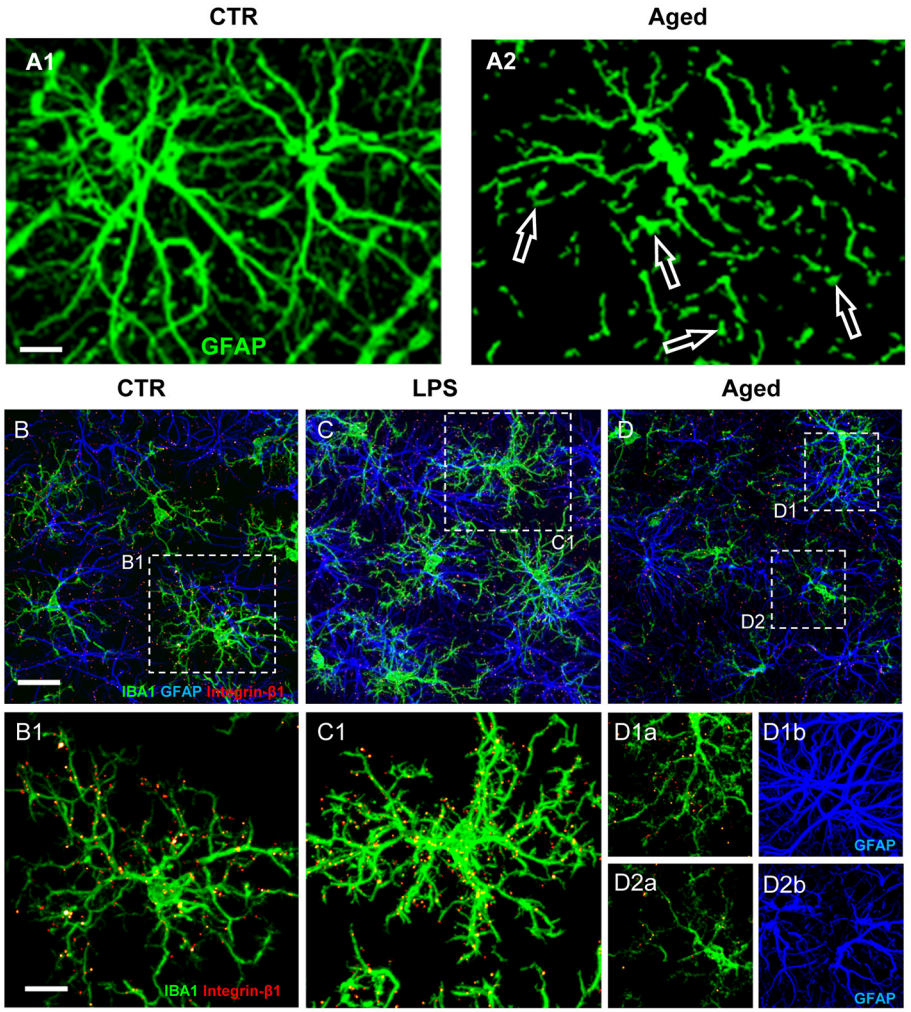


**FIGURE 2 | (A)** Z-projections of confocal stacks acquired in CA1 hippocampus showing GFAP (green) immunostaining in control **(A1)** and aged **(A2)** rats. **(B–D)** Confocal acquisitions of IBA1 (green), GFAP (blue), and integrin-β1 (red) immunostaining in CA1 region of control **(B)**, LPS **(C)**, and aged **(D)** rats. **(B1–D2b)** Immunostaining of Integrin-β1 in microglia of control, LPS-treated and aged rats [magnifications of framed regions in **(B–D)**]; in **(B1,C1,D1b,D2b)** integrin-β1 colocalization on microglial cells is highlighted omitting GFAP immunostaining. Scale bars: **(A1,A2)**: 7.5 μm; **(B–D)**: 20 μm; **(B1–D2b)**: 7.5 μm [Modified from Lana et al., 2019)].

et al., 2015; Lana et al., 2016). Decreased branchings and smaller volume of microglia projections are consistent with previous findings (Wong, 2013; von Bernhardi et al., 2015) and suggest that aged microglia have low efficient cytoskeletal remodeling of their projections and, consequently, decreased migration and phagocytic activity. When microglia cells are in the proximity of intact astrocytes, they display highly branched microglia projections (MPJs), establishing numerous contacts with astrocyte branches (APJs) (**Figures 2B–D**). At contact sites with astrocytes, microglia MPJs accumulate high levels of the mechanosensor Integrin-b1 (Lana et al., 2019) (**Figures 2B1–D2**). Integrin-b1 specifically localized in high density at the contacts MPJ/APJ, possibly influences the dynamic remodeling of MPJs (Lana et al., 2019), as also demonstrated in the spinal cord by Hara et al. (Hara et al., 2017). Mechanical stimuli up-regulate the expression of microglial Integrin-b1 (Milner et al., 2007), a membrane receptor involved in cell mechanosensing (Chen et al., 2017), thus promoting microglia branch extension (Ohsawa et al., 2010). In the aged rat hippocampus, microglia show irregular distribution and MPJs are smaller, shorter and have lower expression of IBA1 than those of young rats microglia (Cerbai et al., 2012; Wong, 2013; Deleidi et al., 2015; Lana et al., 2016). Microglia in the proximity of disrupted astrocyte branches, such as those of clasmatodendrotic astrocytes, show amoeboid morphology, have shorter and enlarged projections, and have low accumulation of Integrin-b1 (Lana et al., 2019). These data suggest that impairment of the direct interaction between astrocytes and microglia may hamper branching and migration of microglia. In acute inflammation, astrocytes form a continuum of APJs

and highly ramified microglia that express increased levels of Integrin-b1, is in close contact with them (**Figures 2C,C1**). Increased expression of Integrin-b1 in microglia may correlate with an efficient microglia-astroglia interaction and ultimately with an efficient microglial targeting and phagocytosis of damaged neurons (Lana et al., 2019).

Nevertheless, it has also been demonstrated that, although microglia actively maintain their protective role during normal aging (Faulkner et al., 2004; Myer et al., 2006; Hanisch and Kettenmann, 2007; Li et al., 2008) by clearing dying neurons (Block et al., 2007), their capability is considerably impaired in an acute proinflammatory context. However, it should be underlined that the model of acute inflammation induced by LPS administration brings about different outcomes and activates different pathways in the diverse animal models used. For instance, in acute inflammatory conditions while activation of inducible NO synthase and expression of TLR4 are higher in mice than in human microglia, siglecs, molecules involved in innate immune responses, are more expresses in human than in mouse microglia (Butovsky et al., 2014). These differences may be responsible for different responses of microglia to noxious stimuli across species (Smith and Dragunow, 2014). In the zebrafish (*Danio rerio*), the scenario is still different since microglia is involved in the high capacity of recovery and regeneration of these animals after a noxious stimulus [for references see Var and Byrd-Jacobs (2020)]. Understanding the diverse microglia behavior in the different species may ultimately help to develop new methods for treating neuroinflammatory processes.

In the aged rat, microglia show impaired mobility and patrolling of brain parenchyma and their decreased phagocytic activity causes increased density of debris in CA1 and CA3 (Cerbai et al., 2012; Lana et al., 2016). Nevertheless, in accordance with previous findings (Koenigsknecht-Talboo and Landreth, 2005), although lesser in number, and less mobile, activated microglia in CA1 still actively scavenge apoptotic neurons, as demonstrated by phagocytosed neuronal debris present in their cytoplasm (Cerbai et al., 2012). On the contrary, under acute inflammatory conditions both microglia and astrocytes secrete Plasminogen activator inhibitor type 1 (PAI-1) that has an important role in microglia migration (Jeon et al., 2012) *via* the low-density lipoprotein receptor-related protein (LRP)-1/Janus kinase (JAK)/STAT1 axis and phagocytic activity *via* vitronectin and Toll-like receptor 2/6 (Jha et al., 2019). These data indicate that astrocytes play a regulatory role in migration and phagocytosis of microglia in an autocrine or paracrine manner.

In the mouse, microglia in normal aging up-regulates immune response signaling receptors (Grabert et al., 2016), neuroprotective signaling pathways (Hickman et al., 2013) and proinflammatory TNFα, IL-1α, and C1q (Liddelow et al., 2017), raising the question of whether these aging-induced modifications could promote the activation of astrocytes. The cytokines TNFα, IL-1α, and C1q are necessary and sufficient to induce the formation of A1 astrocytes, which, releasing a potent neurotoxin, are strongly neurotoxic and rapidly kill neurons (Liddelow et al., 2017). A1 astrocytes are less able to promote the formation of new synapses, causing a decrease in the excitatory function of CNS neurons. Other transcriptional and functional changes may occur in A1 astrocytes that contribute to cognitive decline in normal aging.

**TABLE 1 |** Astrocytes and microglia responses to the same insult are not uniform, but in different stress conditions vary significantly from region to region.

| | | CA1–SR | CA3–SR |
|---|---|---|---|
| GFAP | Adult | 522 ± 8 | 528 ± 27 |
| | Aged | **−20%** | **−17%** |
| | LPS | +5 ns | **+21%** |
| IBA1 | Adult | 127 ± 12 | 239 ± 9 |
| | Aged | **−26%** | **+16%** |
| | LPS | **+70%** | **+56%** |
| OX6 | Adult | 0.3 ± 0.2 | 4.5 ± 0.6 |
| | Aged | **+3,060%** | **+1,160%** |
| | LPS | **+1,900%** | **+570%** |

*Density of astrocytes (GFAP-positive), total microglia (IBA1-positive) and activated microglia (OX-6 positive) in SR of CA1 and CA3 of adult, aged and LPS-treated rats. Significant differences, expressed as percent of adult rats, are shown in bold.* ***CA1–SR:*** *from Cerbai et al., 2012;* ***CA3–SR****: from Lana et al., 2016.*

## COMPARISON BETWEEN CA1 AND CA3

Neuroinflammation is generally believed to be characterized by activation of astroglia, typified by morphological changes, with low-moderate levels of inflammatory mediators in the parenchyma. It has been demonstrated that the responses of astrocytes and microglia to the same insults are diverse not only among different species and CNS areas, but also within the same areas of the hippocampus. The differential reactivity of astrocytes and microglia in normal hippocampus, during inflammaging and acute inflammation is reported in **Table 1** (Cerbai et al., 2012; Lana et al., 2016). In the adult hippocampus, in basal conditions, microglia have much lower density in CA1 than in CA3. During aging, while astrocytes decrease significantly in all hippocampal subregions, total microglia decrease in CA1 and increase in CA3. In acute inflammation, both total and activated microglia increase in all hippocampal subregions, while astrocytes increase in CA3 only. It is of the utmost importance to understand whether the differences of astrocytes/microglia reactivity may explain the diverse susceptibility of the hippocampal areas to aging or to different inflammatory insults (Masgrau et al., 2017).

In CA1 area of the hippocampus of aged rats, microglia cells, although less numerous and less mobile than in young animals, maintain their scavenging activity, phagocytosing damaged, apoptotic neurons or debris. In CA1, fractalkine (CX3CL1) immunostaining is present on neurons actively phagocytosed by microglia (Cerbai et al., 2012). CX3CL1, a transmembrane glycoprotein located in neurons, forms by proteolysis cleavage a soluble chemokine domain that is released in the parenchyma. The two forms have different functions. Membrane bound CX3CL1 is an adhesion molecule, while its cleaved, soluble form, is a chemoattractant that recruits microglia that express its receptor (CX3CR1) to injured neurons (Harrison et al., 1998; Chapman et al., 2000; Cardona et al., 2006; Noda et al., 2011). Furthermore, the soluble form of CX3CL1 regulates microglia

phagocytic activity (Cardona et al., 2006; Bhaskar et al., 2010; Lee et al., 2010; Liu et al., 2010). Since both microglia (Verge et al., 2004 and astrocytes (Dorf et al., 2000) express CX3CR1, fractalkine may be responsible for the recruitment of microglia and astrocytes in a well-organized topographic localization and spatial reciprocal interaction around apoptotic neurons to form triads. CX3CL1 contributes to the maintenance of microglia in a quiescent state (Lyons et al., 2009; Bachstetter et al., 2011). Nevertheless, soluble CX3CL1 increases in cerebral ischemia (Dénes et al., 2008), in response to glutamate stimulation (Chapman et al., 2000), during apoptosis (Fuller and Van Eldik, 2008), and is neuroprotective (Limatola et al., 2005) on cultured rat hippocampal neurons. CX3CL1 is also present in the spinal cord where, mediating the signaling between neurons and glia, it contributes to the insurgence of neuropathic pain (Clark and Malcangio, 2014). Thus, the effects of fractalkine may differ depending upon different stimuli, or upon the spatial localization of the cell (Lana et al., 2017a).

## Neurons Astrocytes Microglia Triads

A triad is defined as a cluster of cells in which a damaged/apoptotic neuron is surrounded by astrocytes that form a "microscar" around it, with the astrocyte branches that not only take contact with the neuronal cell membrane but also infiltrate the neuronal cell body (Cerbai et al., 2012; Lana et al., 2016). In the triad, a microglia cell is in the act of phagocytosing the neuronal cell body (Cerbai et al., 2012; Lana et al., 2016).

A1 astrocytes have been demonstrated *in vitro* to secrete a neurotoxin that induces neurons to undergo apoptosis (Liddelow and Barres, 2017; Liddelow et al., 2017). In addition, it has been demonstrated in a mouse model of Amyotrophic Lateral Sclerosis (ALS) that astrocytes release toxic factors that target specifically motor neurons and mediate cell death (Re et al., 2014). In a less neuronocentric view of neurodegenerative mechanisms, the alterations of astrocytes, in terms of number and morphology, with the consequential loss of their function, loss of maintenance of brain homeostasis, buffering of extracellular glutamate, as well as loss of supply of nutrients to neurons, may contribute to the spread of neuronal damage (Miller et al., 2017). During aging, astrocytes in the hippocampus and striatum of the mouse up-regulate a great number of reactive astrocytic genes (Clarke et al., 2018). The hippocampus and striatum, brain regions known to be most vulnerable in neurodegenerative diseases, are those where astrocytes upregulate A1 reactive genes the most (Burke and Barnes, 2006; Saxena and Caroni, 2011).

In line with these recent results, we had previously demonstrated in CA1, in CA3, as well as in DG of aged rats (Cerbai et al., 2012; Lana et al., 2016, 2017b) that astrocytes send branches to embrace, infiltrate and bisect apoptotic neurons (**Figures 3C1,C2**). This mechanism is finalized to the fragmentation of apoptotic neurons to form cellular debris that can be phagocytosed by microglia. This mechanism can protect and spare the neighboring cells from the damage caused by the release of proinflammatory products by the damaged neurons. Therefore, reactive astrogliosis can be beneficial, while suppression of astroglial reactivity may increase neuronal vulnerability, may exacerbate pathological development and may alter regeneration (Burda and Sofroniew, 2014; Pekny et al., 2014). However, when astrocyte reactivity becomes too intense, it is possible that the release of neurotoxic factors causes an intense neurotoxicity. Thus, astrocytes can behave as passive or active actors in causing or preventing neurodegeneration.

In the triads, astrocytes branches infiltrate and bisect the apoptotic neuron cytoplasm, which is phagocytosed by microglia as shown in **Figure 3A**. Neurons infiltrated by astrocyte branches show signs of degeneration, such as karyorrexhis (**Figures 3E,D1**), and apoptosis (**Figures 3C1,C2,D2**). Apoptotic neurons are in close contact with microglia cells that phagocytose the neuron cell body or cellular debris (**Figures 3D3,D4,F1–F3**, open arrows).

Both microglia and astrocytes can recognize danger signals in the parenchyma, including those released by cellular debris produced from apoptotic cells. Therefore, it appears that microglia and astrocytes can cooperate to help clearing apoptotic neurons or neuronal debris (Medzhitov and Janeway, 2002; Milligan and Watkins, 2009) in a concerted effort to prevent or reduce the release of proinflammatory mediators and damage to neighboring neurons (Nguyen et al., 2002; Turrin and Rivest, 2006). Nevertheless, examples exist of positive effects of active microglia and production of cytokines in early brain development (Salter and Beggs, 2014), in synaptic pruning (Schafer and Stevens, 2013) and in learning and memory mechanisms (Ziv et al., 2006; Derecki et al., 2010).

During normal aging, apoptosis is a physiological mechanism that maintains normal tissue homeostasis through resolution of low-grade inflammation (Gupta et al., 2006). As a consequence, under conditions of physiological aging, the effects of astrocytes and microglia are protective, removing neuronal debris by phagocytosis or entire neurons by phagoptosis (Lana et al., 2017a), pruning dysfunctional synapses and controlling inflammation and the diffusion of cellular damage.

In the hippocampus of aged rats, CA1 and CA3 pyramidal neurons significantly decrease (Cerbai et al., 2012; Osborn et al., 2016), possibly because of increased disposal of degenerating neurons paralleled by decreased neurogenesis (Kuhn et al., 1996) during low-grade but long-lasting inflammaging. In acute inflammation caused by LPS, the neurons dying for apoptotic mechanisms can be replaced by new neurons generated by neurogenesis. Indeed, GFAP/vimentin KO increases cellular proliferation and neurogenesis in the granular layer of the dentate gyrus (Larsson et al., 2004). Furthermore, GFAP null astrocytes support neuronal survival and outgrowth of neurites better than their wild-type counterparts (Menet et al., 2000), indicating that aging of astrocytes associated with increased expression of GFAP can repress the capacity of astrocytes to increase neurogenesis and neuronal protection in the brain. Increased neuron degeneration by aging, paralleled by the age-related decrease of neurogenesis, which decreases physiological neuron replacement, may thus be one of the causes of the age-related decrease of neurons, and may contribute to aging dependent impairments of brain function.

Active and controlled cell death may serve a homeostatic function in regulating the number of cell population in healthy and pathological conditions (Kerr et al., 1972; Becker and

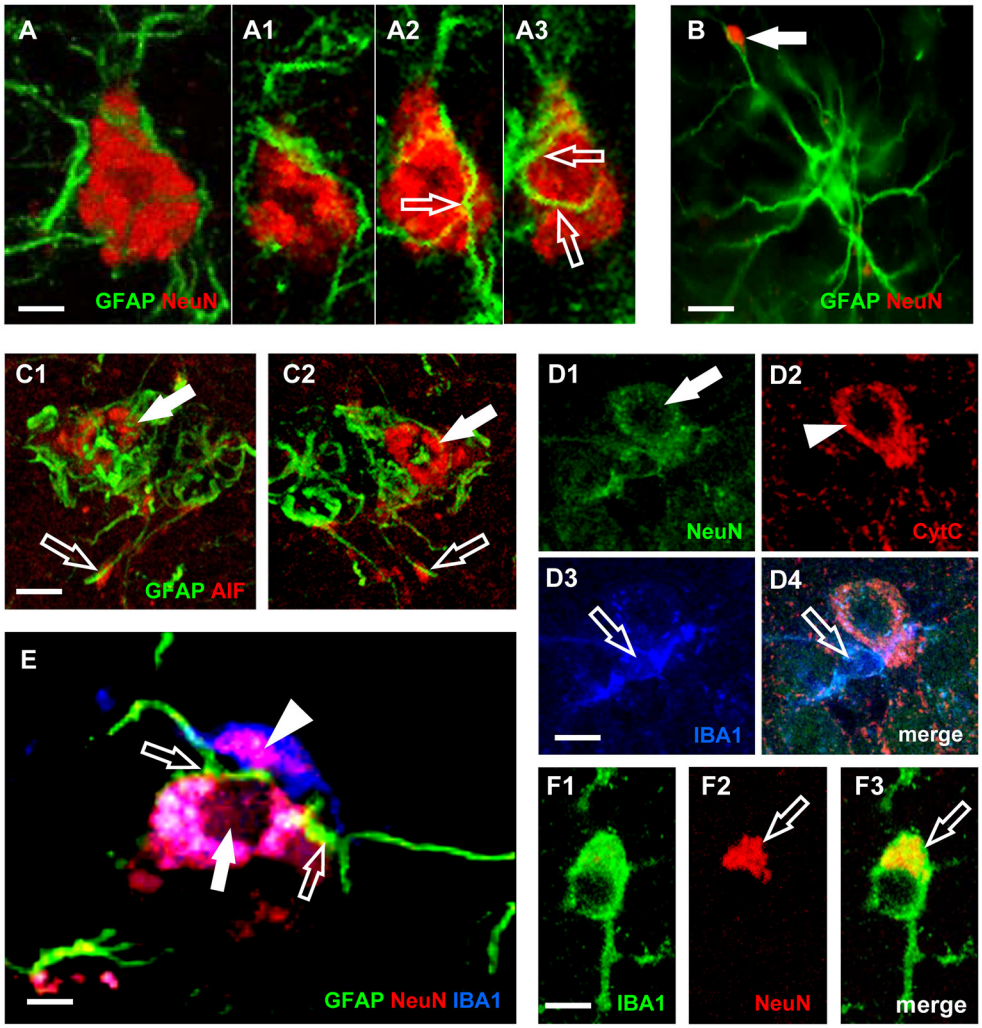


**FIGURE 3 |** Characterization of neurons-astrocytes-microglia interplay and apoptosis. **(A)** Confocal microscopy acquisition of GFAP (green) and NeuN immunostaining (red). Scale bar: 5 μm **(A1–A3)**. Digital subslicing of the neuron in **(A)**. Images are the merge of two contiguous confocal acquisitions. Serial confocal images that digitally sub-slice the neuron cell body **(A1–A3)** clearly demonstrate that astrocyte branches are present inside and infiltrate the neuron body [open arrows in **(A2,A3)**]. Scale bar: 5 μm. **(B)** The arrow shows a neuronal debris closely apposed to an astrocyte branch. The presence in the neuron cytoplasm of AIF **(C1,C2)** or of diffuse CytC immunostaining **(D2)**, are clear signs of apoptosis (Suen et al., 2008). Scale bar: 15 μm. **(C1,C2)** Immunostaining for AIF (red) and GFAP (green). Scale bar: 5 μm. **(D1–D4)** Confocal acquisitions of NeuN (green), CytC (red), and IBA1 (blue) immunostaining acquired in CA3 region of aged rats. Digital subslice, total thickness 0.3 μm, and the merge of the three other images **(D4)**. Scale bar: 15 μm. **(E)** Digital subslicing of a neuron-astrocyte-microglia triad, obtained stacking seven contiguous confocal acquisitions. Open arrows point to astrocytes branches intermingling the body of a neuron. It is possible to appreciate the presence of an engulfed neuronal fragment within the body of the microglial cell (arrowhead). Scale bar: 10 μm. **(F1–F3)** Digital subslicing of the Str. Radiatum of an aged rat of a IBA1-positive microglia (green). It is possible to highlight the presence of a NeuN-positive neuronal fragment [red, **(F2)**] inside the microglial cell body [yellow-orange, **(F3)**, open arrow]. Scale bar: 7 μm [Modified from Cerbai et al. (2012), Lana et al. (2016)].

Bonni, 2004). How apoptosis causes neurons to be disposed of is still a matter of debate. One of the main mechanism is the release of intercellular signals from neurons which induce phagocytic cells such as microglia to engulf and consume the neuron (Noda et al., 2011; Cerbai et al., 2012). Astrocytes and microglia express receptors able to recognize molecules released by neurons (Harrison et al., 1998; Noda et al., 2011), inducing the phagocytosis of damaged cells or neuronal debris. Therefore, triad formation seems a specific mechanism for clearance of degenerating neurons, through phagocytosis, or through phagoptosis (Brown and Neher, 2012; Fricker et al., 2012). Phagoptosis is triggered by cell stress, which is too mild to cause the death of the cell, too serious to allow readaptation of the cell from the damage, and sufficiently strong to recruit astrocytes and microglia for phagocytosis (Kao et al., 2011). During aging, although microglia increase in number, the cells have morphological modifications that cause less neuroprotective and defensive capabilities (Streit et al., 2009; Tremblay et al., 2011; Streit and Xue, 2013). Thus, targeting the triads may represent a therapeutic strategy,

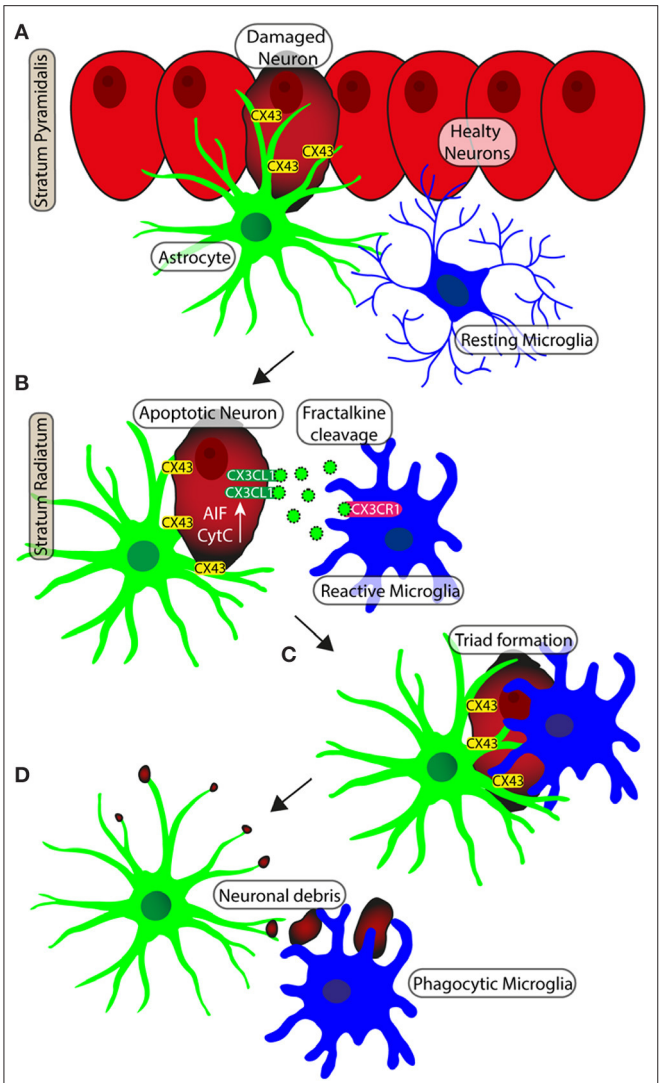


**FIGURE 4 |** Schematic representation of neuron-astrocytes-microglia interplay during neurodegeneration. **(A)** Apoptotic neurons of pyramidal layer show diffuse cytoplasmic staining of CytC or AIF. Gap junctions (Connexine 43) mediate the recruitment of astrocytes on neurons. **(B)** The apoptotic neuron is detached from the Pyramidal layer to becoming an ectopic pyramidal neuron. CX3CL1 (fraktalkine) is released by the ectopic neuron and the astrocyte branches take close contact with the neuron. **(C)** Microglia are recruited and astrocytes branches infiltrate the body of the damaged neuron. **(D)** The damaged neuron is bisected and disgregated in cell debris. Phagocytic microglia mediate the clearance of cell debris.

which may control release of proinflammatory signals from damaged neurons and spread of further cellular damage to neighboring cells.

In normal brain aging and acute neuroinflammation caused by LPS administration in the rat (Cerbai et al., 2012; Lana et al., 2016), many neurons in the Str. Radiatum of hippocampal areas CA1 and CA3 are apoptotic pyramidal neurons that form triads with astrocytes and microglia. They show increased activation of p38MAPK (Xia et al., 1995) and increased immunostaining for CytC (Ow et al., 2008) and AIF (Lorenzo et al., 1999). Some of the apoptotic neurons in the aged rats, located very close to the Str. Pyramidalis but clearly detached from it, are surrounded and infiltrated by astrocyte branches, possibly recruited by overexpression of Connexin43 (Cx43) (Cerbai et al., 2012). Astrocyte branches embrace, infiltrate and wedge the apoptotic neuron and form debris. It appears that the astrocyte branches themselves remove the damaged, apoptotic neuron from the Str. Pyramidalis, signaling via intercellular molecules such as the Cx43 and/or CX3CL1 (Noda et al., 2011). Indeed, it is known that during the first steps of apoptosis caspases break the cell cytoskeleton, allowing the apoptotic cell to detach from the surrounding, healthy cells (Böhm, 2003). This mechanism may explain how pyramidalis apoptotic neurons migrate from the Str. Pyramidalis to the Str. Radiatum to form triads in which phagocytosis may take place. Apoptosis, active and controlled cell death, may serve a homeostatic function, regulating the number of cells, not only in pathological conditions but also in the healthy brain (Becker and Bonni, 2004). For instance, the role of microglia is critical in the early stages of embryonic development in which excess neurons are produced; later, these neurons face programmed cell death and break up into small apoptotic bodies, removed by microglia (Pont-Lezica et al., 2011). Microglia change their morphology in relation to the development of the CNS and in particular pathological conditions.

We first demonstrated in the hippocampus of aged rats that in the triads, astrocyte branches infiltrate a damaged, apoptotic neuron, to bisect the dying neuron and form neuronal debris (Cerbai et al., 2012) which are significantly numerous in aged and LPS-treated rats. Neuronal debris are all closely apposed to astrocyte branches and are phagocytosed by microglia. Therefore, it appears that both microglia and astrocytes can recognize danger signals in the surrounding parenchyma and can interact and help clearing apoptotic cells and the resulting debris (Medzhitov and Janeway, 2002; Milligan and Watkins, 2009), at least in rodents.

Nevertheless, in the contiguous and interconnected CA1 and CA3 subregions of rat hippocampus, astrocytes and microglia show very different reactivity, as reported in **Table 1** (Cerbai et al., 2012; Lana et al., 2016), demonstrating that the responses of astrocytes and microglial to the same insult are not uniform but vary significantly from area to area and in different conditions. It was demonstrated in the rat that activated microglia are seen diffusely scattered throughout the brain after 2 days of LPS infusion (Wenk and Barnes, 2000). During the next weeks, the number of activated microglia gradually decreases in all cerebral regions and later the greatest inflammatory response is concentrated within the hippocampus (Wenk and Barnes, 2000). These findings suggest that in the rat LPS initiates a cascade of biochemical processes that show time dependent, regional and cell specific changes that are maximal after 4 weeks of LPS infusion (Hauss-Wegrzyniak et al., 1998; Wenk and Barnes, 2000).

Rapid neuroprotective effect of microglia is facilitated by its regular distribution in the tissue, which minimizes the distance from possible pro-inflammatory triggers, such as cell debris and entire damaged neurons. Astrocytes constantly contact neuron

surfaces, and interact with neuron debris (Reemst et al., 2016). Therefore, the contiguous meshwork of their projections may be the first structures that enter in contact with possible targets of microglia phagocytosis. We demonstrated that direct cell-cell interactions exist between astrocytes and microglia, which can influence and mediate microglial branching, addressing branch tree extension toward pro-inflammatory triggers. The disruption of astrocyte meshwork that occurs during aging, could be related to dysregulation of microglial defensive activity (Lana et al., 2019). A schematic representation of neuron-astrocytes microglia interplay in the triads is shown in **Figure 4**.

The chronic, sterile, low-grade neuroinflammation that develops during normal brain aging (Franceschi et al., 2018) eventually activates microglia and astrocytes that first cooperate to maintain brain homeostasis. We now know a degree of neuroinflammatory cytokines that act on other cells to influence cellular biochemistry, physiology and development that can represent or not pathological neuroinflammation, but rather demonstrate a way in which signals are communicated within the CNS. When the inflammation becomes more intense, microglia and astrocytes start releasing proinflammatory and neurotoxic mediators and increase the expression of inflammation-related proteins (Liddelow and Barres, 2017). All these mechanisms start a vicious circle that causes progressive impaired interplay between neurons, astrocytes and microglia that, when too intense, may be responsible for derangements from normal brain aging to neurodegenerative diseases (De Keyser et al., 2008; Sofroniew, 2009).

## CONCLUSIONS

The data reported in the present review expand the wealthy panel of interactions that occur among the different cell populations of the CNS and add plausibility to the idea that such interactions bring about a network of morphological and functional reciprocal reliance and dependency. To comprehend the peculiar aspects of the onset and progression of neuroinflammation, it is necessary to understand and take into consideration that any tissue, and mainly the nervous tissue, is a mere collection of single elements but rather by interacting and interdependent cell populations that cooperate to maintain the homeostasis and functionality of the organ. Different types of alterations affecting one population reasonably reverberate to the others either favoring or dysregulating their activities.

It should be pointed out, however, that the majority of the studies reported in this review were performed in rat or mouse experimental models. Many studies indicate that rodent and human microglia and astrocytes react differently to mediators of neuroinflammation, have a different rate of proliferation or activation and express different proteins and enzymes in response to external stimuli such as proinflammatory mediators (Smith and Dragunow, 2014; Streit et al., 2014; Wolf et al., 2017). Not only animal models but also those performed in specimens from human tissue, as all models, have positive and negative aspects. It is of the utmost importance, however, to understand that this experimental field, that dates over 150 years, is still in its infancy.

## AUTHOR CONTRIBUTIONS

MG and DL wrote the paper. FU and DN contributed to the preparation of the figures. MG and GW edited and revised the manuscript. All authors read and approved the final manuscript.

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frontiers in Aging Neuroscience

MINI REVIEW
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# *In vivo* Direct Conversion of Astrocytes to Neurons Maybe a Potential Alternative Strategy for Neurodegenerative Diseases

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Partly because of extensions in lifespan, the incidence of neurodegenerative diseases is increasing, while there is no effective approach to slow or prevent neuronal degeneration. As we all know, neurons cannot self-regenerate and may not be replaced once being damaged or degenerated in human brain. Astrocytes are widely distributed in the central nervous system (CNS) and proliferate once CNS injury or neurodegeneration occur. Actually, direct reprogramming astrocytes into functional neurons has been attracting more and more attention in recent years. Human astrocytes can be successfully converted into neurons *in vitro*. Notably, *in vivo* direct reprogramming of astrocytes into functional neurons were achieved in the adult mouse and non-human primate brains. In this review, we briefly summarized *in vivo* direct reprogramming of astrocytes into functional neurons as regenerative strategies for CNS diseases, mainly focusing on neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD). We highlight and outline the advantages and challenges of direct neuronal reprogramming from astrocytes *in vivo* for future neuroregenerative medicine.

**Keywords: astrocytes, neurons, neurodegenerative diseases, *in vivo*, conversion**

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## INTRODUCTION

Partly because of extensions in lifespan, the incidence of neurodegenerative diseases such as Alzheimer's disease (AD), (Alzheimer's disease facts and figures, Alzheimers Dement, 2021), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), is increasing, and these neurodegenerative diseases result in a heavy social and economic burden in the world. However, the pathological mechanisms underlying neurodegenerative diseases are unclear. A variety of factors such as genetic, environmental, and aging factors, are considered to participate in the neurodegeneration (Przedborski, 2017). Abnormal protein aggregation (Spires-Jones and Hyman, 2014; Wang et al., 2020), oxidative stress with reactive oxygen species (ROS) (Xu et al., 2016; Bi et al., 2018; Singh et al., 2019; Wang et al., 2020), iron deposition (Jiang et al., 2017; Levi et al., 2019; Chen et al., 2021b), mitochondrial dysfunction (Przedborski, 2017; Wang et al., 2020),

autophagy (Chen et al., 2017, 2021a; Ren et al., 2021), decreasing of neurotrophins (Allen et al., 2013), and neuroinflammatory responses (Kwon and Koh, 2020), are recognized as common pathophysiological mechanisms, and all of those contribute to the neuronal loss in brains. Thus far, there are no effective strategies to slow neuronal degeneration, or prevent the progression of them. Logically, replenishing the lost neurons can repair the neuronal function by endogenous neurogenesis or cell transplantation (Chen et al., 2019). As we all know, neurons cannot self-regenerate, while glial cells such as astrocytes can proliferate upon injury or disease (Li and Chen, 2016; Chen et al., 2019; Lei et al., 2019). Currently, neurons may not be replaced once being damaged or degenerated in human brain (Li and Chen, 2016; Boldrini et al., 2018; Lei et al., 2019), and there is a debate on whether adult human brains have endogenous neurogenesis (Lei et al., 2019). The newborn neurons are largely restricted in a few regions such as the hippocampus and the subventricular zone (Boldrini et al., 2018; Sorrells et al., 2018), and the progenitor cells migrating toward injury sites often differentiate into glia rather neurons (Faiz et al., 2015), which has disadvantageous effect on repairing damaged or degenerative brains. Nevertheless, numerous studies have shown that embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs)-derived transplants can survive, innervate, produce neurotransmitters, and promote functional recovery in animal models (De Gioia et al., 2020; Gantner et al., 2020; Song et al., 2020), and even clinical trials with those transplants have achieved some success (Kefalopoulou et al., 2014; Schweitzer et al., 2020). There are several serious problems that are difficult to overcome including supply and availability, ethical issues, immune rejection for ESCs, and the risk of tumor formation, genomic stability for iPSCs (Trounson and McDonald, 2015; Martin, 2017).

Shinya Yamanaka's team was pioneered in the ground-breaking iPSC technology that mouse fibroblasts could be reprogrammed into the pluripotent stem cells by delivering four transcription factors (TFs) (Oct4, Sox2, Klf4, and c-Myc) *in vitro* (Takahashi and Yamanaka, 2006). Then, it was reported that human somatic cells can be converted into human iPSCs (hiPSCs) in the following years (Takahashi et al., 2007; Park et al., 2008). HiPSCs technology has no ethical concern and immune rejection, showing great promise in the regenerative strategies by mass-generating patient-specific stem cells (Stadtfeld and Hochedlinger, 2010). Based on the hiPSCs technology (Stadtfeld and Hochedlinger, 2010), direct lineage reprogramming, which converts a specific somatic cell type to another, is developed rapidly. The cells from direct lineage reprogramming are not passing through a pluripotent state, therefore, they mostly have no the ability of tumor formation (Chambers and Studer, 2011). Thereafter, it has been reported that various types of functional cells, including neurons, that can be successfully generated by a direct reprogramming strategy *in vitro* and even *in vivo* (Vierbuchen et al., 2010; Niu et al., 2013), which leads to the understanding of direct reprogramming technology as an alternative approach for rescuing neurodegeneration.

The primary consideration is the starting cell type in generating neurons via direct lineage reprogramming. Given that fibroblasts have large quantity and extensive distribution in the body, they are promising candidate cells for reprogramming, and indeed by introducing the combined expression of neural lineage-specific TFs, direct reprogramming of mice and human fibroblasts into neurons was first reported *in vitro* (Vierbuchen et al., 2010). However, considering the fact that (1) glial cells are proximal in lineage distance to neurons and ubiquitous distribution in adult mammalian brains; (2) they constitute over half of the total brain cells; (3) they have intrinsic proliferative capability, glial cells can serve as ideal source cells for reprogramming into neurons to achieve neuroregeneration (Amamoto and Arlotta, 2014; Guo et al., 2014). Astrocytes serve with multiple important physiological functions in the central nervous system (CNS), such as the constitution and maintenance of the blood-brain barrier (Figley and Stroman, 2011), producing and releasing of neurotrophic factors (Anderson et al., 2016), adjusting the density of ion in the extracellular space (Walz, 2000), modulating neuroinflammation (Kwon and Koh, 2020; Vadodaria et al., 2021), and even regulating neuronal activity, synaptic transmission, and neural circuit function (Bazargani and Attwell, 2016; Li et al., 2020; Vadodaria et al., 2021; Yin et al., 2021). Although astrocyte proliferation largely stops after 1 month of age in rodents (Ge et al., 2012), in response to insults, including brain trauma, stroke and neurodegeneration, astrocytes often are under a state that called astrogliosis, in which astrocytes accelerate proliferation (Liddelow and Barres, 2017). For the first time, the approach for reprogramming astrocytes into neurons has been found by high expression of the neurogenic TF Pax6 *in vitro* (Heins et al., 2002). Indeed, astrocytes originating from the same progenitor cells with neurons (Kriegstein and Alvarez-Buylla, 2009) are efficiently reprogrammed into functional neurons with one TF (Heins et al., 2002; Berninger et al., 2007). The above studies indicate that astrocytes may be an ideal starter cells for reprogramming into neurons to replace the lost neurons in neurodegenerative diseases.

It is fascinating that these reactive astrocytes are *in situ* reprogrammed into functional neurons for neural circuit reestablishment and functional recovery. Actually, direct reprogramming astrocytes into functional neurons has been attracting more and more attention in recent years (Amamoto and Arlotta, 2014; Chen et al., 2015). It has been confirmed that human astrocytes can be successfully converted into neurons or neuroblasts *in vitro* (Corti et al., 2012; Zhang et al., 2015; Li et al., 2016; Rivetti di Val Cervo et al., 2017; Yin et al., 2019; Qian et al., 2020). These studies provide a potential alternative approach to regenerate functional new neurons in CNS of adult mammalian by *in situ* directly reprogramming astrocytes into neurons. Notably, *in vivo* direct reprogramming of astrocytes into functional neurons were achieved in the adult mouse brain, and even in the adult non-human primate brains (Guo et al., 2014; Chen et al., 2015, 2020; Liu et al., 2015; Niu et al., 2015; Li and Chen, 2016; Srivastava and DeWitt, 2016; Brulet et al., 2017; Rivetti di Val Cervo et al., 2017; Qian et al., 2020; Wu et al., 2020; Zhou et al., 2020). In this review, we briefly summarized *in vivo* direct reprogramming of astrocytes into functional neurons as regenerative strategies for CNS diseases, mainly focusing on neurodegenerative diseases such as PD, AD,

HD, and ALS. We highlight and outline the advantages and challenges of direct neuronal reprogramming *in vivo* for future neuroregenerative strategies.

## *IN VIVO* ASTROCYTE-TO-NEURON CONVERSION FOR PD

Parkinson's disease (PD) is the second common progressive neurodegenerative after AD, and its main pathological characteristics are the progressive loss of dopaminergic neurons in the substantia nigra region and the depletion of dopamine in the striatum, which cause some major clinical motor symptoms of PD including rest tremor, rigidity, bradykinesia, and postural instability (Przedborski, 2017). So far, there is no effective treatment strategy that can protect dopaminergic neurons from neurodegeneration.

Evidence has been provided that the transplantation of dopaminergic neurons derived from the human fetal ventral midbrain, were able to partially improve the clinical motor function in PD patients (Kefalopoulou et al., 2014; Li and Chen, 2016). However, the clinical transplantation of those dopaminergic neurons is limited because of the ethical issues in harvesting human fetal tissue, immune rejection, and the risk of transplantation-induced dyskinesia (Olanow et al., 2003). In order to obtain standardizing dopaminergic neurons for clinical application, an alternative approach is to induce iPSCs into dopaminergic neurons *in vitro*. Those dopaminergic neurons have no immune rejection for transplantation, but have the risk of tumor if not properly controlled (Kwon and Koh, 2020). These induced dopaminergic neurons are capable to restore certain function after transplantation in animal models of PD (Chen et al., 2019). Furthermore, the transplantation of dopaminergic neurons derived from iPSCs has been achieved in a clinical trial (Schweitzer et al., 2020).

Initially, Caiazzo et al. (2011) reported that mouse and human fibroblasts can be directly converted into functional dopaminergic neurons called 'induced dopamine-releasing' (iDA) neurons *in vitro* by forced expression of three transcription factors: Mash1 (also called Ascl1), Lmx1a and Nurr1 (also called Nr4a2), together referred as ALN, in which lentiviruses expressing three factors were employed. Ascl1, Lmx1a, and Nurr1 are critical for midbrain dopaminergic neurons development (Arenas et al., 2015), and the reprogramming of astrocytes into dopaminergic neurons has been successful *in vitro* by delivering these three transcription factors in a single polycistronic lentiviral vector (Addis et al., 2011). However, the ALN combination converted astrocytes or NG2 glia into GABAergic neurons, rather into dopaminergic neurons in mouse brains by using the Cre-recombinase-dependent AAV vectors (Torper et al., 2015). Afterward, Rivetti di Val Cervo et al. (2017) reported a strategy that was able to generate induced dopaminergic neurons from human astrocytes with reprogramming efficiency reaching up to 16% *in vitro* by using overexpression of three transcription factors in a lentiviral vector, NeuroD1, Ascl1, and Lmx1a, and the microRNA miR-218, collectively called NeAL218. Moreover, in a mouse model of PD, adult striatal astrocytes transfected by lentiviruses with overexpression of the NeAL218 factors were also converted into dopaminergic neurons, and those induced dopaminergic neurons promoted the motor improvement (Rivetti di Val Cervo et al., 2017; Fyfe, 2017).

Two recent studies now provide a very simple strategy to efficiently reprogram astrocytes into iDA neurons *in vivo*, which only need to deplete an RNA-binding protein called PTB (polypyrimidine tract-binding protein) encoded by a single gene, polypyrimidine tract-binding protein 1 (*Ptbp1*) in astrocytes (Arenas, 2020; Qian et al., 2020; Zhou et al., 2020; Jiang et al., 2021). Previous studies have shown that *Ptbp1* (PTB) expression is observed in most cell types, and the other two PTB family members in mammalian genomes are *Ptbp2* (also called nPTB or brPTB) which is exclusively expressed in the nervous system, and *Ptbp3* (also called as ROD1) which is mainly detected in immune cells (Hu et al., 2018). It has been certified that PTB-regulated loop is very important for neuronal induction, and nPTB-regulated loop for neuronal maturation (Hu et al., 2018; Qian et al., 2020). In non-neuronal cells, such as fibroblasts, astrocytes, PTB-regulated loop is closely related to neuronal induction. In this loop, RE1-silencing transcriptional factor (REST) complex prevents the expression of multiple neuron-specific transcriptional genes (for example Ascl1, NeuroD1) as well as miR-124 in non-neuronal cells. Although REST is also the target of miR-124 in such loop, such targeting is potently inhibited by PTB via direct competition of miR-124 targeting on REST components (Xue et al., 2013, 2016; **Figure 1**). Thus, knockdown of *Ptbp1* can promote miR-124 to efficiently target REST. Considering that miR-124 also is able to target PTB, the derepression of miR-124 can further inhibits PTB and REST, leading to the expression of neuron-specific transcription factors for neurogenesis. The PTB-miR-124-REST loop, which is self-sustainable and conserved in mammals, can be triggered by *Ptbp1* knockdown (Xue et al., 2016). Cultured mouse embryonic fibroblasts can be converted into functional neurons by downregulation of the expression of *Ptbp1* (Xue et al., 2013), however, human adult fibroblasts are only converted into immature neurons according to the same protocol (Xue et al., 2016). The mechanism is that in adult human fibroblasts, downregulation of PTB increases the expression of nPTB, and meanwhile, high expression nPTB suppresses the transcription activator BRN2 (encoded by *Pou3f2*) and miR-9, both of which are required for neuronal maturation (Xue et al., 2013, 2016). Therefore, adult human fibroblast-to- neuron conversion requires both PTB-miR124-REST loop for neuronal induction and nPTB-BRN2-miR-9 loop for neuronal maturation *in vitro* (Xue et al., 2016). In astrocytes, during neurogenesis from neural stem cells, nPTB induced by PTB knockdown may be immediately counteracted by miR-9 (Qian et al., 2020). Further investigations found that the PTB-regulated loop in astrocytes is similar to the one in fibroblasts and the nPTB-regulated loop in astrocytes is similar to the one in neurons, and transient high expression of nPTB is observed in PTB-deficient astrocytes. These results suggest that astrocytes can be converted into neurons by *Ptbp1* knockdown (Qian et al., 2020). Indeed, both isolated mouse and human astrocytes are successfully converted into mature neurons by transfection with a lentivirus vector

expressing shRNA against *Ptbp1* (shPTB) *in vitro* (Qian et al., 2020). The same effect is achieved in astrocytes isolated from the mouse cortex by using the genome-editing technique CRISPR-CasRx to deplete *Ptbp1* mRNA (Zhou et al., 2020).

These induced dopaminergic neurons from reprogramming of astrocytes in the substantia nigra or striatum can improve motor function in a mouse model of PD (Arenas, 2020; Qian et al., 2020; Zhou et al., 2020; Jiang et al., 2021). Qian et al. transduced mouse astrocytes in the substantia nigra with an adeno-associated virus (AAV; serotype2) vector to express a small hairpin RNA (shRNA) silencing *Ptbp1* expression (shPTB) (Qian et al., 2020). Qian et al. (2020) also synthesized PTB antisense oligonucleotides (ASOs) which is essential short nucleic acids and can bind to *Ptbp1* mRNA, thus preventing its translation into PTB protein (Qian et al., 2020). Notably, the local transient injection of ASOs in the substantia nigra also could generate dopaminergic neuron like cells and promoted the recovery of motor functions in PD mice (Qian et al., 2020). By contrast, Zhou et al. (2020) designed an AAV expressing CasRx and two guide RNAs (gRNAs) targeting *Ptbp1* mRNA to transfect astrocytes in the striatum. The results of Qian et al. (2020) suggested that the brain-region-specific transcription factors play crucial roles in the astrocyte-to-dopaminergic neuron conversion. However, because the region-specific transcription factors in the striatum are different from in the substantia nigra, this mechanism is not suitable to explain that striatal astrocytes can be converted into dopaminergic neurons in Zhou and colleagues' study (Arenas, 2020; Qian et al., 2020; Zhou et al., 2020). Additionally, another important promoter on progress of astrocyte-to-dopaminergic neuron conversion is the local brain environment including various local brain-derived factors. Qian et al. (2020) results showed that the efficiency of astrocyte-to-dopaminergic neuron conversion was higher *in vivo* than *in vitro*. Beyond that, Zhou et al. (2020) found that Müller glia also could be converted into functional retinal ganglion cells in a mouse model of NMDA-induced retinal injury by AAV-GFAP-CasRx-*Ptbp1*, leading to alleviate symptoms caused by the loss of retinal ganglion cells. Besides, *in vitro* human fetal cortical astrocytes could be converted into different neurons by depleting *Ptbp1*, such as glutamatergic neurons (Qian et al., 2020). The above results indicate that glia-to-neuron conversion by PTB deleption may have a therapeutic potential for PD or other neurodegenerative diseases, even neuronal injuries such as trauma, tumor, or stroke.

However, many questions of *in situ* direct conversion of astrocytes to functional dopaminergic neurons remain to be answered (Arenas, 2020). For instance, astrocytes with depletion of PTB are also converted to other neuron types, such as glutamatergic neurons, GABAergic neurons and interneurons, in addition to dopaminergic neurons (Qian et al., 2020; Zhou et al., 2020). Given that *Ptbp1* is also expressed in other midbrain cell types (La Manno et al., 2016; Arenas, 2020), such as endothelial and pericyte cells, ependymal cells and microglia, therefore, there is an intriguing question whether these cells can also been converted into dopaminergic neurons by PTB depletion in animal models of PD. Moreover, although more than half of the fibers project to the striatum were from induced dopaminergic neurons, the vast majority of converted dopaminergic neurons project to the septum, rather than the striatum (Arenas, 2020; Qian et al., 2020). Besides, ectopic location or project of induced dopaminergic neurons might cause side effects, such as dyskinesia because of random and irregular release of dopamine. Although the simplicity of this gene-therapy approach to neuron replacement *in vivo* makes it very attractive, many questions remain to be answered, and it must be cautiously optimistic for the development of regenerative medicine for neurological disorders such as PD.

## *IN VIVO* ASTROCYTE-TO-NEURON CONVERSION FOR AD

Alzheimer's disease (AD), is the most prevalent neurodegenerative disease accounting for 60–80% of dementia cases, and it is manifested by memory loss and a decline in cognitive functions (Alzheimer's disease facts and figures, Alzheimers Dement, 2021). Generally, the typical pathological characteristics of AD occur many years before the onset of clinical symptoms with the progressive accumulation of extracellular amyloid β (Aβ) plaques outside neurons and hyperphosphorylated tau (p-tau)-composed neurofibrillary tangles (NFTs) inside neurons, accompanied by neuronal death, synapses loss and global brain atrophy (Spires-Jones and Hyman, 2014; Zhang T. et al., 2021). It has been shown that there are several mechanisms involved in AD, such as inflammation and immune activation, lipid metabolism, endosomal vesicle recycling, and autophagy (Zhang T. et al., 2021). So far, no effective treatment is available to stop or even slow the progression of AD. Because AD is a disorder with extensive neurodegeneration, it is very difficult for cell transplantation to every area of the degenerating brain.

As for *in vivo* astrocyte reprogramming, it has been reported that a retrovirus expressing NeuroD1 under the control of human GFAP promoter, was constructed, and overexpression of NeuroD1 was capable of reprogramming reactive astrocytes into functional neurons in the adult mouse cortex in a mouse model of AD (Guo et al., 2014). NeuroD1-converted neurons had the ability of spontaneous and evoked synaptic responses by electrophysiological recordings. Interestingly, astrocytes were mainly converted into glutamatergic neurons whereas NG2 cells were reprogrammed into GABAergic neurons except glutamatergic neurons following NeuroD1 expression, suggesting that different glial cells may be involved in different neuronal fate due to lineage differentiation (Guo et al., 2014). Surprisingly, reactive astrocytes are much more competent and easily reprogrammed into neurons than quiescent astrocytes (Guo et al., 2014). Moreover, when cultured human astrocytes were infected with NeuroD1-retrovirus, they also can be efficiently reprogrammed into neurons, and the majority of NeuroD1-converted neurons were glutamatergic neurons (Guo et al., 2014). Unfortunately, the *in vivo* reprogramming of glia cells to neurons could not ultimately rescue behavioral deficits in a mouse model of AD, such as cognitive impairment.

Generally, the master transcription factors including NeuroD1, Sox2, Ngn2, and Ascl1 are closely related to guide the glia converted neuronal fate (Berninger and Jessberger, 2016;

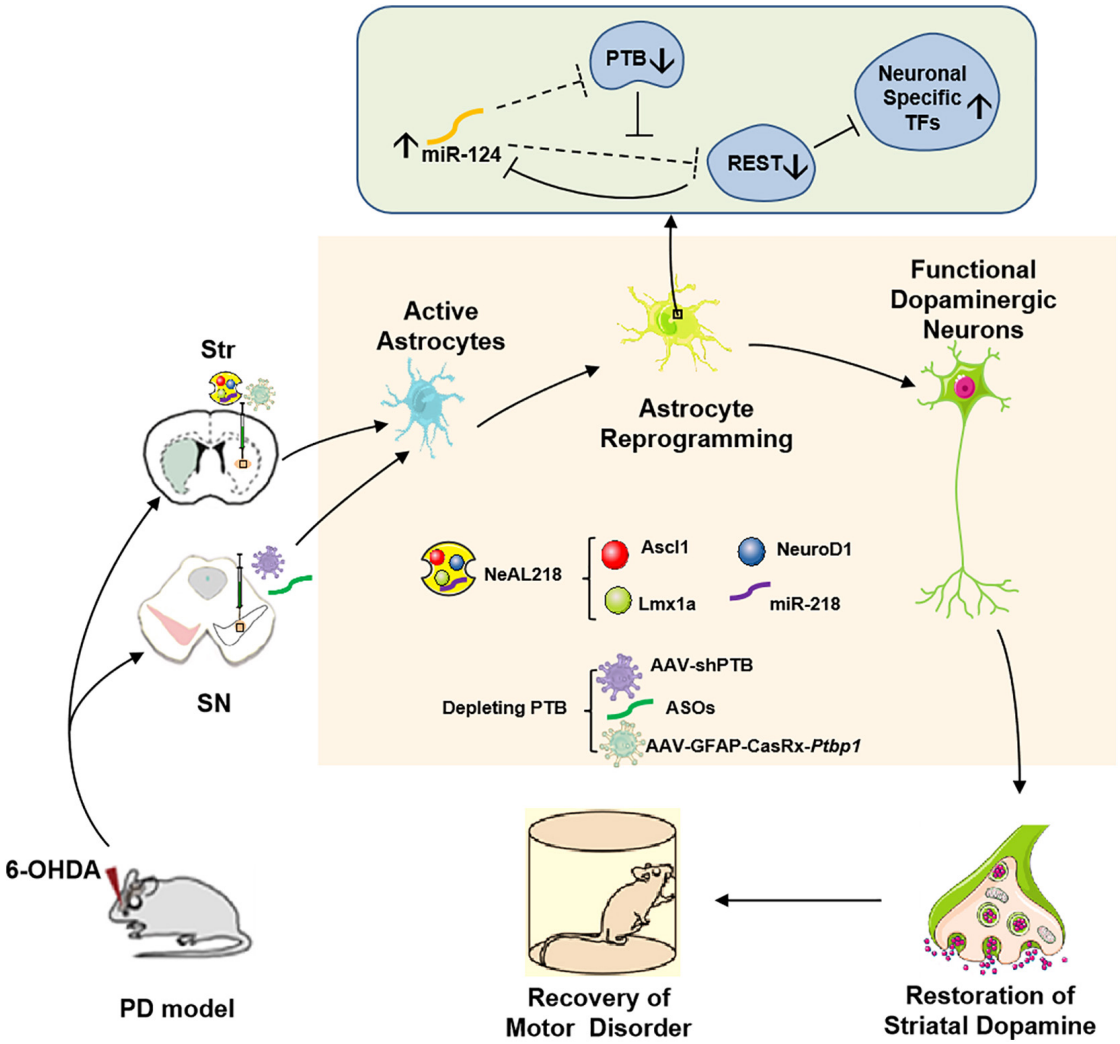


**FIGURE 1 |** *In vivo* reprogramming of astrocytes into functional dopaminergic neurons promotes behavioral improvement in a mouse model of PD. Astrocytes can be converted into dopaminergic neurons by high expressing three transcription factors (NeuroD1, Ascl1, and Lmx1a), and miR-218, collectively referred to as NeAL218 in mouse striatum (Fyfe, 2017; Rivetti di Val Cervo et al., 2017) or by depleting an RNA-binding protein called PTB in mouse striatum or substantia nigra (Qian et al., 2020; Zhou et al., 2020), which leads to behavioral improvement in a mouse model of PD. The molecular mechanism of astrocyte-to- dopaminergic neuron conversion by depletion PTB is that RE1-silencing transcriptional factor (REST) complex prevents the expression of multiple neuron-specific transcriptional genes (for example Ascl1, NeuroD1) as well as miR-124 in astrocytes (Hu et al., 2018). Although REST also is the target of miR-124 in such loop, such targeting is potently inhibited by PTB via direct competition of miR-124 targeting on REST components. Thus, depletion of PTB can promote miR-124 to efficiently target REST (Qian et al., 2020). Considering that miR-124 also is able to target PTB, the derepression of miR-124 can further inhibits PTB and REST, leading to the expression of neuron-specific transcription factors for neurogenesis (Qian et al., 2020). Furthermore, striatal astrocyte-to- dopaminergic neuron conversion also can be achieved by overexpression the neuron-specific transcriptional genes such as Ascl1, NeuroD1, Lmx1a, and miR-218 (Fyfe, 2017; Rivetti di Val Cervo et al., 2017). Str, Striatum; SN, Substantia Nigra; PTB, Polypyrimidine Tract-Binding protein; TFs, Transcriptional Factors; ASOs, PTB antisense oligonucleotides.

Li and Chen, 2016). NeuroD1 as a bHLH proneural transcription factor, is essential for embryonic brain development and adult neurogenesis (Cho and Tsai, 2004; Gao et al., 2009; Kuwabara et al., 2009), and can induce terminal neuronal differentiation (Boutin et al., 2010). The potentially mechanism is associated with the activation of downstream neural transcription factors (Gao et al., 2009; Kuwabara et al., 2009; Boutin et al., 2010), regulation of the Sonic hedgehog (SHH) signaling pathway (Sirko et al., 2013; Yang et al., 2019), and epigenetic modulation (Matsuda et al., 2019).

## *IN VIVO* ASTROCYTE-TO-NEURON CONVERSION FOR HD

Huntington's disease (HD) is an autosomal dominant disease characterized by the degeneration of GABAergic medium spiny neurons (MSNs) in the striatum and other brain regions, leading to progressive motor, cognitive, and psychiatric symptoms (Wu et al., 2020). It has been shown that 95% of the total neurons within the human striatum are MSNs (Reinius et al., 2015), and MSNs are susceptible to mutant huntingtin protein (mHtt)

(Rikani et al., 2014). The accumulation and aggregation of mHtt in MSNs was discovered during early degeneration in the striatum of the patients with HD (Rikani et al., 2014). Previous studies have shown that both cell transplantation (Ma et al., 2012) and gene therapy (Yang S. et al., 2017) that can reduce the mHtt level, can promote neurological function recovery in animal models of HD.

To date, MSNs have been reprogrammed from human fibroblast cells *in vitro* (Victor et al., 2014). It has been reported that Dlx2 is important for generating GABAergic neurons (McKinsey et al., 2013; Yang N. et al., 2017). A lentiviral vector with high expression of miR-9/9*-124 plus transcription factors Dlx1 and Dlx2 under a doxycycline (Dox)-inducible promoter was generated, and human fibroblasts were converted to striatal neurons after infection with these lentiviral vector (Victor et al., 2014). Recent report has shown that the AAV (serotype 2/5, rAAV2/5) with Cre-FLEx system was constructed, and it contained a vector expressing Cre recombinase under the control of GFAP promoter and FLEx vectors possessing an inverted coding sequence of NeuroD1-P2A-mCherry or Dlx2-P2A-mCherry. Notably, *in vivo* direct conversion of striatal astrocytes into MSNs has been achieved by this AAV-mediated ectopic expression of NeuroD1 and Dlx2 transcription factors (Wu et al., 2020). Those converted MSNs are electrophysiologically functional and forming synaptic circuits with other neurons, also can project their axon nerve terminals to substantia nigra pars reticulata and the external globus pallidus (Wu et al., 2020). Moreover, *in vivo* regeneration of MSNs in the striatum could reduce the striatum atrophy, and improve the motor functions in the R6/2 HD mouse model. In addition, the life span of the R6/2 HD mouse was significant extension after NeuroD1 + Dlx2 gene therapy treatment (Wu et al., 2020).

## *IN VIVO* ASTROCYTE-TO-NEURON CONVERSION FOR ALS

Amyotrophic lateral sclerosis (ALS), also called Lou Gehrig disease, is a motor neuron degenerative disease, affecting mainly upper and lower motor neurons (MNs) in the motor cortex, brain stem, and spinal cord (Ferraiuolo et al., 2011; Meyer et al., 2014). Transactivation response DNA-binding protein 43 kDa (TDP-43)-positive cytoplasmic inclusions and bunina bodies are often presented in the lost motor neurons of patients with ALS (Neumann et al., 2006; Barp et al., 2020). The typical clinical symptoms are muscular atrophy and paralysis, ultimately leading to death caused by respiratory failure (Ferraiuolo et al., 2011; Meyer et al., 2014; Brown and Al-Chalabi, 2017). Indeed, ALS is caused by multiple factors, such as older age, household heredity factors, environmental and lifestyle factors (Brown and Al-Chalabi, 2017), and so far, ALS has been considered as a multi-system disorder, not just a motor neuron disease, and the pathogenesis of ALS still remains unclear. It has been demonstrated that many factors, such as neuroinflammation, glutamate-induced excitotoxicity, mitochondrial dysfunction, and so on, can damage the neuro-muscular junction integrity and retrograde axonal degeneration, ultimately resulting in motor neuronal degeneration (Brown and Al-Chalabi, 2017; Liu and Wang, 2017). The technology application of astrocyte-to-neuron-conversion may bring hope to develop new therapeutic strategies for ALS.

Recently, Zhao et al. (2020) reported that both human astrocytes and mouse astrocytes from an ALS mouse model carrying a SOD1 mutation could be rapidly and efficiently converted into motor neuron-like cells by treatment with defined small molecules (Kenpaullone, Forskolin, Purmorphamine, Retinoic acid) *in vitro*. These induced motor neuron-like cells expressed motor neuron markers and had the electrophysiological properties of neurons (Zhao et al., 2020).

## *IN VIVO* ASTROCYTE-TO-NEURON CONVERSION FOR OTHER CNS DISEASES

Given astrocytes are widely distributed in the CNS, *in vivo* astrocyte-to-neuron conversion is associated with other CNS disease. For example, the single neural transcription factor NeuroD1-based gene therapy can successfully reprogram astrocytes into functional neurons in damaged spinal cord (Puls et al., 2020). More recently, AAV-based NeuroD1 gene therapy can regenerate a large number of functional neurons, reduce microglia and macrophage activation, and protect parvalbumin interneurons in the converted areas, leading to restoring brain functions after ischemic injury in adult mice and non-human primates (Chen et al., 2020; Ge et al., 2020; Zhang X. et al., 2021). Many groups have also successfully converted astrocytes into proliferative neuroblasts by overexpression of transcription factor Sox2, and then further can be differentiated into neurons in mouse brain and injured adult spinal cord (Niu et al., 2013, 2015; Su et al., 2014; Islam et al., 2015; Wang et al., 2016).

## ADVANTAGES AND CHALLENGES OF *IN VIVO* ASTROCYTE-TO-NEURON CONVERSION

Recently, *in vivo* astrocyte-to-neuron conversion technology provides an alternative approach to regenerate functional new neurons in adult mammalian brains by directly reprogramming local astrocytes into neurons (Fyfe, 2017; Rivetti di Val Cervo et al., 2017; Qian et al., 2020; Zhou et al., 2020). This *in situ* neuron conversion technology not only provides an alternative strategy for regenerating functional new neuron by directly converting local astrocytes into neurons, but also avoid the disadvantages from the transplantation process in CNS. Given that astrocytes are abundant in CNS, the reprogramming of astrocytes into functional neurons *in situ* provides a new potential strategy to restore lost neuronal function, and this approach has no immunosuppression, no ethical concerns, as well as no cell transplantation. Beyond that, *in vivo* astrocyte-to-neuron conversion provide the possibility of targeting larger astrocyte numbers, perhaps it is possible that replace the widespread loss

of neurons in neurodegenerative diseases, such as AD, which transplantation can hardly do.

Although *in vivo* astrocyte-to-neuron conversion technology have a promising future in the development of regenerative medicine for neurodegenerative diseases, there are some challenges and limitations. Indeed, these approaches are still in their infancy (Gascón et al., 2017). It is unclear whether human astrocytes can also be converted into neurons by these approaches *in vivo*, if it success, whether the converted neurons are the wanted neuron type, can survive over long periods and integrate to neighbor neurons. As for the neurodegeneration caused by gene mutations, such as familial neurodegenerative diseases (Meyer et al., 2014; Przedborski, 2017; Singleton and Gasser, 2020; Wu et al., 2020; Zhang T. et al., 2021), the gene mutation is still present under certain genetic background, and these converted neurons from astrocytes might eventually degenerate. The efficiency of the existing direct lineage reprogramming strategy *in situ* still needs to be improved to meet clinical requirements. There are many varieties of influencing factors on reprogramming astrocytes into neurons *in vivo*. First, pathological environments, such as in AD or PD, are filled with various deleterious factors, such as ROS, inflammatory cytokines (Xu et al., 2016; Singh et al., 2019; Kwon and Koh, 2020) that all affect the neuronal reprogramming, survival and integration *in vivo*. Second, given that reprogramming astrocytes into neurons *in vivo* has to take place in a complex mixture of many various cell types, and the influences of the neighboring cells play an important role in the neuronal conversion and correct integration of the converted neurons (Gascón et al., 2017). Third, considering that neurodegenerative disorders tend to occur in older people, the age-related limitations of reprogramming must be taken into consideration, for example, astrocyte senescence is observed in patients of AD (Bhat et al., 2012), and PD (Chinta et al., 2018). Fourth, the mechanisms underpinning fate conversion and neuronal subtype identity is still unclear. Moreover, it remains to be a question whether it is possible to use small molecule strategy, rather than gene therapy, to direct the neuronal reprogramming *in vivo*. Thus, much more work remains to be done to develop a non-invasive manner approach for fully using the power of neuronal reprogramming in neuronal replacement therapies. Last but not least, it is exciting for *in vivo* astrocyte-to-neuron conversion has been successful in rodent models of PD (Rivetti di Val Cervo et al., 2017; Qian et al., 2020; Zhou et al., 2020), AD (Guo et al., 2014), and HD (Wu et al., 2020), even in non-human primates with ischemic stroke (Ge et al., 2020; Zhang X. et al., 2021), and it will be looking forward to the clinical application of direct neuronal reprogramming in neurodegenerative diseases.

## AUTHOR CONTRIBUTIONS

YW wrote the first draft. XZ and FC designed the figure. NS and JX reviewed and critiqued the manuscript. All authors contributed to the article and approved the submitted version.

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# Preservation Analysis on Spatiotemporal Specific Co-expression Networks Suggests the Immunopathogenesis of Alzheimer's Disease

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The occurrence and development of Alzheimer's disease (AD) is a continuous clinical and pathophysiological process, molecular biological, and brain functional change often appear before clinical symptoms, but the detailed underlying mechanism is still unclear. The expression profiling of postmortem brain tissue from AD patients and controls provides evidence about AD etiopathogenesis. In the current study, we used published AD expression profiling data to construct spatiotemporal specific coexpression networks in AD and analyzed the network preservation features of each brain region in different disease stages to identify the most dramatically changed coexpression modules and obtained AD-related biological pathways, brain regions and circuits, cell types and key genes based on these modules. As result, we constructed 57 spatiotemporal specific networks (19 brain regions by three disease stages) in AD and observed universal expression changes in all 19 brain regions. The eight most dramatically changed coexpression modules were identified in seven brain regions. Genes in these modules are mostly involved in immune response-related pathways and non-neuron cells, and this supports the immune pathology of AD and suggests the role of blood brain barrier (BBB) injuries. Differentially expressed genes (DEGs) meta-analysis and protein–protein interaction (PPI) network analysis suggested potential key genes involved in AD development that might be therapeutic targets. In conclusion, our systematical network analysis on published AD expression profiling data suggests the immunopathogenesis of AD and identifies key brain regions and genes.

**Keywords: Alzheimer's disease, spatiotemporal specific coexpression networks, network preservation analysis, immune response-related pathways, non-neuron cells, key genes**

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## INTRODUCTION

As the most common form of dementia, Alzheimer's disease (AD) is a major public health concern. The occurrence and development of AD is a continuous clinical and pathophysiological process, and the National Institute of Aging and Alzheimer's Association (NIA-AA) research framework categorizes AD into three continuous stages: preclinical, mild cognitive impairment (MCI), and

dementia (Albert et al., 2011; McKhann et al., 2011; Sperling et al., 2011). A large number of studies have pointed out that in AD, changes in molecular biological processes and brain function networks often appear before clinical symptoms, brain metabolic homeostasis, such as nerve growth factor metabolic pathway is impaired before clinical AD; a substantial proportion of nondemented older adults have amyloid-beta accumulation and amyloid plaque; lower functional connectivity was observed before cognitive changes by using resting-state MRI (Price et al., 2009; Sperling et al., 2011; Buckley et al., 2017; Pentz et al., 2020), but the detailed underlying mechanism is still unclear.

Brain transcriptome analysis is considered a powerful method for studying AD mechanisms, and many studies conducted to date have focused on the expression profiling of postmortem brain tissue from AD patients and controls (Blalock et al., 2004; Mirnics et al., 2005; Haroutunian et al., 2009; Kim et al., 2012; Zhang et al., 2013). In addition to finding differentially expressed genes (DEGs) that are significantly changed in AD patients, expression profiling can also provide more evidence about the systematic molecular processes underlying the etio-pathogenesis of AD. Based upon the associations between coexpressed gene modules and AD traits, several previous studies identified AD-related gene modules, which suggests that the biological processes that these genes contribute to may be affected in AD (Wang et al., 2016; Tang and Liu, 2019; Hu et al., 2020; Kelly et al., 2020). By using spatial-temporal expression pattern analysis, transcriptome data can also provide evidence about specific brain regions and cell types that are possibly related to AD (Wang et al., 2016). It has been reported that the spatial-temporal pattern of gene expression in the brain shows strong correspondence with brain function (Richiardi et al., 2015; Anderson et al., 2018), so expression profiling of AD patients may provide more information about brain functional changes during AD development.

In the current study, we used published AD expression profiling data to construct 57 spatiotemporal specific (19 brain regions by three disease stages) coexpression networks in AD. By analysing the network preservation features of each brain region in different disease stages, the most dramatically changed coexpression modules were identified. Based on these modules, AD-related biological pathways, brain regions and circuits, cell types and key genes were analyzed.

# DATA AND METHODS

## Gene Expression Profiling and Data Normalization

Dataset GSE84422 was downloaded from the National Biotechnology Information Center (NCBI) comprehensive gene expression database.[1] This dataset contains the expression data of 1,054 brain tissue samples distributed in 19 brain regions of 125 subjects (Wang et al., 2016). Based on their Clinical Dementia Rate (CDR) score, subjects in GSE84422 were divided into three groups: control group, CDR 0–0.5; mild group, CDR 1–2, severe group, CDR 3–5 (as shown in **Table 1**). The raw microarray data were preprocessed by using RMA with quantile normalization (Irizarry et al., 2003). According to different platforms, hgu133a.db, hgu133b.db, and hgu133plus2.db are used for ID conversion. The average expression value of the probe set for each gene was used as its expression value.

[1]https://www.ncbi.nlm.nih.gov/geo/

## Coexpression Network in Different Brain Regions

Weighted gene coexpression network analysis (WGCNA) was performed on dataset GSE84422 to identify the gene modules with coordinated expression patterns for each brain region in different disease severities (Zhang and Horvath, 2005). After data normalization, the top 25% of the expressed genes in each brain region at each disease stage were taken as input genes, and coexpression networks were constructed using the R package WGCNA (Langfelder and Horvath, 2008). Briefly, Pearson's correlation coefficients were calculated between all pairs of genes after microarray data normalization. Next, the correlation matrix was converted into an adjacency matrix using a power function $f(x) = x\beta$, where x was the element of the correlation matrix and parameter $\beta$ was determined such that the resulting adjacency matrix was approximately scale-free (Zhang and Horvath, 2005). The appropriate power value was estimated by a gradient test (power value ranging from 1 to 20) and determined when the scale independence value was equal to 0.85. The adjacency matrix was subsequently transformed into a topological overlap matrix (TOM), which captured both the direct and indirect interactions between each pair of genes (Ravasz et al., 2002). Average linkage hierarchical clustering was then employed to cluster the genes based on the TOM. Finally, a tree cutting algorithm was used to dynamically cut the hierarchical clustering dendrogram branches into highly connected modules, each of which was assigned a distinct colour code.

## Module Preservation Analysis

For each brain region, the preservation of coexpression modules across different disease stages was analyzed by using the R package NetRep (Ritchie et al., 2016). Coexpression networks of the control group were used as the discovery dataset, networks of the mild and severe groups were regarded as the tested datasets, and 10,000 permutations were performed. The NetRep statistics module preservation using seven statistical test methods, as recommended by the software, was applied. Modules whose *P*-value was less than 0.0001 in all seven methods were identified as strong preservation modules; those with *P*-values less 0.0001 in 1–6 methods were identified as weak preservation methods; and those with *P*-values not less than 0.0001 in any method were identified as non-preservation modules(NPMs).

## Functional Enrichment Analysis

To identify the biological processes in which NPM genes are involved, the Cytoscape plug-in ClueGO genes were used to provide a system-wide view (Bindea et al., 2009). The set including all NPM genes was used as the input gene set, and

**TABLE 1 |** Brain regions and disease groups information of samples used in co-expression analysis.

| Region | Full region name | Microarray Platform | Control | Mild | Severe |
|---|---|---|---|---|---|
| AC | Anterior cingulate | Affy 133 A and B | 23 | 18 | 18 |
| AMY | Amygdala | Affy 133Plus2 | 17 | 12 | 22 |
| CN | Caudate nucleus | Affy 133 A and B | 15 | 16 | 21 |
| DPC | Dorsolateral prefrontal cortex | Affy 133 A and B | 24 | 16 | 17 |
| FP | Frontal pole | Affy 133 A and B | 21 | 17 | 25 |
| HIPP | Hippocampus | Affy 133 A and B | 17 | 17 | 21 |
| IFG | Inferior frontal gyrus | Affy 133 A and B | 18 | 17 | 18 |
| ITG | Inferior temporal gyrus | Affy 133 A and B | 20 | 18 | 20 |
| MTG | Middle temporal gyrus | Affy 133 A and B | 22 | 14 | 22 |
| NAC | Nucleus accumbens | Affy 133Plus2 | 17 | 12 | 22 |
| OVC | Occipital visual cortex | Affy 133 A and B | 21 | 18 | 14 |
| PCC | Posterior cingulate cortex | Affy 133 A and B | 18 | 15 | 24 |
| PCG | Parahippocampal gyrus | Affy 133 A and B | 15 | 14 | 20 |
| STG | Superior temporal gyrus | Affy 133 A and B | 17 | 19 | 20 |
| PG | Precentral gyrus | Affy 133 A and B | 21 | 20 | 19 |
| PUT | Putamen | Affy 133 A and B | 16 | 18 | 18 |
| SFG | Superior frontal gyrus | Affy 133 A and B | 23 | 18 | 19 |
| SPL | Superior parietal lobule | Affy 133 A and B | 20 | 14 | 16 |
| TP | Temporal pole | Affy 133 A and B | 19 | 16 | 23 |

the ClueGo parameters were set as indicated: GO biological process, cellular component, and molecular function terms; display pathways with *P*-values $\leq$0.05; GO tree interval, three min level and eight max level; GO term minimum # genes, 5; threshold of 10% of genes per pathway; and a kappa score of 0.9. Pathway *P*-values were adjusted with Benjamini-Hochberg to 0.0100. The pathways were then represented, taking advantage of Cytoscape's complex visualization environment as kappa score-based functional groups and named by the most significant term of each group. For each NPM, a specific pathway cluster enrichment analysis was performed by using the online analysis tool DAVID (Huang da et al., 2009). As recommended in DAVID, the cut-off for pathway cluster enrichment was set at a score > 1.3. The representative biological terms associated with significant clusters were manually selected.

## 3-D Brain Region Module

3-D modules for brain regions affected by NPMs were formulated by using Mango image processing software (Lancaster, Martinez).[2] The labels of brain regions were obtained from the Talairach Atlas[3] (Lancaster et al., 2000).

[2]http://ric.uthscsa.edu/mango

[3]http://www.talairach.org/

## Cell-Type Enrichment Analysis

Cell-type enrichment analyses of NPM genes were performed with the web-based tool Brain Expression Spatio-Temporal pattern (BEST) in http://best.psych.ac.cn (Guo et al., 2019). Cell type-specific expression profiles, which provide specific expression gene sets for astrocytes, endothelial cells, microglia, neurons, and oligodendrocytes, were obtained from http://www.brainrnaseq.org (Zhang et al., 2016). Fisher exact tests (FETs) were performed between each NPM and cell type, and the negative logarithm of the FET *P*-value was defined as the enrichment score.

## Meta-Analysis of DEGs

Gene expression studies of AD that utilized tissue samples from the middle temporal gyrus (MTG) and total temporal cortex (TC) were searched in GEO by keyword searches and manual selection. In total, six datasets were selected for meta-analysis: GSE132903, GSE5281, and GSE84422 for MTG; GSE131617, GSE36980, and GSE118553 for TC. Sample statuses in different studies are heterogeneous, so only data of defined controls and AD patients were used, and data from patients with probable AD and other diseases were excluded. The sample information is summarized in **Table 3**. Data quality control and meta-analysis were performed with the online tool ImaGEO[4] (Toro-Dominguez et al., 2019). The maximum *P*-value method was selected, allowed missing values (%) was set at 10, and the adjusted *P*-value threshold was set at 0.05.

## Protein–Protein Interaction Analysis

DEGs and NPM genes in MTG were combined in the MTG gene set; DEGs in TC and NPM genes in ITG, MTG, and STG were combined in the TC gene set. The two gene sets were used as input data to perform protein–protein interaction (PPI) analysis in the online PPI network analysis platform STRING (Szklarczyk et al., 2019). The full STRING network was used to generate the MTG and TC networks by adding evidence edges between the input genes, and the minimum required interaction score of edges was 0.4. The generated networks were imported into Cytoscape, and the topological properties of the nodes were calculated using the plug-in "Network Analyzer" (Shannon et al., 2003; Assenov et al., 2008).

# RESULTS

## 57 Coexpression Gene Networks of 19 Brain Regions in Three Different Disease Stages

According to the topological structure of the coexpression network, the differences in networks with different organizations can be compared to analyze the spatial distribution of a disease. Therefore, we divided the gene set selected from 19 brain regions into 57 expression matrices according to the different brain regions and disease degrees, an unbiased gene

[4]http://bioinfo.genyo.es/imageo/

coexpression network of expression matrices was constructed, and coexpression modules were identified. The selection of the soft threshold and clustering results of each coexpression network can be seen in **Supplementary Figure 1**. The numbers of coexpression modules in each network are shown in **Figure 1**.

## Evidence of Preservation of the Coexpression Network in 19 Brain Regions

As shown in **Figure 2**, in the control groups, there were 233 coexpression modules in 19 brain regions; among them, 164 were less preserved in the process of AD, accounting for 73.54% of the total. Furthermore, eight modules in seven brain regions were identified as not preserved. Three modules were non-preserved in mild AD: the 12th in ITG (ITG-12), the 13th in SPL (SPL-13), and the 13th in STG (STG-13). Three modules were not preserved in severe AD: the 11th module in MTG (MTG-11), the 8th module in PUT (PUT-8) and the 9th module in PG (PG-9). Two modules are non-preserved in both mild and severe AD cases: the 12th module of the PUT brain regions (PUT-12) and the 22nd module of the PCG brain regions (PCG-22). Detailed gene lists of each NPM are shown in **Supplementary Table 1**.

## Functional Enrichment of the NPM Genes

We observed the functional distribution of the NPM genes by GO term network analysis using ClueGo. Finally, these genes were found to be enriched in 44 GO term groups (constructed by 85 GO terms). As shown in **Figure 3**, most of the enriched GO groups were related to the immune response, and functions related to cell differentiation, vesicle transport, and lipid metabolism were also involved. Functional enrichment analyses were also performed for genes in each NPM, as shown in **Table 2**. Genes in four NPMs were significantly enriched in the functional pathway clusters, and the enriched clusters were mainly related to the immune response.

## Brain Region Distribution of Non-preservation

**Figure 4** shows the brain region distribution of the NPMs in different disease stages. NPMs in mild stages are located in two gyri of the temporal cortex (superior temporal gyrus, inferior temporal gyrus) and a lobule of the parietal cortex (superior parietal lobule). NPMs in the severe stage are located in the temporal cortex (middle temporal gyrus), primary motor cortex (precentral gyrus), and basal nuclei (putamen). NPMs in both disease stages are located in the basal nuclei (putamen) and limbic system (parahippocampal gyrus).

## Cell-Type Enrichment of NPM Genes

Gene sets associated with five kinds of brain cells were used in enrichment analysis of genes in each NPM. **Figure 5** shows the enrichment scores of the NPMs in different cell types. According to the cut-off of 1.3 (equivalent to a *P*-value of 0.05 in FET), 2 modules were significantly enriched in astrocytes, 5 in endothelial cells, 4 in microglia, 2 in neurons, and 3 in oligodendrocytes. Strong significance appears in astrocytes, endothelial cells, microglia, and oligodendrocytes.

**TABLE 2 |** Pathway clusters significantly enriched by genes of non-preservation modules (enrichment score > 1.3).

| Non-preservation modules | Annotation cluster | Representative annotation terms | Enrichment score |
|---|---|---|---|
| ITG-12 | 1 | Immune response | 1.69 |
| SPL-13 | | None | |
| STG-13 | | None | |
| MTG-11 | | None | |
| PUT-8 | 1 | Defense response to virus | 10.78 |
| | 2 | Virus infection | 1.85 |
| | 3 | Proteolysis involved in cellular protein catabolic process | 1.51 |
| | 4 | Hydrolase | 1.4 |
| PG-9 | 1 | Defense response to virus | 5.76 |
| | 2 | Interferon-gamma-mediated signaling pathway | 2.04 |
| | 3 | Negative regulation of transcription from RNA polymerase II promoter | 1.90 |
| | 4 | CUB domain | 1.79 |
| | 5 | Golgi apparatus | 1.66 |
| | 6 | EGF-like domain | 1.56 |
| | 7 | Complement pathway | 1.43 |
| PUT-12 | | None | |
| PCG-22 | 1 | Cholesterol metabolism | 1.48 |

## PPI Network of DEGs and NPM Genes in MTG and TC

Since all three temporal gyri are involved in disease development, meta-analyses were performed to identify DEGs in the temporal cortex or specific temporal gyrus. Finally, six data sets were selected, and the results of the meta-analyses are summarized in **Table 3**. Detailed information on the meta-analysis is shown in **Supplementary Tables 2, 3** and **Supplementary Figures 2–5**. No overlap was observed between the DEGs and NPM genes, but there were universal PPIs among them (as shown in **Figures 6A,B**). As shown in **Table 4**, 177 of 241 MTG genes (including DEGs and NPM genes) can be found in STRING, 342 interactions

**TABLE 3 |** Datasets used in the meta-analysis.

| Brain region | dataset | cases | controls | DEGs | DEGs after meta-analysis |
|---|---|---|---|---|---|
| MTG | GSE132903 | 97 | 98 | 6,908 | 192 |
| | GSE5281 | 16 | 12 | 2,253 | |
| | GSE84422 | 20 | 14 | 0 | |
| Temporal cortex | GSE131617 | 58 | 13 | 0 | 62 |
| | GSE36980 | 10 | 19 | 258 | |
| | GSE118553 | 45 | 26 | 2,475 | |

**TABLE 4 |** Summary of nodes in the PPI networks of MTG and TC.

| | MTG | | | | TC | | | |
|---|---|---|---|---|---|---|---|---|
| | DEGs | | NPM genes | | DEGs | | NPM genes | |
| Total number | 192 | | 49 | | 62 | | 145 | |
| Numbers in PPI networks | 137 | | 40 | | 44 | | 97 | |
| Average degree | 3.88 | | 3.88 | | 2.41 | | 4.3 | |
| **High degree genes (degree ≥ 10)** | **Gene symbol** | **Degree** | **Gene symbol** | **Degree** | **Gene symbol** | **Degree** | **Gene symbol** | **Degree** |
| | NOTCH1 | 31 | SOX9 | 18 | PSMC3 | 11 | ISG15 | 20 |
| | GFAP | 15 | EZR | 13 | | | CXCL10 | 18 |
| | YAP1 | 14 | NTRK2 | 11 | | | STAT1 | 18 |
| | CXCL12 | 13 | VCAN | 10 | | | GBP1 | 14 |
| | NEUROD1 | 12 | | | | | CXCL11 | 13 |
| | DCN | 11 | | | | | PSMB10 | 12 |
| | ASCL1 | 11 | | | | | GBP2 | 12 |
| | ABL1 | 11 | | | | | ICAM1 | 11 |
| | COL1A2 | 10 | | | | | MX1 | 11 |
| | MYH11 | 10 | | | | | NEDD8 | 11 |
| | | | | | | | AGT | 10 |
| | | | | | | | HERC6 | 10 |
| | | | | | | | IFIT1 | 10 |

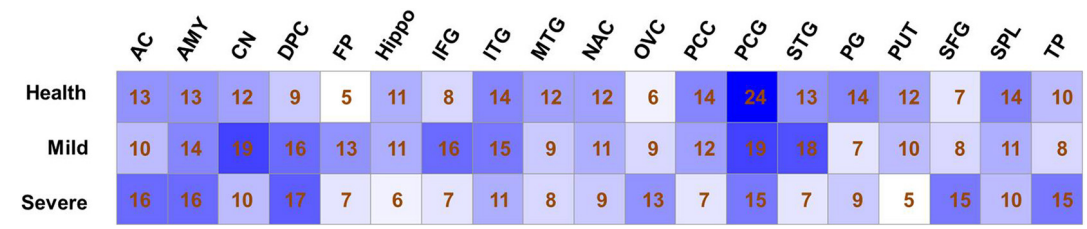


**FIGURE 1 |** Modules number of co-expression networks in different sample sets. The number of co-expression modules that identified by Weighted gene coexpression network analysis analysis is shown for each sample set.

between them can be found, significantly ($P = 2.83E\text{-}14$) higher than the predicted interaction number 222, predicted by the average interaction number in the STRING network. A total of 141 of 207 TC genes were found in STRING, and 262 interactions were found among them, which was significantly ($P = 0.00145$) higher than the predicted number of 216. The average degree, degree distribution, and high degree genes (degree ≥ 10) are shown in **Table 4** and **Figure 6C**.

## DISCUSSION

In the current study, we explored AD-related biological processes by analysing the coexpression gene modules of different brain regions in different disease stages. The coexpression network features and key genes of AD peripheral blood or brain have been reported in several previous studies (Seyfried et al., 2017; Liang et al., 2018; Sweeney et al., 2018; Zhang et al., 2018; Hu et al., 2020; Kelly et al., 2020; Soleimani Zakeri et al., 2020). Among these studies, Wang et al. performed a pan-cortical brain region genomic analysis, obtained and ranked 44,692 gene probesets, 1,558 coexpressed gene modules and 19 brain regions based upon their association with AD; through these analyses temporal lobe gyri were identified as sites associated with the greatest and earliest gene expression abnormalities, abnormal expression was specific to cell type of oligodendrocytes, astrocytes, and neurons, and neurobiological pathways (included actin cytoskeleton, axon guidance, and nervous system development) were enriched by abnormally expressed genes and modules (Wang et al., 2016); however, the changes in coexpression modules in sub-brain regions during AD development have not been fully studied. We constructed 57 coexpression networks by using this expression dataset from 1,053 postmortem brain samples across 19 cortical regions, evaluated network conservation during disease pathology (from healthy to mild and severe AD stages) in each brain region, and deduced disease-related biological processes based on the network features.

As we expected, in the development of AD, there is a wide range of coexpression pattern changes in the whole brain. This suggested that dysfunctions of expression appear in multiple brain regions, not only in brain regions that are

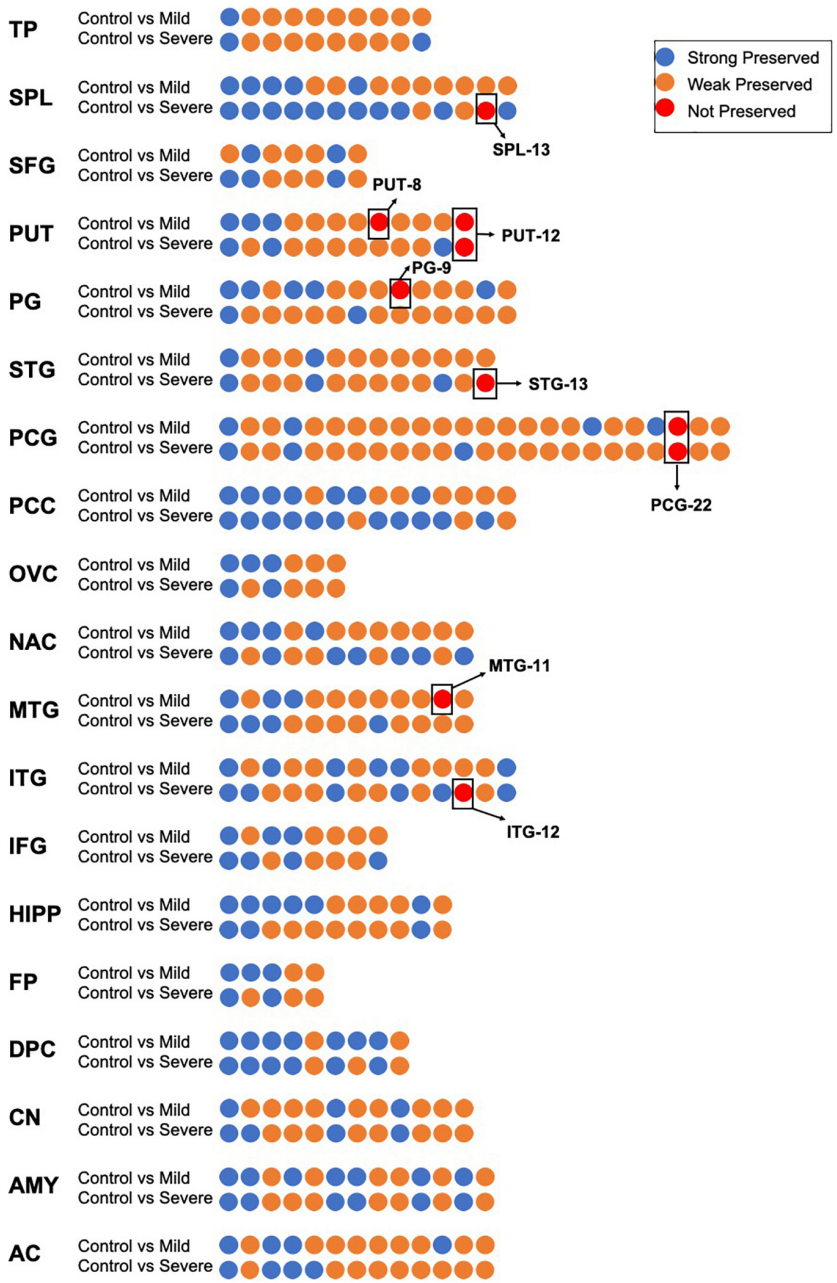


**FIGURE 2 |** The preservation evidence of control co-expression modules in different disease stages and different brain regions. For each brain region, co-expression networks in control group was compared with networks in mild and severe groups, co-expression modules are show as dots, which are sorted according to module size and colored according to their preservation evidence. Blue: strong preservation evidence; yellow: weak preservation evidence; red: none preservation evidence.

traditionally associated with memory. We focused on the eight most dramatically changed coexpression modules. Functional pathway analysis suggested that genes in these modules are mainly involved in the immune response instead of transmitters or other pathways that directly affect neuronal function. These results supported the neuroimmunopathogenesis of AD. In recent years, AD has no longer been considered a neural-centric disease, and the critical role played by neuroinflammation in the pathogenesis of AD has been implicated in many genetic, functional, and neuroimaging studies (Cao and Zheng, 2018; Jansen et al., 2019; Kunkle et al., 2019; Passamonti et al., 2019; Bis et al., 2020; Burgaletto et al., 2020).

The most dramatic coexpression pattern changes occurred in seven brain regions. These results are consistent with previous brain structure or functional studies. Coexpression patterns in five regions began to change dramatically in the mild stage, including two subregions of the temporal cortex, one lobule of the parietal cortex, part of the dorsal striatum, and the

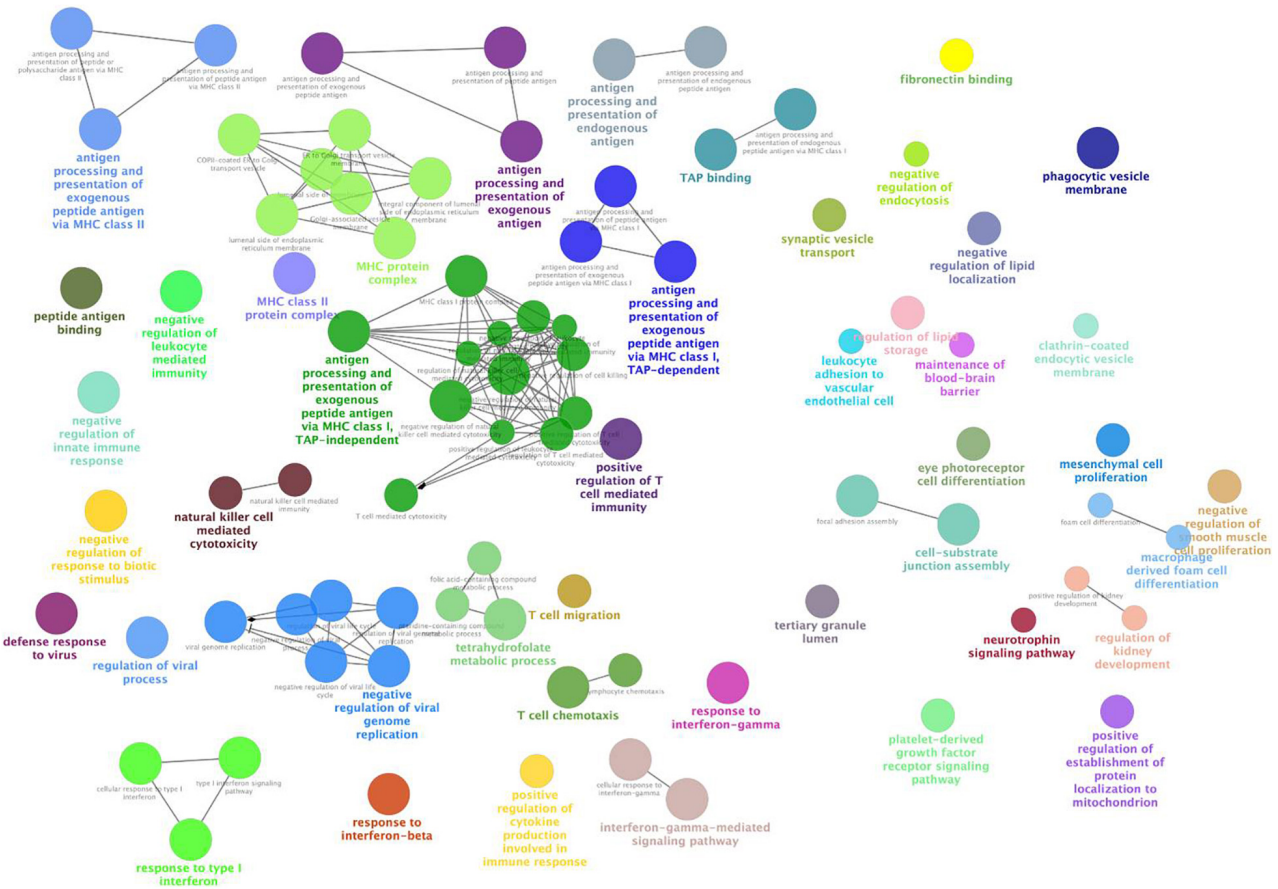


**FIGURE 3 |** Functional GO groups enriched by genes in non-preserved co-expression modules. This figure illustrates the functionally grouped network that constructed by GO terms (nodes) that associated to non-preserved co-expression module genes. The size of the nodes reflects the statistical significance of the terms. The degree of connectivity between terms (edges) is calculated using kappa statistic and functional groups are defined using the kappa score. The name of the group is given by the most significant term in the group and nodes in the same group are represented with same color.

parahippocampal gyrus in the limbic system. The associations of structural or functional changes in such brain regions and early AD pathology have been widely reported. It has been reported that tau pathology spreads hierarchically from the inferior temporal lobe throughout the cortex (Franzmeier et al., 2019), and neural activity increases in the superior parietal lobule of patients with MCI (Jacobs et al., 2012). Associations between dysfunction of the limbic system or basal ganglia and early AD have also been reported (Hopper and Vogel, 1976; Nestor et al., 2003; de Jong et al., 2008; Botzung et al., 2019). In severe AD, dysfunction spreads to the middle temporal gyrus and precentral gyrus. The relationship between the middle temporal gyrus and AD has been emphasized in many studies (Galton et al., 2001b; Dong et al., 2021). Although it has not been fully studied, the relationship between the precentral gyrus and AD has been reported in several studies (Peters et al., 2009).

Considering the correlation between brain expression patterns and brain functional connections (Richiardi et al., 2015; Anderson et al., 2018), the expression changes suggested potential connectivity changes in AD, and the evidence in the current study corresponds to previous reports. Regional tau PET levels within major functional networks showed a medial temporal limbic network-specific distribution (Franzmeier et al., 2019), and limbic network and striatal connectivity alternated in patients with AD and MCI (Badhwar et al., 2017), and an increased effectiveness of temporoparietal connectivity has been reported in AD patients (Jacobs et al., 2012).

In addition to brain region distribution features, violent coexpression pattern changes present a cell type-specific distribution. In general, all NPM genes were most significantly enriched in non-neuron cells (astrocytes, endothelial cells, microglia, and oligodendrocytes), and only NPM genes in the SPL and putamen showed an enrichment trend in neurons. Considering the roles of glia in AD and the immune response (Gonzalez-Reyes et al., 2017; Fakhoury, 2018; Leng and Edison, 2021), the enrichment of astrocytes and microglia may highlight the immunopathology in AD. Oligodendrocytes are located in the white matter, recent neuroimaging studies have implicated micro- and macrostructural abnormalities in white matter in the risk and progression of AD (Nasrabady et al., 2018), it's reported that age and severity of dementia were significantly associated with white matter changes in AD patients (Kao et al., 2019),

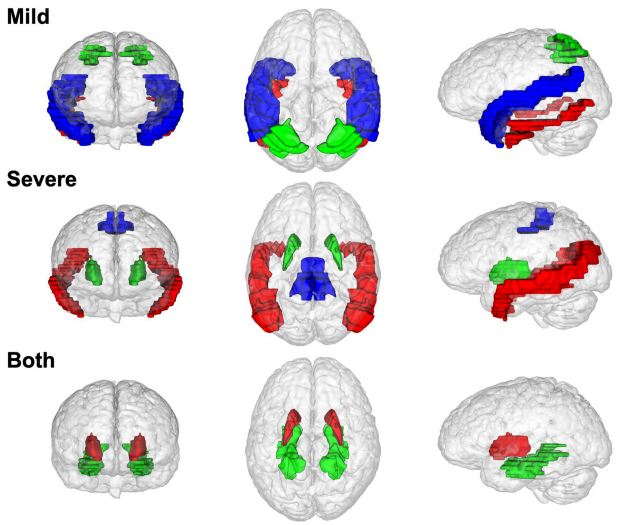


**FIGURE 4 |** The brain region distribution of non-preserved modules in different Alzheimer's disease stages. Modules that non-preserved in mild stages are located in superior temporal gyrus (blue), inferior temporal gyrus (red), and superior parietal lobule (red). Modules that non-preserved in severe stage are located in middle temporal gyrus (red), putamen (green), precentral gyrus (blue). Modules that non-preserved in both two disease stages are located in putamen (red) and parahippocampal gyrus (green).

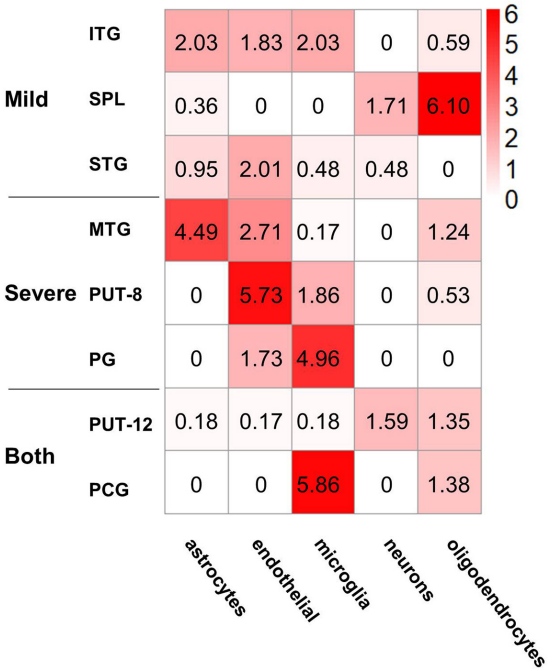


**FIGURE 5 |** Cell type enrichment of non-preserved modules in different brain regions. The heatmap shows the enrichment results of five kinds of cell type-specific genes in the eight non-preserved modules, the color in heatmap illustrated the negative logarithm of P-value of the enrichment, from white (-log(*P*-value) = 0) to red (-log(*P*-value) > 6). The significant cutoff is defined as 1.3, which is equivalent to *P*-value 0.05.

and white matter may also play an important role in the pathogenesis and diagnosis of AD, besides of intact with gray matter, demyelination of the white matter is reported to occur prior to the presence of amyloid-β plaques and neurofibrillary tangles in the presymptomatic stages of AD (Sachdev et al., 2013). Oligodendrocytes may affect AD pathogenesis in both neuropathological and immunopathological manners, oligodendrocytes are regulated by Aβ oligomers in differentiation and maturation (Quintela-Lopez et al., 2019), oligodendrocyte precursor cells present antigens and may be involved in perpetuating the autoimmune response (Kirby et al., 2019). Endothelial cells participate in the formation of the blood brain barrier (BBB), and Aβ influences endothelial mitochondrial dysfunction pathways and contributes to the progression of neurovascular dysfunction in AD. The enrichment results in the current study also support BBB-related pathology in AD (Parodi-Rullan et al., 2019). Additionally, the functional enrichment analysis also suggested that the dysfunction of NPM genes influences the maintenance of the BBB.

The temporal lobe, especially the MTG, is an important brain area involved in cognition and memory (Kornblith et al., 2017; Naya et al., 2017; Vaz et al., 2019) and has been a focus in AD pathology (Galton et al., 2001a; Visser et al., 2002; Dickerson and Sperling, 2008; de Flores et al., 2020). Several genes have been reported to be differentially expressed in the temporal lobe between patients with AD and healthy controls, but the results of different studies are heterogeneous (Patel et al., 2019). In the current study, we searched transcriptomics data of MTG or TC and performed a meta-analysis. As shown in **Table 3**, articles in both groups presented high heterogeneity. When the cut-off of DEGs was set as a *P*-value less than 0.05 and a fold change of more than two or less than 0.5, both meta-analyses failed to identify DEGs. This may be due to the high degree of heterogeneity among the studies. When the cut-off was adjusted to a *P*-value less than 0.05, some stable but not violent DEGs were identified. None of these DEGs were in NPMs, but they were extensively connected to NPM genes in PPI networks. This suggests that AD pathology is not caused by drastic changes in a few genes but is related to changes in the entire expression pattern. A large number of genes were involved in the pattern change, but for a single gene, the change was not dramatic.

Network topology analysis showed that the degree of NPM genes was higher than that of DEGs, but this trend was not significant. According to the hypothesis that disease genes tend to have higher degrees in the network (Jonsson and Bates, 2006; Sun et al., 2010), high degree genes were identified and may play more important roles in the functional network and AD pathology. Several high degree genes have been reported to participate AD pathology related pathways or related to AD, *ABL1,SOX9, STAT1, PSMB10, NEDD8, HERC6*, and *IFIT1* have been reported as participants of amyloid- or Tau-signaling (Jing et al., 2009; Chen et al., 2012; Orre et al., 2013; Woodling et al., 2014; Li et al., 2019; Vong et al., 2021); *DCN, CXCL10, CXCL11*, and *ICAM1* play roles in amyloid plaque formation (Frohman et al., 1991; Snow et al., 1992; Krauthausen et al., 2015); *NOTCH1, GFAP, YAP1, CXCL10,CXCL12, ASCL1, STAT1, GBP1, GBP2*, and *AGT*

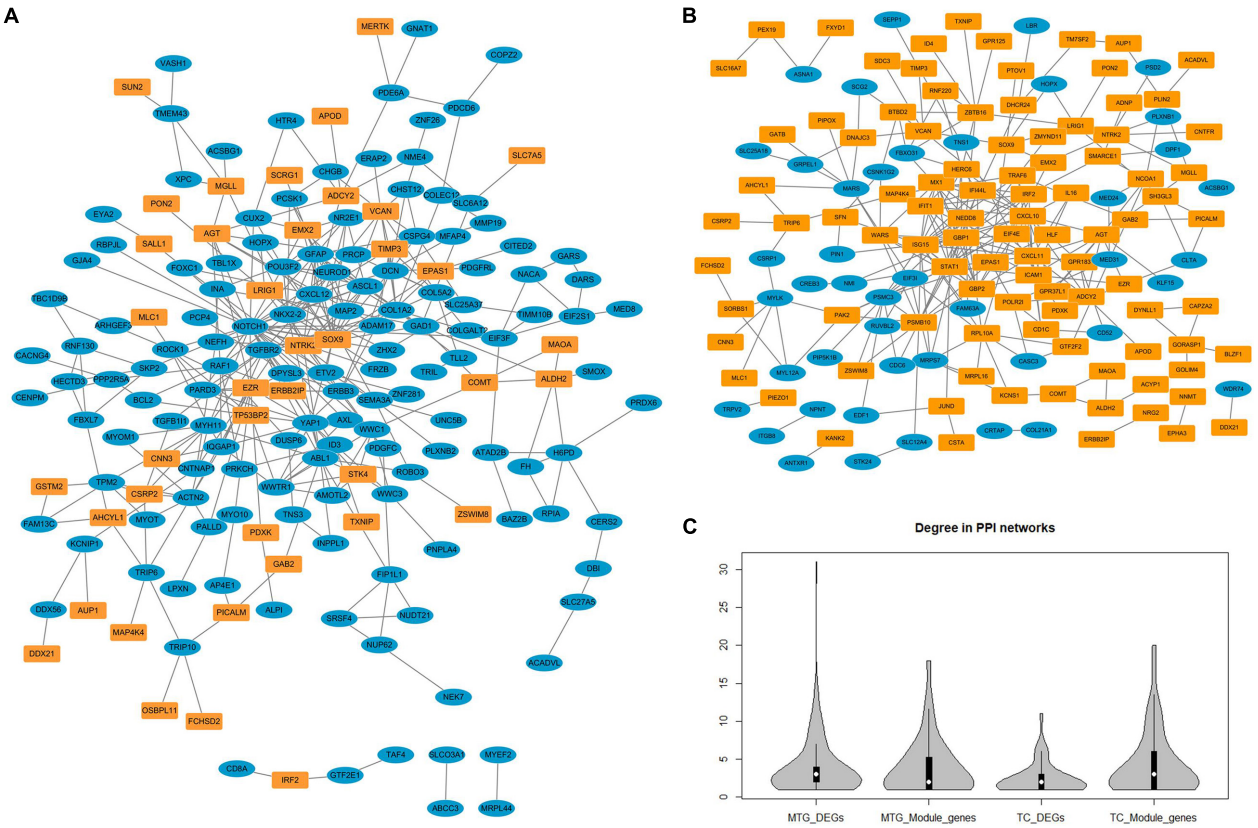


**FIGURE 6 |** The protein–protein interaction networks constructed by differentially expressed genes and genes in non-preserved modules. **(A)** Protein-protein interaction network constructed by middle temporal gyrus differentially expressed genes (blue nodes) and non-preserved module genes (yellow nodes). **(B)** Protein–protein interaction network constructed by temporal cortex differentially expressed genes (blue nodes) and non-preserved module genes (yellow nodes). **(C)** The degree distribution of different types of genes in protein-protein interaction network.

are altered expression in plasma, CSF, or brain of AD patients (Kitamura et al., 1997; Galimberti et al., 2006; Laske et al., 2008; Mateos et al., 2011; Xu et al., 2018; Cho et al., 2019; Meyer et al., 2019; Oeckl et al., 2019; Kuan et al., 2021); AD association of variants in or surrounding *MYH11, NTRK2, PSMC3* and *ISG15* have been detected (Chen et al., 2008; Roy et al., 2020; Blue et al., 2021; Novikova et al., 2021). Relationships between AD and high degree genes *COL1A2, EZR,* and *VCAN* haven't been reported, their roles in AD pathology need further study.

In conclusion, in the current study, we constructed 57 spatiotemporal specific coexpression networks in AD. By using network preservation analysis, we observed universal expression changes in all 19 brain regions. The eight most dramatically changed coexpression modules were identified in seven brain regions. Genes in these modules are mostly involved in immune response-related pathways, this supports the immune pathology of AD. The distribution of NPMs provides evidence of the brain functional mechanism of AD. The cell type distribution of NPMs also suggests the role played by the immune response and BBB injuries. In addition to revealing information about the potential etiopathogenesis of AD, our analysis suggested potential key genes involved in AD development that might be therapeutic targets.

Comprehensive analysis in this study provides new evidence for the immunopathological mechanism of AD, reveals the potential key brain regions, cells and molecular pathways in the development of AD. It provides new clues for the mechanism and intervention study of AD. In spite of above results, this study also has some limitations. Analysis in this study is based on public data, and the new results have not been verified in new samples or animal models, subsequent studies are needed to validate their stability. Our analyses only use expression data, genetic factors (such as APOE genotype) and demographic factors (such as gender and age) have not been considered, so the results should be further validated in more diverse populations. Although several high degree genes we identified have been reported altered expression in AD patients, more systematic validation and consequence functional researches should be performed to confirm their value.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

JW and LG designed the study and drafted the manuscript. LG and YL performed the analysis. All authors have read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.727928/full#supplementary-material

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REVIEW
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# Role of Glia-Derived Extracellular Vesicles in Neurodegenerative Diseases

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Extracellular vesicles (EVs), as nano-sized vesicles secreted by almost all cells, have been recognized as the essential transmitter for cell-to-cell communication and participating in multiple biological processes. Neurodegenerative diseases (ND), such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, share common mechanisms of the aggregation and propagation of distinct pathologic proteins among cells in the nervous systems and neuroinflammatory reactions mediated by glia during the pathogenic process. This feature indicates the vital role of crosstalk between neurons and glia in the pathogenesis of ND. In recent years, glia-derived EVs have been investigated as potential mediators of signals between neurons and glia, which provides a new direction and strategy for understanding ND. By a comprehensive summary, it can be concluded that glia-derived EVs have both a beneficial and/or a detrimental effect in the process of ND. Therefore, this review article conveys the role of glia-derived EVs in the pathogenesis of ND and raises current limitations of their potential application in the diagnosis and treatment of ND.

**Keywords: extracellular vesicles, glia, neurodegenerative diseases, astrocyte, microglia**

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## INTRODUCTION

Neurodegenerative diseases (ND) are a varied assortment of central nervous system (CNS) disorders characterized by the progressive loss of neurons and appearance of abnormal proteinaceous assemblies in the nervous system, such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Rachakonda et al., 2004; Jucker and Walker, 2018; DeTure and Dickson, 2019). Although significant achievements in the knowledge on pathogenic mechanisms of ND have been made, there is still an urgent need and has been actively pursued in the field for reliable biomarkers for early diagnosis and management of the disease course (Li and Le, 2017; Zetterberg and Biennow, 2021). An even more serious concern is that ND, as a major health problem for the world's aging population, has no cure in sight. To overcome these obstacles, a growing body of research is focusing on the role of cell-to-cell communications in the pathogenesis of ND (Mathieu et al., 2019).

It has been shown that glia-neuron crosstalk in the CNS is fundamental for various biological functions, ranging from brain development, neural circuit maturation, and homeostasis maintenance. Understanding how to assess and modulate glia-neuron interactions is essential for developing effective therapies for ND (Bartels et al., 2020).

The crucial role of extracellular vesicles (EVs) in glia-to-neuron communications has been recognized in recent years. EVs are cystic vesicles with a double-layer membrane structure, first identified from the blood (Wolf, 1967). Later studies further discovered that almost all eukaryotic cells could secrete EVs, such as stem cells, various organ cells, and cells in the CNS, including neurons, astrocytes, microglia, and oligodendrocytes (Budnik et al., 2016). EVs can be classified according to cell sources or the origin of their membrane. Using the latter categorization, EVs can be divided into microvesicles (100–1,000 nm in diameter) and exosomes (30–50 nm diameter) (van Niel et al., 2018). The emerging data from electron microscopy, flow cytometry, high-throughput proteomics, and genomics have confirmed that EVs contain plentiful amounts of cell components and can migrate to specific organs or cells, thereby diverting the contents into the recipient to modulate the state of the cell (Vinuesa et al., 2019). The characteristics of EVs determine the functions of glia-derived EVs on the glia-neuron crosstalk, which provides enormous potential for investigating the pathogenesis and new therapeutic targets for ND.

In this review, we will summarize the advances of EV research in the field of ND, with a specific focus on glia-derived EVs. Effects of glia-derived EVs in the inter-cellular communications among astrocytes, microglia, oligodendrocytes, and neurons are described. Furthermore, two distinct roles: beneficial and detrimental roles of glia-derived EVs in the pathogenesis of typical ND, including AD, PD, and ALS, are particularly discussed. We also provide an update on the glia-derived EVs as potential biomarkers for ND. Recent developments on the application of glia-derived EVs as new nanotherapeutics are also briefly reviewed here.

## EXTRACELLULAR VESICLES IN GLIA-NEURON COMMUNICATION

### Astrocyte-Derived Extracellular Vesicles on Neurons

Astrocytes are the most abundant cell type in the brain. They actively contribute to various essential functions in the CNS, including the formation and maintenance of the blood-brain barrier (BBB), synapse formation and plasticity, ion homeostasis, neurotransmitter buffering, and the secretion of neuroactive agents (Freeman, 2010; Linnerbauer et al., 2020). Neurons and astrocytes orchestrate CNS homeostasis through multiple mechanisms of transcellular communication, which range from local cell-to-cell direct contact to the release of neurotransmitters and EVs (Huang et al., 2018).

Constitutively secreted astrocyte-derived EVs have now emerged as crucial players in maintaining normal neuronal functions, including promoting neurite outgrowth and neuronal survival (Chaudhuri et al., 2018). For example, prion protein (PrP) in astrocytes involves the perception of oxidative stress. The release of EVs carrying PrP from astrocytes may improve the survival of neurons under hypoxic and ischemic conditions (Guitart et al., 2016). Apolipoprotein D (ApoD), a classical neuroprotective protein, can be exclusively transported by EVs from astrocytes to neurons, thereby promoting functional integrity and survival of neurons against oxidative stress (Pascua-Maestro et al., 2019). In addition, *Clostridium botulinum* C3 transferase (C3bot) has vimentin-dependent axonotrophic effects and may participate in the molecular crosstalk between astrocytes and neurons. A study presented evidence that the astrocytic release of vimentin by EVs has neuro-regenerative and plasticity augmenting effects through the interaction of C3bot with neuronal membranes after spinal cord injury (Adolf et al., 2019). Besides, there is evidence that astrocytes can regulate the dendritic development of neurons by the cargo miRNA and mtDNA of their derived EVs (Guescini et al., 2010; Luarte et al., 2020).

What's more, astrocytes can alter the cargo of astrocyte-derived EVs in response to different stimuli, such as neuroinflammation (Chaudhuri et al., 2020; You et al., 2020). Recent evidence showed that human astrocyte-derived EVs activated by interleukin (IL)-1β could reduce the neurite outgrowth, branching, and neuronal firing of the primary cultured mouse cortical neurons (You et al., 2020). Astrocytes-derived EVs secreted in response to IL-1β and tumor necrosis factor (TNF)-α were enriched with miRNAs that target proteins involved in neurotrophin signaling. Downregulation of the target genes of these miRNAs in neurons was associated with reductions in dendritic growth, dendritic complexity, reduced spike rates, and burst activity (Chaudhuri et al., 2020). However, contrary to the above, astrocyte-derived EVs have neuroprotective effects when triggered by certain conditions. It has been shown that astrocytes, when subjected to hyperthermia, can secrete EVs containing heat shock protein Hsp/c70, as well as intracellular signaling components including activated forms of extracellular-signal-regulated kinase, Akt, and Jun N-terminal kinase (JNK)/SAPK that may have implications for the survival of neurons (Taylor et al., 2007). Studies have also demonstrated that astrocytes-derived EVs can inhibit autophagy and ameliorated neuronal damage in oxygen and glucose deprivation conditions by releasing specific microRNAs (Pei et al., 2020; Xu et al., 2019). A quantitative proteomic profile analysis aims to explore the cargo of astrocyte-derived EVs in response to various stimuli and found that EVs from astrocytes treated with ATP and IL-10 contains a series of proteins that play a role in enhancing neurite outgrowth, regulation of synaptic transmission, dendritic branching, and promoting neuronal survival. On the contrary, astrocyte-derived EVs released in response to IL-1β comprise proteins that regulate peripheral inflammatory response and immune cell trafficking to the CNS (Chaudhuri et al., 2018). From the evidence above, it can be concluded that different types of stimuli have opposed effects on the survival of the neurons. The impact degree and mechanisms of these stimuli on EVs releasing remain to be further studied.

## Microglia-Derived Extracellular Vesicles on Neurons

Microglia account for approximately 10% of cells in the CNS, which usually exist in a resting state and contribute to modulating the strength of synaptic transmissions and sculpting neuronal synapses (Colonna and Butovsky, 2017). Microglia appear to be heterogeneous with diverse functional phenotypes that range from pro-inflammatory M1 phenotypes to immunosuppressive M2 phenotypes (Tang and Le, 2016; Zhang et al., 2017; Wolters et al., 2021). Once sensing damage signals or toxic aggregated proteins inside or outside the cell, microglia will switch from resting to active (Zhong et al., 2018; Dong et al., 2020). The continuous crosstalk between microglia and neurons is dependent on microglia housekeeping functions and contributes to the homeostasis of CNS (Marinelli et al., 2019). In addition to the chemokines and cytokines, an increasing body of evidence indicates that EVs are also crucial to microglia-neuron communication.

Microglia-derived EVs have been shown to participate in the miscellaneous physiological functions, including metabolic supporting of neurons, regulating synaptic activity and transmission, as well as neuronal survival. For instance, a proteomic profile of the microglial-derived EVs from primary microglia following treatment with recombinant carrier-free Wnt3a showed that the altered proteins are involved in cellular architecture, metabolism, protein synthesis, and degradation (Hooper et al., 2012). In a lipotoxic context emulated by incubating primary microglia with palmitate, microglial-derived EVs induced the morphologic alterations of the dendritic spine in primary hippocampal neurons (Vinuesa et al., 2019). Concerning the function of neuronal survival, M2 microglia-derived EVs were demonstrated to attenuate ischemic brain injury and promoted neuronal survival via miR-124 and its downstream target USP14, which have been shown to participate in the regulation of brain-derived neurotrophic factor and fibroblast growth factor (Song et al., 2019). As neuroprotective cargos, other miRNAs have also been reported to be altered in the microglial-derived EVs. And they could be the EVs-dependent connections between microglia and neurons, which is worth researching further (Lemaire et al., 2019).

Plenty of evidence indicates that different subsets of microglial-derived EVs have diverse functional properties, and specific signaling pathways may regulate the trafficking of EVs. On the one hand, EVs from pro-inflammatory microglia (M1 phenotype) have been shown to contribute to the pro-neuroinflammation response. EVs contained pro-inflammatory molecules initially released by microglia following the external stimulus can activate more microglia that may contribute to the progressive neuroinflammatory response (Kumar et al., 2017). Besides, the crosstalk between EVs and inflammasome is becoming one of the hotspots in inflammatory reactions (Noonin and Thongboonkerd, 2021). It has been discovered that lipopolysaccharide (LPS)-primed microglia exposed to manganese secrete more EVs that contain ASC, a component of the inflammasome complex. And ASC, in turn, leads to the increase of NLRP3 and pro-IL-1β in microglia, thus promoting the inflammasome activation (Sarkar et al., 2019). What's more, a distinct profile of proteins was identified in EVs released from LPS treated microglia compared to the control (Yang et al., 2018). The research aims to explore the relationships among inflammatory microglia, their released EVs, and the synaptic defects of neurons, has identified miR-146a-5p, a microglia-specific miRNA, targets two kinds of adhesion protein that play a crucial role in dendritic spine formation and synaptic stability. The results also show that inflammatory EVs transfer miR-146a-5p cargo to neurons and significantly decrease dendritic spine density in hippocampal neurons (Prada et al., 2018).

On the other hand, microglia-derived EVs may also have a protective effect on neurons through the anti-inflammatory response (M2 phenotype). There's research shows that microglia-derived EVs carrying inflammation-related genes can transfer signals to the glioma cells in the brain, thereby playing a role in reducing neuronal death and promoting the recovery of brain homeostasis (Grimaldi et al., 2019). When cultured with brain extracts of traumatic brain injury mice, EVs released from microglia have a significantly higher level of miR-124-3p, promoting the anti-inflammatory M2 polarization in microglia and ameliorating the inflammatory reaction in scratch-injured neurons (Huang et al., 2018). The above evidence indicates the influence of microglia-derived EVs subjected to an inflammatory environment can be detrimental or beneficial is depended on the context, supporting the need to investigate the involvement of microglial-derived EVs in different pathological conditions in more detail.

## Oligodendrocyte-Derived Extracellular Vesicles on Neurons

The role of oligodendrocytes in the CNS is to insulate axons, maintain axonal health and transmit the rapid impulse with a multilayered myelin sheath. The formation of functional myelin sheath-axon entity depends on the interactions between the oligodendrocyte and neuron. Neurons can internalize oligodendrocyte-derived EVs in response to multiple neuronal signals and present neuroprotective properties in the CNS (Krmer-Albers, 2020). Previous research found that oligodendrocyte-derived EVs carrying specific protein and RNA cargo can be secreted under the stimulation of neurotransmitter glutamate triggers mediated by $Ca^{2+}$ entry through oligodendroglial NMDA and AMPA receptors. Notably, these oligodendrocyte-derived EVs have multiple effects on neurons under conditions of cell stress, including improving neuronal viability, modulating neuronal outgrowth and metabolism (Frühbeis et al., 2013). The same research team went on to show that oligodendrocyte-derived EVs contribute to resisting oxidative stress and promoting neuronal survival through the diversion of superoxide dismutase and catalase during oxygen-glucose deprivation (Fröhlich et al., 2014). Analyses of two mouse models deficient in oligodendrocyte-specific genes showed that wild-type oligodendrocyte-derived EVs improve the metabolic state and promote axonal transport in nutrient-deprived neurons. In contrast to that, mutant oligodendrocytes release fewer EVs

along with underrepresented proteins cargo. Notably, mutant oligodendrocyte-derived EVs lost the function of maintaining the nutrient-deprived neurons and promoting axonal transport (Frühbeis et al., 2020).

## EXTRACELLULAR VESICLES IN GLIA-TO-GLIA COMMUNICATION

In addition to acting on neurons, recent studies have shown that glia-derived EVs also play a vital role in intercellular glia-to-glia communications. It has been demonstrated that EVs secreted from young astrocytes, but not those from aged astrocytes, can convey support for the differentiation of oligodendrocytes through altering the expression of proteins in oligodendrocyte progenitor cells, resulting in the maturation and survival of oligodendrocyte (Willis et al., 2020). A recent study discovered that EVs derived from activated astrocytes could accelerate the transformation of the microglial anti-inflammatory phenotype. And miR-873a-5p, as one of the components of these astrocyte-derived EVs, may play a vital role in the interactions between astrocytes and microglia (Long et al., 2020). There is also evidence that EVs released by pro-inflammatory microglia can block remyelination.

In contrast, EVs from microglia and mesenchymal stem cells co-cultured systems contribute to the recruitment of oligodendrocyte precursor cells (OPC) and myelin repair. Moreover, astrocytes play an essential role in regulating the inflammatory reaction of microglia-derived EVs on OPC (Lombardi et al., 2019). Typically, transmitting EVs between astrocytes and microglia can form an inflammatory positive feedback loop, leading to dysregulation and amplification of the neuroinflammatory response.

## ROLE OF GLIA-DERIVED EXTRACELLULAR VESICLES IN NEURODEGENERATIVE DISEASES

In recent years, glia-derived EVs have been investigated as a potential transmitter of information between neurons and glia, which involves the pathological processes of ND (**Table 1**). However, the role of glia-derived EVs in the process of ND is complicated. Evidence suggests that activated glial cells accelerate the clearance of pathological proteins by releasing the EVs and thus inhibit the spreading of pathological seeds from cell to cell. On the contrary, the results of more recent studies suggest that glia-derived EVs facilitate the spreading of pathological proteins and promote neurodegeneration (Peng et al., 2020).

### Glia-Derived Extracellular Vesicles in Alzheimer's Disease

Alzheimer's disease is characterized by the extracellular plaque deposits of the Aβ and the neurofibrillary tangles of the microtubule-binding protein tau, which activates microglia, induces neuroinflammation, and drives neurodegeneration (DeTure and Dickson, 2019). Glia-derived EVs are now recognized as important mediators in the pathogenesis of AD. Many studies have shown that EVs derived from Aβ or aggregated tau stimulated glia are involved in Aβ aggregation and propagation of tau pathology. When fluorescently labeled Aβ protofibrils were added to a co-culture system of primary neurons and glia, astrocytes could rapidly engulf and store large amounts of Aβ protofibrils. However, no labeled Aβ was found in neurons. The incomplete digestion of Aβ leads to severe endosomal/lysosomal dysfunction in the astrocytes. Eventually, this high intracellular load of toxic results in the secretion of astrocyte-derived EVs containing N-terminally truncated Aβ and the apoptosis of neighboring neurons (Söllvander et al., 2016). A study further analyzed the protein content of EVs released from the neurons and glia co-culture system and found a three-fold increase of apolipoprotein E (apoE) in EVs from Aβ-protofibril-exposed cells than those from unexposed cells. More particularly, these apoE contained EVs are primarily derived from the astrocytes in the co-culture system (Nikitidou et al., 2017).

In addition to the astrocytes, evidence also indicates that microglia can convert aggregated Aβ into neurotoxic forms through the shedding of EVs (Joshi et al., 2014). It has been shown that soluble pre-fibrillar Aβ species are far more toxic than the insoluble fibrils species. After Aβ internalization into microglia, neurotoxic Aβ can be trafficked into EVs. Then EVs' lipids promote soluble Aβ species from extracellular insoluble aggregates (Joshi et al., 2014). A recent study found that Aβ-associated microglia hyper-secrete EVs to extracellular regions in a humanized APP mouse model, and the expression level of Itgax, a well-established integrin that forms a complex with Integrin beta2 as inactivated-C3b receptor 4, is increased explicitly in the EVs secreted from the microglia, suggesting the contribution of neurodegeneration-induced microglia EVs secretion and the extracellular deposits of Aβ in the pathogenesis of AD (Muraoka et al., 2021).

There is evidence indicating that tau spreading depends on the direct transmission of EVs between neurons and glia. A study *in vivo* used nSMase2- deficient 5XFAD mice with reduced ceramide generation to assess AD-related pathology and demonstrated the release of astrocyte-derived EVs containing tau phosphorylation and Aβ42 plaque burden was increased in the AD mouse model (Dinkins et al., 2016). But contrary to that, evidence from organotypic hippocampal slices shows that neurons and microglia assimilate tau-containing EVs, but not astrocytes (Wang et al., 2017). Ruan et al. have reported that depleting microglia suppresses tau propagation and reduces neuron excitability in the dentate gyrus *in vivo*. Meanwhile, inhibiting the synthesis of microglia-derived EVs reduced tau propagation (Asai et al., 2015; Ruan et al., 2020). Then this team's most recent study utilized a mouse model of humanized APP mutant knock-in homozygote and found that the activated microglia surrounding the plaque can secrete more EVs that carry more phosphorylated tau (p-tau), comparing to resting microglia. Neurons absorb p-tau contained in EVs and trigger abnormal aggregation of tau protein. Moreover, tau propagation slows down-regulated when microglia are consumed, which is consistent with their previous findings (Clayton et al., 2021).

**TABLE 1 |** Biological properties and effects of glia-derived EVs on neurodegenerative diseases.

| Disease | Source of EVs | Stimulation/Culture condition | Main components | Effects of EVs | Markers of EVs | References |
|---|---|---|---|---|---|---|
| AD | Microglia | Aβ1-42 | Aβ neurotoxic species | Neurotoxicity | IB4 | Joshi et al., 2014 |
| AD | Microglia | Aβ and tau protein | Tau | Accelerating the spread of tau pathology | CD9 | Clayton et al., 2021 |
| AD | Astrocyte | Aβ protofibrils | N-terminally truncated Aβ | Neuronal apoptosis and Aβ spreading | Flotillin-1 | Söllvander et al., 2016 |
| AD | Astrocyte | Aβ protofibrils | apoE | Intercellular transfer of Aβ pathology by apoE | Flotillin-1, TSG101 and CD9 | Nikitidou et al., 2017 |
| AD | Astrocyte | Aβ | Tau/p-tau | Accelerating the spread of tau pathology and forming pre-tangles and NFTs | NA | Chiarini et al., 2017 |
| PD | BV2 | Aggregative α-synuclein | MHC-II, TNF-α | Activation of inflammatory response and leading to the neuronal death | CD63 | Chang et al., 2013 |
| PD | BV2 | Plasma EVs derived from PD patients | Human α-synuclein | Phosphorylated α-synuclein is spread to multiple brain regions of the mouse model | TSG101 | Xia et al., 2019 |
| PD | Microglia | Human α-synuclein preformed fibrils | α-synuclein | Promoting the dissemination of α-synuclein in the brain and increasing α-synuclein aggregation in neurons | Tsg101, Alix and CD6 | Guo et al., 2020 |
| PD | Microglia | Monomeric α-synuclein | NA | Mitochondrial fission and reducing neurotoxicity | Alix, CD9 and TSG 101 | Li et al., 2019 |
| PD | Astrocytes | MPP+ | Downregulation of miR-200a-3p | Reducing neuronal apoptosis. | Flotillin-1 | Shakespear et al., 2020 |
| PD | Astrocytes | LPS | Upregulation of miR34a | Heightening the vulnerability of DAergic neurons to the neurotoxin | β1 integrin, ribophorin, CD63 and HSP70 | Mao et al., 2015 |
| ALS | Astrocytes | C9orf72 mutation | Downregulation of miR-494-3p | Neurite/axonal shortening and neuronal degeneration | NA | Varcianna et al., 2019 |
| ALS | Astrocytes | Overexpressing SOD1 in astrocyte | SOD-1 | Inducing selective motor neuron death | Flotillin-1 | Basso et al., 2013 |
| ALS | Astrocytes | – | IL-6 | Regulation of neuroinflammatory reaction | CD81 | Chen et al., 2019 |

*AD, Alzheimer's disease; PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis; NA, not analysis. EVs, extracellular vesicles; Aβ, amyloid-β; apoE, apolipoprotein E; TSG101, Tumor susceptibility gene 101; NFTs, Neurofibrillary Tangles; SOD1, Cu/Zn superoxide dismutase 1.*

Microglia-derived EVs have been found to participate in the neuroinflammation in AD. There is evidence that neurons with APP695 mutant can significantly enhance the expression of inflammatory markers, along with higher APP and Aβ1-40 production. Then under the APP695 mutant neurons-microglia co-culture condition, microglia exerted a clearance effect on extracellular APP and Aβ accumulation, probably through internalizing EVs released from neurons in the early stage. However, microglia gradually lose such clearance property and express both pro-inflammatory (iNOS, IL-1β, TNF-α, MHC class II, IL-6) and pro-resolving genes (IL-10 and Arginase 1) in the final stage (Fernandes et al., 2018).

## Glia-Derived Extracellular Vesicles in Parkinson's Disease

There is considerable evidence supporting that abnormal glia-neuron crosstalk contributes to the progressive death of dopaminergic (DAergic) neurons in the substantia nigra (SN) and the aggregation of α-synuclein in the SN and other areas in the brain and peripheral tissues in PD patients (Meade et al., 2019). α-synuclein released from neurons can be taken up by glia through endocytosis and form inclusion bodies (Tremblay et al., 2019; Marchetti et al., 2020). The glia-derived EVs may exist in distinct phenotypes by carrying various molecules to the DAergic neurons, resulting in neuronal death or neuroprotective functions.

Under inflammatory or neurotoxic conditions, glia generally turns into a harmful phenotype accompanied by the release of EVs, contributing to the increase of DAergic neuron vulnerability and the exacerbation of neuronal death (Wang et al., 2021). Results from the α-synuclein overexpression mouse model indicate that the abnormity accumulated α-synuclein can alter the pro-inflammatory gene expression profile in the glial. What's more, the increased levels of pro-inflammatory cytokines were associated with the extent of glial accumulation of α-synuclein (Lee et al., 2010). The EVs derived from microglia exposed to aggregated α-synuclein lead to more severe neurotoxicity (Li et al., 2019). When microglia-derived EVs were combined with pro-inflammatory cytokines, the aggregation of α-synuclein

would be more significant, suggesting that the internalization of microglia-derived EVs in neurons was accompanied by an immunologically synergic effect, resulting in enhanced neurotoxicity (Guo et al., 2020).

Besides, a growing number of studies focus on microglia-derived EVs as a mediator of α-synuclein transmission in the pathology of PD. EVs-contained α-synuclein oligomers are more easily absorbed by recipient cells and induce more toxicity than the free α-synuclein oligomers outside the cell (Danzer et al., 2012). A recent study reported that when treated with human α-synuclein preformed fibrils, microglia-derived EVs containing α-synuclein contribute to the protein aggregation in the neurons (Guo et al., 2020). What's more, by stereotaxic injection of α-synuclein preformed fibrils treated microglia-derived EVs into the mouse striatum, phosphorylated α-synuclein can be found in multiple brain regions of the mouse model, including cortex, hippocampus, cerebellum, and SN (Xia et al., 2019).

On the other side, glial have also been discovered to affect neuroprotection against the degeneration of DAergic neurons by releasing specific EVs. For example, astrocytes-derived EVs are shown to reduce MPP+-induced cell death in primary DAergic neuron cultures, with the decreased level of mitogen-activated protein kinase kinase 4, a vital kinase in the c-JNK cell death pathway (Shakespear et al., 2020). Under the stimulation of LPS, astrocytes-derived EVs could heighten the vulnerability of DAergic neurons to neurotoxin by up-regulating the expression of miR-34a, which was shown to targeting anti-apoptotic protein Bcl-2 in DAergic neurons. Whereas inhibition of miR-34a in astrocytes-derived EVs can postpone DAergic neuron loss under the inflammatory stimuli (Mao et al., 2015).

### Glia-Derived Extracellular Vesicles in Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis is a progressive ND characterized by motor neuron degeneration and death, with gliosis replacing lost neurons (Zhang et al., 2014). Although the mechanisms underlying ALS remain unclear, many pathologic processes have been implicated, such as protein aggregation, impaired protein degradation, and neuroinflammation (Suk and Rousseaux, 2020). The mutual effect between motor neurons and glia is essential in the outcome of ALS. Cu/Zn superoxide dismutase 1 (SOD1) was the first gene associated with ALS, accounting for about 10–20% of familial ALS. It has been shown that mutant SOD1 overexpression primary astrocytes can release mutant SOD1-containing EVs, leading to selective motor neuron death (Basso et al., 2013). Studies in animal models also indicate that astrocyte-derived EVs contribute to the spreading of pathogenic SOD1 (Silverman et al., 2019). Astrocyte-derived EVs from brains and spinal cords of the SOD1-G93A ALS mouse model and the spinal cord of ALS patients with SOD1 mutant have been found to contain abundant misfolded and non-native disulfide-cross-linked aggregated SOD1 (Silverman et al., 2019). In addition, human-induced astrocytes from ALS patients carrying C9orf72 mutations were used to explore the role of astrocyte-derived EVs in the neurotoxicity of ALS. miRNA cargo is altered in the astrocyte-derived EVs of C9orf72-ALS patients, contributing to dysregulation of neurite network maintenance and motor neuron survival (Varcianna et al., 2019). As neuroinflammation participates in the pathogenesis of ALS, a study evaluated the expression level of IL-6 in the astrocyte-derived EVs from the plasma of sporadic ALS patients. And the results showed that the level of IL-6 in astrocyte-derived EVs was increased in ALS patients and positively associated with the disease progression, suggesting the increased inflammatory cascade in the CNS of ALS patients (Chen et al., 2019). The involvement of glia-derived EVs in glia-to-neuron communication in the context of ALS has not been explored in-depth, which deserves further investigation.

## EXTRACELLULAR VESICLES AS BIOMARKERS FOR NEURODEGENERATIVE DISEASES

One of the clinical challenges of ND is the difficulties in making a definitive diagnosis at the early stages and predicting the disease progression. The unmet demand to identify reliable biomarkers for early diagnosis and management of the disease course has attracted much attention (Li and Le, 2020). EVs harbor proteins and nucleic acids that are likely to indicate pathogenic processes occurring in hardly accessible tissues, such as the CNS, for their potential of tracking down the donor cell. Therefore, peripheral EVs may hold promise for biomarker discovery in ND.

Peripheral glia-derived EVs were shown to be enriched in the plasma and cerebrospinal fluid (CSF) of AD patients with similar characteristics to those derived from the CNS, such as containing glia-specific cargo proteins or miRNAs, which involves the cellular transmission of AD-related pathology. For instance, plasma astrocyte-derived EVs levels of BACE-1, γ-secretase, soluble Aβ42, soluble APPβ, P-T181-tau, and P-S396-tau were markedly increased compared with those in neuron-derived EVs. What's more, levels of BACE-1 and soluble APPβ were significantly higher in astrocyte-derived EVs of AD patients than in those of healthy controls (Goetzl et al., 2016). Goetzl et al. have found that astrocyte-derived EVs from AD patients contain high levels of multiple complement components (C1q, C4b, C3b, Bb, C3d, factor B, and factor D), which is known to contribute to the formation of the membrane attack complex (MAC) (Goetzl et al., 2018). Besides, Muraoka et al. (2020a) performed the first proteomic profiling of EVs isolated from post mortem AD brain tissues and found high glia-specific factors in the CNS- derived EVs from AD patients. ANXA5, VGF, GPM6A, and ACTZ were identified by machine learning quantitative proteomics datasets, which can distinguish AD and control CNS-derived EVs with 88% accuracy. Another recent study also examined the proteomic profiling of the EVs separated from the CSF of AD, cognitive impairment (MCI) patients, and healthy controls. The results indicated that astrocyte-specific molecules were enriched in AD compared to MCI, and HSPA1A, NPEPPS, and PTGFRN can be applied to monitor the progression of MCI to AD (Muraoka et al., 2020b).

It has been reported that the level of CNS-derived EVs contained α-synuclein is significantly higher in the plasma of PD patients, which may serve as a potential biomarker for

PD (Shi et al., 2014; Si et al., 2019). A longitudinal study reported that α-synuclein level in the plasma EVs is significantly higher in patients with early stage PD than healthy controls, and the increased α-synuclein is associated with a higher risk for motor symptom progression in PD (Niu et al., 2020). It has to be taken into account that the outcome depends on the donor cells of the EVs carrying α-synuclein in the plasma. However, so far, no data relevant to α-synuclein in specific glia-derived EVs as potential biomarkers for PD has been reported. A recent study evaluated the plasma levels of neuron-derived, astrocyte-derived, and oligodendrocyte-derived EVs in patients with PD, multiple system atrophy (MSA), progressive supranuclear palsy. The neuron-derived EVs showed a significant increase in PD compared to control and MSA. And the plasma levels of oligodendrocyte-derived EVs and the ratio of oligodendrocyte/neuron-derived EVs showed a substantial correlation with UPDRS part III scores in the patients with MSA ($r^2$ = 0.57) and PD ($r^2$ = 0.51), respectively (Ohmichi et al., 2019). In addition, alterations of other proteins, nucleic acids, and miRNAs have also been detected in the peripheral EVs from PD patients (Fraser et al., 2016; Zhao et al., 2019). Proteomic analysis of urinary EVs has shown that the combination of SNAP23 and calbindin reached the diagnostic performance of PD with 77% sensitivity and 85% specificity (Wang et al., 2019). It has been shown that circulating EVs have altered levels of miR-195, miR-24, let-7-c-3p, miR-331-5p, and miR-505 in PD patients compared with controls (Cao et al., 2017; Yao et al., 2018). Nevertheless, little was known about the peripheral EVs, carrying a cargo of protein or miRNAs as potential biomarkers derived from what kind of donor cells (cells in the blood, neurons, or glia). Within this context, more efforts in identifying the specific EV-donor cell are needed to clarify the role of glia-derived EVs in the diagnosis and prognosis of PD.

Plasma-derived EVs have also been analyzed recently in human samples as well as in animal models by Pasetto et al. (2021). EVs from ALS patients and two ALS-related mouse models show a distinct size distribution with smaller mean diameters and lower amount of Hsp90 than that of controls (Pasetto et al., 2021). In this study, the level of cyclophilin A in EVs, together with EV size distribution show potentials in identifying patients with fast or slow disease progression (Pasetto et al., 2021).

## EXTRACELLULAR VESICLES FOR THERAPY OF NEURODEGENERATIVE DISEASES

Efforts to study EVs for therapeutic applications in diseases are rapidly increasing. EVs bear the great advantage of being stable in the blood, the ability to overcome the BBB, and possessing specific cell-targeting capabilities, therefore delivering their cargoes within the CNS (Aryani and Denecke, 2016). Therapeutic EVs usually comprise diverse cargoes include RNA, proteins, and drugs. The key strategic issues that need to be addressed are the choice of therapeutic cargoes, promoting EV stability, tissue targeting, and functional cargo delivery to recipient cells (György et al., 2015). The identification of EVs as tools for delivering cargo molecules to diseased tissues makes these circulating shuttles possible targets for therapeutic development.

Accumulating evidence of preclinical therapeutic efficacy indicates that EVs could be a potential regenerative substance in the treatment of ND. Several types of stem cells-derived EVs have been used for nerve regeneration as EV donors in preclinical trials through induction of regenerative phenotypes, immune regulation, and apoptosis inhibition (Yin et al., 2020; Lee and Kim, 2021). Mesenchymal stem cells (MSCs)-derived EVs could promote neurogenesis and endogenous angiogenesis and reduce the neuroinflammation in the brain injury model (Zhang et al., 2015). It was shown that adipose MSCs-derived EVs contain neprilysin, an enzyme that degrades Aβ, which effectively reduces intracellular and secreted Aβ levels, suggesting a possible treatment application for AD (Katsuda et al., 2013; De Godoy et al., 2018). A study using a 3D culture model of stem cells derived from the dental pulp of human exfoliated deciduous teeth (SHEDs) and DAergic neurons and found that EVs derived from SHEDs rescued DAergic neurons from 6-hydroxy-dopamine (6-OHDA)-induced apoptosis and may be considered as a potential therapeutic tool in the treatment of PD (Jarmalavičiūtė et al., 2015). In addition to *in vitro*, intranasal administration of EVs derived from SHEDs also presented the improvements in motor function and normalization of tyrosine hydroxylase (TH) expression in the SN of 6-OHDA treated PD rat model (Narbute et al., 2019). Lombardi et al. (2019) evaluated the effects of EVs released from either pro-inflammatory or pro-regenerative microglia on the demyelinated lesions of OPCs, and the results showed that pro-inflammatory microglia-derived EVs blocked remyelination, whereas EVs released by microglia co-cultured with immunosuppressive MSCs accelerated OPC recruitment and myelin repair.

Since the beneficial effects of glia-derived EVs and their potential therapeutic actions on ND, it would be crucial to engineering EVs with a beneficial role. Many studies have been conducted to engineer EVs to express beneficial biomolecules such as proteins, mRNA, and miRNAs by using various donor cells to treat ND. The therapeutic potential of EVs-mediated interfering RNA (siRNA) delivery was firstly demonstrated by the knockdown of BACE1, a therapeutic target of AD, in EVs derived from autologous dendritic cells. After intravenous injection, EVs are explicitly delivered to neurons, microglia, oligodendrocytes in the brain, resulting in the specific BACE1 gene knockdown (Alvarez-Erviti et al., 2011). Haney et al. (2013) revealed that EVs contained catalase genetic material, active catalase, and NF-κb, which were released from the transfected macrophages, can efficiently transfer their contents to contiguous neurons resulting in a reduction of inflammation and neuroprotection in a mouse model of PD. The following administered to the PD mice macrophages overexpressing glial-derived GDNF, again with the analog results of improving neuroinflammation and neurodegeneration (Zhao et al., 2014).

Given two opposite effects of glia-derived EVs in ND, treatment strategies utilizing EVs may bring a wrong side at the same time. Therefore, some studies focused on inhibiting glia-derived EVs secretion from diminishing the propagation

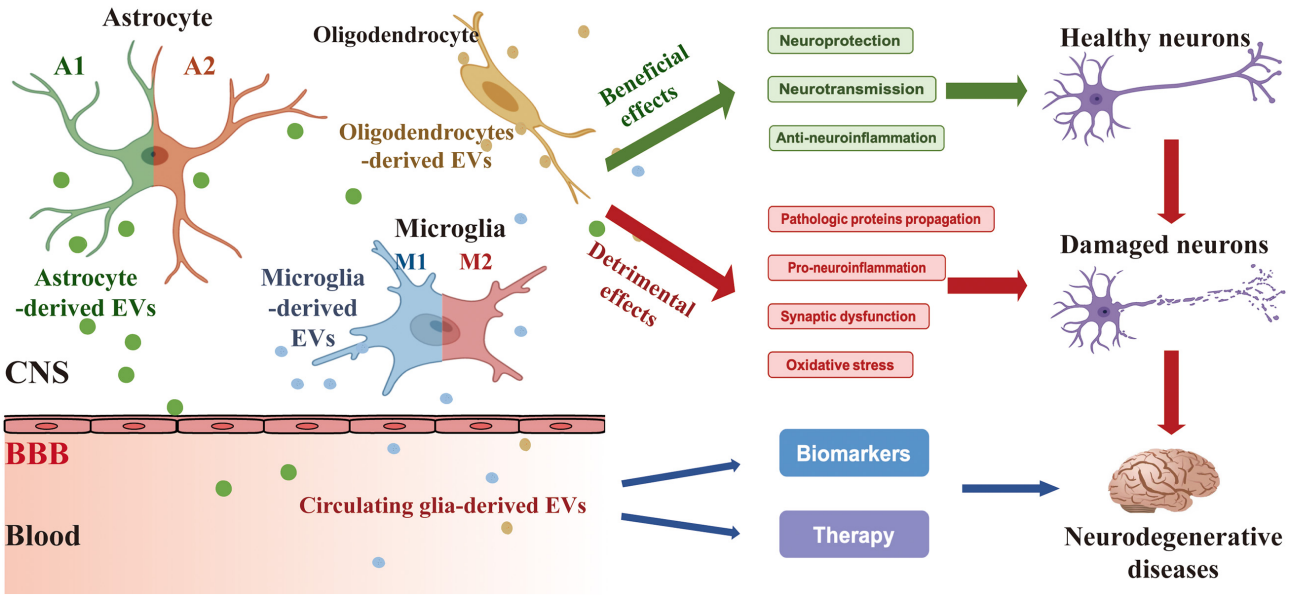


**FIGURE 1 |** Secretion of EVs derived from glia (astrocyte, microglia, and oligodendrocyte) and their effects on neurons and ND. Glia-derived EVs have a beneficial and/or detrimental impact on the process of ND like a "double-edged sword." Under condition normal circumstances, glia-derived EVs contribute to neuroprotection, neurotransmission, and anti-neuroinflammation. However, in response to the different stimuli such as toxic aggregated proteins and neuroinflammation, glia-derived EVs modify their cargo content and have detrimental effects on pathologic proteins propagation, pro-neuroinflammation, synaptic dysfunction, and oxidative stress. In addition, circulating glia-derived EVs hold considerable potential for clinical applications in the diagnosis and treatment of ND. CNS, central nervous system; BBB, blood-brain barrier.

of pathological protein in ND. For instance, ceramide-enriched EVs have been shown to exacerbate AD-related brain pathology by promoting the aggregation of Aβ. A study reported that reducing the secretion of astrocyte-derived EVs by nSMase2 improves pathology and cognition in an AD mouse model (Dinkins et al., 2016). Besides, it has been shown that blocking dynamin-related protein 1 can reduce the release of EVs and spread of α-synuclein pathology from neurons to neurons and from microglia to neurons, which plays a role in neuroprotection through both mitochondrial and autophagy-lysosomal pathways (Fan et al., 2019). MSCs-derived EVs were shown to modulate the detrimental effect of glia in neurodegenerative conditions. A study found that the intranasal route of administration of cytokine-preconditioned MSCs-derived EVs contributes to the immunomodulation and neuroprotection in an AD transgenic mouse model. MSC-EVs can deliver into the brain, thereby increasing the dendritic spine density and depressing microglia activation (Losurdo et al., 2020). Furthermore, it was shown that MSCs-derived EVs could effectively trigger the microglia polarization from M1 to an M2 phenotype and improve the survival of neurons through down-regulation of the pro-inflammatory cytokines (Sun et al., 2018).

Although many studies in the mouse model have shown promising results of the application of EVs in the treatment of ND, crucial species differences in the function of the BBB and targets of EVs to the brain may exist between humans and mice. Little research so far has dealt with the effect of EVs on the human brain. Overall, it will be of great value to clarify the glia-derived EVs content and distinct functional impacts on ND and improve the techniques of EVs engineering. These efforts will open up a new field, implementing the characterization of glial as a potential producer of a novel generation of ND nanotherapeutics.

## CONCLUSION AND FUTURE PERSPECTIVES

Glia is abundant in the CNS and contributes to supporting the essential functions of neurons, including synapse formation and plasticity, neurotransmitter buffering, secretion of neuroactive agents, and modulation of neuroinflammatory reactions. The crucial role of glia-derived EVs in glia-to-neuron communications has been widely noted in recent years. Under normal circumstances, glia-derived EVs play an important part in maintaining the normal neuronal function, promoting neurite outgrowth and neuronal survival. However, in response to the different stimuli such as toxic aggregated proteins and neuroinflammation, glia-derived EVs modify their cargo content and have dual effects on the survival of the neurons like a "double-edged sword." In addition, as microglia are heterogeneous with diverse phenotypes of pro-inflammatory M1 and anti-inflammatory M2 phenotypes, EVs from M1 or M2 phenotypes of microglia also present various functional properties triggered by different stimuli (**Figure 1**).

In recent years, glia-derived EVs have been shown to participate in the pathological processes of ND. It is worth noting that the role of glia-derived EVs in the ND also results in totally different outcomes. On the one hand, the glial can

promote clearance and inhibit the spreading of pathological proteins by releasing the EVs. On the other hand, glia-derived EVs have been shown to facilitate the propagation of pathological proteins and ultimately result in neurodegeneration. The sources of heterogeneity may be attributable to the differential expression of the main components in the glia-derived EVs.

Glia-derived EVs hold considerable potential for various clinical applications. EVs can overcome the BBB, be stable in the blood, and have the possibility of tracking down the donor cell, therefore delivering their cargoes within the CNS. For this reason, various studies have been conducted in discovering potential biomarkers and therapeutic targets for ND by focusing on the CNS-derived EVs. However, investigations on the glia-specific EVs in identifying biomarkers for ND are rare, which may be due to the mix of different EVs released from various donor cells that exist in the peripheral circulation. Approaches to label different cell-specific EVs are required in future studies on glia-derived EVs *in vivo*. Moreover, concerning the beneficial and detrimental roles of the glia-derived EVs in the pathogenesis of ND, further investigations of the specific associated biological molecules in the glia-derived EVs are necessary. Based on more understanding of the core molecule expressed in the glia-derived EVs, engineering EVs to express beneficial biomolecules will provide a novel and efficient tool for the therapeutic applications on ND.

## AUTHOR CONTRIBUTIONS

WL, TL, and SL designed the project of this manuscript. TL, XT, SL, MA-N, and WL contributed to the drafting of the manuscript. TL, MA-N, and WL revised the manuscript. All the authors edited and approved the final version of the manuscript.

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# The Anti-diabetic Drug Gliquidone Modulates Lipopolysaccharide-Mediated Microglial Neuroinflammatory Responses by Inhibiting the NLRP3 Inflammasome

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The sulfonylurea drug gliquidone is FDA approved for the treatment of type 2 diabetes. Binding of gliquidone to ATP-sensitive potassium channels (SUR1, Kir6 subunit) in pancreatic β-cells increases insulin release to regulate blood glucose levels. Diabetes has been associated with increased levels of neuroinflammation, and therefore the potential effects of gliquidone on micro- and astroglial neuroinflammatory responses in the brain are of interest. Here, we found that gliquidone suppressed LPS-mediated microgliosis, microglial hypertrophy, and proinflammatory cytokine COX-2 and IL-6 levels in wild-type mice, with smaller effects on astrogliosis. Importantly, gliquidone downregulated the LPS-induced microglial NLRP3 inflammasome and peripheral inflammation in wild-type mice. An investigation of the molecular mechanism of the effects of gliquidone on LPS-stimulated proinflammatory responses showed that in BV2 microglial cells, gliquidone significantly decreased LPS-induced proinflammatory cytokine levels and inhibited ERK/STAT3/NF-κB phosphorylation by altering NLRP3 inflammasome activation. In primary astrocytes, gliquidone selectively affected LPS-mediated proinflammatory cytokine expression and decreased STAT3/NF-κB signaling in an NLRP3-independent manner. These results indicate that gliquidone differentially modulates LPS-induced microglial and astroglial neuroinflammation in BV2 microglial cells, primary astrocytes, and a model of neuroinflammatory disease.

**Keywords: neuroinflammation, microgliosis, LPS (lipopolysaccharide), gliquidone, proinflammatory cytokine, NLRP3 inflammasome**

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## INTRODUCTION

Diabetes mellitus is a serious metabolic disorder characterized by elevated blood glucose levels (hyperglycemia) due to improper insulin function (Ramos-Rodriguez et al., 2014). Long-term hyperglycemia can impair macrovascular and microvascular function and cause brain atrophy, neuropathy, and neuroinflammation (Wrighten et al., 2009; Ramos-Rodriguez et al., 2014).

Moreover, type 2 diabetes and insulin resistance can lead to peripheral meta-inflammation and an overproduction of proinflammatory cytokines (Ferreira et al., 2014). Clusters of proteins that are differentially regulated in type 2 diabetes have also been linked to neuroinflammation and related neurodegenerative diseases, including Alzheimer's disease (AD) (Mittal et al., 2016). Clinical studies have shown that some classes of diabetes medications can rescue both glycemic control and inflammation-linked deficits, providing benefits for cognitive function and reducing dementia risk (Chatterjee and Mudher, 2018; Weinstein et al., 2019; Akimoto et al., 2020). However, the molecular mechanisms by which diabetes medications affect neuroinflammatory responses have not been fully elucidated.

Therapeutic interventions for type 2 diabetes include the class of drugs known as sulfonylureas, such as gliquidone and glibenclamide. These drugs bind the sulfonylurea receptor on the surface of pancreatic β-cells, which functions as a subunit of ATP-sensitive potassium (K-ATP) channels (Aquilante, 2010; Sola et al., 2015). Gliquidone inhibits $K^+$ efflux in the cell membrane, resulting in depolarization, activation of voltage-gated calcium channels and calcium influx (de Wet and Proks, 2015). The resulting increase in calcium conductance leads to insulin expression and release (Yang and Berggren, 2006). Isotope tracing studies using [$^3$H]glibenclamide ([$^3$H]Grb) have demonstrated that glibenclamide crosses the blood-brain barrier (BBB) in wild-type mice. In addition, glibenclamide reaches the brain more easily after cerebral ischemic insult (Angel and Bidet, 1991; Ortega et al., 2012). As further evidence that sulfonylurea drugs can cross the BBB, gliquidone injection decreases the immobility time of mice in the forced swimming test (Galeotti et al., 1999), and gliquidone significantly decreases cromakalim-evoked potassium efflux in slices of the rat substantia nigra (Schmid-Antomarchi et al., 1990). These observations suggest that the sulfonylurea family of type 2 diabetes drugs may provide additional potential therapeutic benefits by reducing insulin-mediated neuroinflammation.

As part of the neuroinflammatory response, NLRP3/IL-1β/caspase-1 inflammasome formation is promoted. NLRP3 is one of four known NLRP inflammasome types, named Nod-like receptor (NLRP) 1 to 4 (Franchi et al., 2009). NLRP3 is dominantly involved in neuroinflammatory responses *in vitro* and *in vivo*. For instance, NLRP3 activates TLR4 signaling, which leads to the induction of neuroinflammatory responses in an NF-κB-dependent manner (Paik et al., 2021). Interestingly, $K^+$-channel agonists (e.g., Leu-Leu-*O*-methyl ester) and/or Kir6.2 siRNA have been reported to modulate neuroinflammatory responses through the NLRP3 inflammasome (Munoz-Planillo et al., 2013; Qu et al., 2017; Yang et al., 2019). However, it is unclear whether gliquidone, an antagonist of K-ATP channels (specifically, the SUR1 and Kir6 subunits), modulates neuroinflammatory responses by regulating NLRP3 inflammasome activation.

To address this gap, in this study we investigated whether gliquidone affects lipopolysaccharide (LPS)-stimulated micro- and astroglial neuroinflammatory responses. We found that gliquidone suppressed LPS-induced microglial activation and proinflammatory cytokine levels by altering NLRP3 inflammasome activation in wild-type mice, with smaller effects on astrogliosis. In BV2 microglial cells, gliquidone significantly suppressed the LPS-induced increase in proinflammatory cytokine levels by altering ERK/STAT3/NF-κB signaling. Importantly, gliquidone downregulated LPS-mediated neuroinflammatory responses in BV2 microglial cells by inhibiting NLRP3 inflammasome formation. In primary astrocytes, gliquidone reduced LPS-induced neuroinflammatory responses by regulating STAT3/NF-κB signaling but not NLRP3 inflammasome activation. Taken together, these data suggest that the anti-diabetic drug gliquidone may be a new therapeutic intervention for neuroinflammation-mediated neuropathy in the brain.

# MATERIALS AND METHODS

## Ethics Statement

All experiments were approved by the institutional biosafety committee (IBC) and performed in accordance with approved animal protocols of the Korea Brain Research Institute (KBRI, approval no. IACUC-19-00042).

## Gliquidone and Lipopolysaccharide

Gliquidone was purchased from Selleck Chemicals (S3151, Houston, TX, United States) and Tokyo Chemical Industry Co., Ltd. (G0332, Tokyo, Japan). Gliquidone was used at a concentration of 5 μM (in 1% DMSO) in *in vitro* assays and injected at a dose of 10 or 20 mg/kg (i.p., dissolved in 5% DMSO, 10% PEG, 20% Tween 80) in *in vivo* experiments.

## C57BL6/N Mice

Male C57BL6/N mice (wild-type; Hana Company, Busan, South Korea) were used in *in vivo* experiments. A pathogen-free facility with a 12 h light/dark cycle and a temperature of 22°C was used to house all mice. Daily injections of gliquidone (10 or 20 mg/kg) or vehicle (5% DMSO) were administered intraperitoneally (i.p.) on 3 consecutive days. On the third day, 10 mg/kg LPS (i.p.) or PBS was injected. The mice were sacrificed 8 h after the last injection and fixed in 4% paraformaldehyde. Subsequently, mouse brain slices with a thickness of 30 μm were prepared using a Leica CM1850 cryostat (Leica Biosystems, Buffalo Grove, IL, United States).

## Immunofluorescence Staining

Mouse brain slices were incubated in 10% normal goat serum (Vector Laboratories) at room temperature for 1 h, followed by immunostaining with rabbit or mouse anti-target antibody at 4°C overnight. The sections were subsequently incubated with an Alexa Fluor 488- or 555-labeled goat IgG secondary antibody for 2 h at room temperature. Sections were mounted on glass slides were covered with a DAPI (Vector Laboratories)-containing mounting solution, and images were obtained by fluorescence microscopy (DMi8, Leica Microsystems, Wetzlar, Germany) for analysis by Image J software (NIH). Detailed information on the primary and secondary antibodies used for IF and immunocytochemistry (ICC) is provided in **Table 1**.

**TABLE 1 |** List of antibodies used for Immunofluorescence Staining (IF) or immunocytochemistry (ICC).

**Primary antibodies**

| Immunogen | Host species | Dilution | Manufacturer | Catalog no. | Application |
|---|---|---|---|---|---|
| Iba-1 | Rabbit | 1:500 | Wako | 019-19741 | IF |
| GFAP | Rabbit | 1:500 | Neuromics | RA22101 | IF |
| NLRP3 | Goat | 1:100 | Abcam | AB4207 | IF, ICC |
| COX-2 | Rabbit | 1:100 | Cell Signaling | 89332 | IF |
| pERK | Rabbit | 1:200 | Cell Signaling | 9101 | ICC |
| IL-6 | Mouse | 1:50 | Santa Cruz | SC-57315 | IF |
| CD11b | Rat | 1:200 | Abcam | AB8878 | ICC |
| GFAP | Chicken | 1:500 | Millipore | AB5541 | ICC |
| p-STAT3$^{S727}$ | Rabbit | 1:200 | Abcam | AB86340 | ICC |
| p-NF-κB$^{S536}$ | Rabbit | 1:200 | Cell Signaling | 3033S | ICC |

**Secondary antibodies**

| Antibody | Dilution | Manufacturer | Catalog no. | Application |
|---|---|---|---|---|
| Goat anti-rabbit IgG, 555 | 1:200 | Invitrogen | A21428 | IF |
| Goat anti-rabbit IgG, 488 | 1:200 | Invitrogen | A11008 | IF, ICC |
| Goat anti-mouse IgG, 488 | 1:200 | Invitrogen | A11001 | IF |
| Donkey anti-goat, 555 | 1:200 | Invitrogen | A21432 | IF |
| Goat anti-chicken IgG, 488 | 1:200 | Abcam | A150169 | IF |
| Goat anti-rat IgG, FITC | 1:200 | Invitrogen | A18866 | ICC |

## Enzyme-Linked Immunosorbent Assay

Wild-type mice were injected daily with gliquidone (10 or 20 mg/kg, i.p.) or vehicle (5% DMSO + 10% PEG + 20% Tween80) for 3 days, followed by injection of LPS (10 mg/kg, i.p.) or PBS 30 min after the last injection on day 3. Eight hours later, blood was sampled and centrifuged at 2,000 rpm for 20 min, and the supernatant (serum) collected and stored at −80°C until analysis. Peripheral IL-1β, IL-6, and TNF-α levels were measured using the appropriate ELISA kit (IL-1β, Cat. no. 88-7013-88; IL-6, Cat. no. 88-7064-88; and TNF-α, Cat no. 88-7324-88; Invitrogen, Waltham, MA, United States) as described by the manufacturer.

## BV2 Microglial Cell Line

BV2 microglial cells (generously provided by Dr. Kyung-Ho Suk) were cultured at 37°C and 5% $CO_2$ in DMEM high glucose (Invitrogen, Carlsbad, CA, United States) containing 5% fetal bovine serum (FBS, Invitrogen).

## Mice Brain-Derived Primary Astrocyte Culture

Primary astrocytes were prepared from postnatal day 1 (P1) C57BL6/N mice as previously described (Ryu et al., 2019). Briefly, whole brains were dissected and minced through 70 μm mesh, and the mixed cells were grown in low-glucose DMEM (1,000 mg/l glucose) supplemented with 10% FBS, 100 unit/ml penicillin, and 100 μg/ml streptomycin for 2 weeks. On day 14, primary microglial cells were detached by shaking at 250 rpm at room temperature overnight, followed by dissociation of primary astrocytes using trypsin-EDTA. After 3 rounds of washing and centrifugation for 10 min at 2,000 rpm, the pellet containing primary astrocytes was used for experiments. The cells were seeded at $7.0 \times 10^5$ cells/well in 12-well plates for q-PCR and $2.5 \times 10^5$ cells/well for ICC. After 2 days, the primary astrocytes were fixed in 4% paraformaldehyde at room temperature for 10 min and immunostained with an antibody against GFAP (a marker of astrocytes) at 4°C overnight. Next, the cells were washed thrice with PBS, and secondary antibodies were added for 1 h. Finally, the cells were washed with PBS, incubated with DAPI, and mounted. Images were acquired by fluorescence microscopy (DMi8, Leica Microsystems, Wetzlar, Germany), and primary astrocyte purity was calculated as follows: [Astrocyte purity (%) = (GFAP- and DAPI-positive cells/DAPI-positive cells) × 100].

## Cell Counting Kit-8 (CCK-8) Assay

Cell viability and cytotoxicity were assessed with the CCK-8 assay (Dongin Biotech Co., Ltd., Seoul, South Korea) as recommended by the manufacturer. After seeding in 96-well plates at a density of $2 \times 10^4$ cells/well and incubation overnight at 37°C, BV2 cells were treated with gliquidone (0.1, 1, 5, 10, 25, and 50 μM) or vehicle (0.001, 0.01, 0.05, 0.1, 0.25, and 0.5% DMSO) for 6 h at 37°C. Next, CCK solution was added and incubated in the dark for 0.5 h, followed by detection at 450 nm in a SPECTROstar Nano microplate reader (BMG Labtech, Germany).

## MTT Assay

The cytotoxicity of gliquidone was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. BV2 cells seeded into 96-well plates without FBS at a density of $4 \times 10^4$ cells/well were treated for 24 h with gliquidone (0.1, 1, 5, 10, 25, and 50 μM) or vehicle (0.001, 0.01, 0.05, 0.1, 0.25, and 0.5% DMSO), followed by incubation with 0.5 mg/ml

MTT for 3 h in the dark. Finally, DMSO was added to dissolve the formazan crystals with shaking, and the absorbance at 570 nm was measured using a SPECTROstar Nano microplate reader (BMG Labtech, Germany).

## Reverse Transcription-Polymerase Chain Reaction

Reverse transcription-polymerase chain reaction was performed to assess the effects of gliquidone on LPS-evoked microglial and astroglial inflammatory cytokine levels. First, total RNA was isolated from BV2 microglial cells or primary astrocytes using QIAzol Lysis Reagent (Qiagen, Cat No. 79306) and reverse transcribed into cDNA. The cDNA was subsequently used as the template in RT-PCR using Prime Taq Premix (GeNet Bio) as previously described with the primers shown in **Table 2**. The amplicons were separated by electrophoresis on a 1.5% agarose gel containing EcoDye (1:5000, Biofact, Daejeon, South Korea), and images were analyzed using the software Fusion Capt Advance (Vilber Lourmat, Eberhardzell, Germany).

## Real-Time PCR (q-PCR)

The effects of gliquidone on LPS-mediated microglial and astroglial NLRP3 inflammasome activation and subsequent proinflammatory cytokine production were assessed in BV2 microglial cells and mouse primary astrocytes. In the preventive/pretreatment experiments, cells were first treated for 30 min with gliquidone (5 μM) or vehicle (1% DMSO) and then with LPS (200 ng/ml) or PBS for 5.5 h or 23.5 h. In the curative/post-treatment experiments, cells were first treated for 30 min with LPS (200 ng/ml) or PBS and then with gliquidone (5 μM) or vehicle (1% DMSO) for 5.5 h or 23.5 h. Total RNA was then extracted using TRIzol (Invitrogen, Waltham, MA, United States) as recommended by the manufacturer and reverse-transcribed (1 μg) to synthesize cDNA (GeNet Bio, Chungcheongnam-do, South Korea). The cDNA was used with Fast SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, United States) to perform real-time q-PCR in a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, United States). The cycle threshold (*C*t) values of the mRNA levels of factors related to inflammasome and inflammation were normalized to the *C*t value for *gapdh*, and the fold change relative to the vehicle-treated control was quantified. Used primers are shown in **Table 3**.

**TABLE 2 |** Sequences of primers used for reverse transcription-polymerase chain reaction (RT-PCR).

| Gene name | | Sequence |
|---|---|---|
| *il-1β* | Sense | 5′-AGC TGG AGA GTG TGG ATC CC-3′ |
| | Antisense | 5′-CCT GTC TTG GCC GAG GAC TA-3′ |
| *il-6* | Sense | 5′-CCA CTT CAC AAG TCG GAG GC-3′ |
| | Antisense | 5′-GGA GAG CAT TGG AAA TTG GGG T-3′ |
| *cox-2* | Sense | 5′-GCC AGC AAA GCC TAG AGC-3′ |
| | Antisense | 5′-GCC TTC TGC AGT CCA GGT TC-3′ |
| *inos* | Sense | 5′-CCG GCA AAC CCA AGG TCT AC-3′ |
| | Antisense | 5′-GCA TTT CGC TGT CTC CCC AA-3′ |
| *gapdh* | Sense | 5′-CAG GAG CGA GAC CCC ACT AA-3′ |
| | Antisense | 5′-ATC ACG CCA CAG CTT TCC AG-3′ |

**TABLE 3 |** Sequences of primers used for real time-PCR.

| Gene name | | Sequence |
|---|---|---|
| *il-1β* | Sense | 5′-TTG ACG GAC CCC AAA AGA TG-3′ |
| | Antisense | 5′-AGG ACA GCC CAG GTC AAA G -3′ |
| *il-6* | Sense | 5′-CCA CGG CCT TCC CTA CTT C-3′ |
| | Antisense | 5′-TTG GGA GTG GTA TCC TCT GTG A-3′ |
| *cox-2* | Sense | 5′-CCA CTT CAA GGG AGT CTG GA -3′ |
| | Antisense | 5′-AGT CAT CTG CTA CGG GAG GA-3′ |
| *inos* | Sense | 5′-GGA TCT TCC CAG GCA ACC A-3′ |
| | Antisense | 5′-TCC ACA ACT CGC TCC AAG ATT-3′ |
| *pro-il-1β* | Sense | 5′- TCT TTG AAG TTG ACG GAC CC -3′ |
| | Antisense | 5′- TGA GTG ATA CTG CCT GCC TG -3′ |
| *nlrp3* | Sense | 5′-TCC ACA ATT CTG ACC CAC AA-3′ |
| | Antisense | 5′-ACC TCA CAG AGG GTC ACC AC-3′ |
| *sur1* | Sense | 5′-GGA GAG GAA AGC CCC AGA AC-3′ |
| | Antisense | 5′-GTC ATC TTC CTC GCT CTC GG-3′ |
| *gapdh* | Sense | 5′-TGG GCT ACA CTG AGG ACC ACT-3′ |
| | Antisense | 5′-GGG AGT GTC TGT TGA AGT CG-3′ |

## Western Blotting

To determine the effects of gliquidone on LPS-induced ERK signaling, western blotting was conducted. BV2 cells or primary astrocytes were lysed using lysis buffer (ProPrep, iNtRON Biotechnology, Inc., Seongnam, South Korea), followed by centrifugation at 12,000 rpm for 15 min. After quantifying the protein concentration in the supernatant, 15 μg of protein sample was separated by 8% SDS gel electrophoresis. The proteins in the gel were subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk or 5% BSA at room temperature for 1 h, the membrane was incubated with anti-p-ERK (1:1000, Cell Signaling), anti-ERK (1:1000, Cell Signaling), or β-actin (1:1000, Santa Cruz Biotechnology) at 4°C. Finally, HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG (both 1:1000, Enzo Life Sciences, Farmingdale, NY, United States) was added to the membrane and incubated for 1 h, and detection was performed using ECL Reagent (GE Healthcare, Chicago, IL, United States). Fusion Capt Advance software was used for image acquisition and analysis (Vilber Lourmat).

## Immunocytochemistry

BV2 microglial cells and primary astrocytes were fixed in 4% paraformaldehyde for 10 min, washed thrice with PBS, and incubated overnight with anti-target antibody in GDB buffer as described previously (Nam et al., 2018). After washing with PBS, the cells were incubated for 1 h with an Alexa Fluor 488- or 555-conjugated antibody at room temperature. Finally, images of cells mounted in DAPI (Vector Laboratories, CA, United States) were captured using a fluorescence microscope (DMi8, Leica

Microsystems, Wetzlar, Germany) and analyzed using ImageJ software.

## Statistical Analyses

GraphPad Prism 7 software (GraphPad Software, San Diego, CA, United States) was used for data analyses. Comparisons between two groups were performed using the unpaired two-tailed *T*-test with Welch's correction; one-way ANOVA was employed for multiple comparisons (except for hippocampal NLRP3 IF). Tukey's test or the Newman–Keuls multiple comparisons test was used for multiple comparisons with significance set at $p < 0.05$. Means ± SD are presented ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

# RESULTS

## Gliquidone Decreases Lipopolysaccharide-Stimulated Microgliosis and Hypertrophy in a Model of Neuroinflammatory Disease

The anti-diabetic drug gliquidone was recently shown to promote a beneficial microglial phenotype via the Kir6-containing ATP-dependent potassium channel in a mouse model of Parkinson's disease (PD) (Du et al., 2018). However, the effects of gliquidone on LPS-induced proinflammatory responses are unknown. Therefore, we investigated whether gliquidone modulates LPS-mediated microglial activity by injecting wild-type mice daily with gliquidone (10 or 20 mg/kg, i.p.) or vehicle for 3 days; after the final administration of gliquidone, LPS (10 mg/kg, i.p.) or PBS was injected. Eight hours after LPS or PBS injection, IF staining of brain slices was performed with an anti-Iba-1 antibody, and Iba-1 intensity, Iba-1-positive cells, and Iba-1-labeled area were analyzed in the cortex and hippocampus (**Figures 1A–E**). Treatment with 20 mg/kg gliquidone significantly decreased the LPS-induced increases in Iba-1 immunoreactivity, Iba-1-positive cells, and Iba-1-labeled area in the cortex and hippocampal CA1 and CA3 regions (**Figures 1A–E**). By contrast, administration of 10 mg/kg gliquidone significantly reduced the LPS-induced increases in Iba-1 intensity, Iba-1-positive cells, and Iba-1-labeled area only in the hippocampal CA3 region (**Figures 1C–E**). These results indicated that a gliquidone dose of 20 mg/kg more effectively downregulates LPS-mediated microgliosis and changes in microglial kinetics and morphology in the wild-type mouse brain.

To determine if gliquidone alters LPS-evoked astrocyte activation *in vivo*, we treated wild-type mice according to same paradigm described above and performed IF staining of brain slices with an anti-GFAP antibody. GFAP intensity, GFAP-positive cells, and GFAP-labeled area were measured (**Figures 2A–E**). At doses of 10 and 20 mg/kg, gliquidone significantly reduced the LPS-induced increases in GFAP immunointensity and GFAP-positive cells in the cortex but not the hippocampus (**Figures 2C–E**). In addition, administration of 10 or 20 mg/kg gliquidone did not alter GFAP-labeled area in the cortex and hippocampus (**Figure 2E**). These data indicate that gliquidone modulates LPS-stimulated astrocyte activation in the mouse brain, albeit less effectively than microglial activation.

## Gliquidone Eliminates Lipopolysaccharide-Induced Increases in COX-2 and IL-6 Proinflammatory Cytokine Levels in Wild-Type Mice

Since gliquidone downregulated LPS-induced micro- and astrogliosis in wild-type mice, we next investigated whether gliquidone modulates LPS-stimulated proinflammatory cytokine levels *in vivo*. Wild-type mice were treated as described above, and brain slices were subjected to IF staining with anti-COX-2 or anti-IL-6 antibodies. Treatment with 20 mg/kg gliquidone significantly diminished LPS-stimulated levels of COX-2 in the cortex and hippocampal CA3 region but not the hippocampal CA1 and DG regions (**Figures 3A–C**). At a dose of 10 mg/kg, gliquidone significantly decreased the LPS-evoked increase in COX-2 levels in the cortex and hippocampal CA3 region (**Figures 3A–C**). Moreover, injection of 10 or 20 mg/kg gliquidone following LPS administration markedly suppressed IL-6 levels in the cortex and hippocampus in wild-type mice, and the effects of 10 mg/kg gliquidone were significantly greater than those of 20 mg/kg gliquidone (**Figures 4A–C**). These findings suggest that gliquidone suppresses LPS-stimulated COX-2 and IL-6 levels in a model of neuroinflammatory disease.

## Gliquidone Inhibits Lipopolysaccharide-Stimulated NLRP3 Inflammasome Activation in Wild-Type Mice

The induction of proinflammatory cytokines by LPS is closely linked to microglia- and/or astrocyte-associated inflammation as well as inflammasome formation (Su et al., 2021; Zhao et al., 2021). Thus, we examined whether gliquidone affects LPS-induced NLRP3 inflammasome activation *in vivo*. Wild-type mice were treated as described above, and IF staining of brain slices was conducted with an anti-NLRP3 antibody. Injection of 10 mg/kg gliquidone significantly decreased LPS-induced NLRP3 activation in the cortex and hippocampus (**Figures 5A–C**), whereas 20 mg/kg gliquidone effectively diminished LPS-induced NLRP3 levels in the cortex but not the hippocampus (**Figures 5A–C**). These data indicate that gliquidone decreases LPS-stimulated NLRP3 inflammasome formation in the mouse brain.

## Gliquidone Downregulates Lipopolysaccharide-Induced Peripheral Proinflammatory Cytokine Levels in Wild-Type Mice

To investigate whether gliquidone alters peripheral proinflammatory cytokine levels *in vivo*, wild-type mice

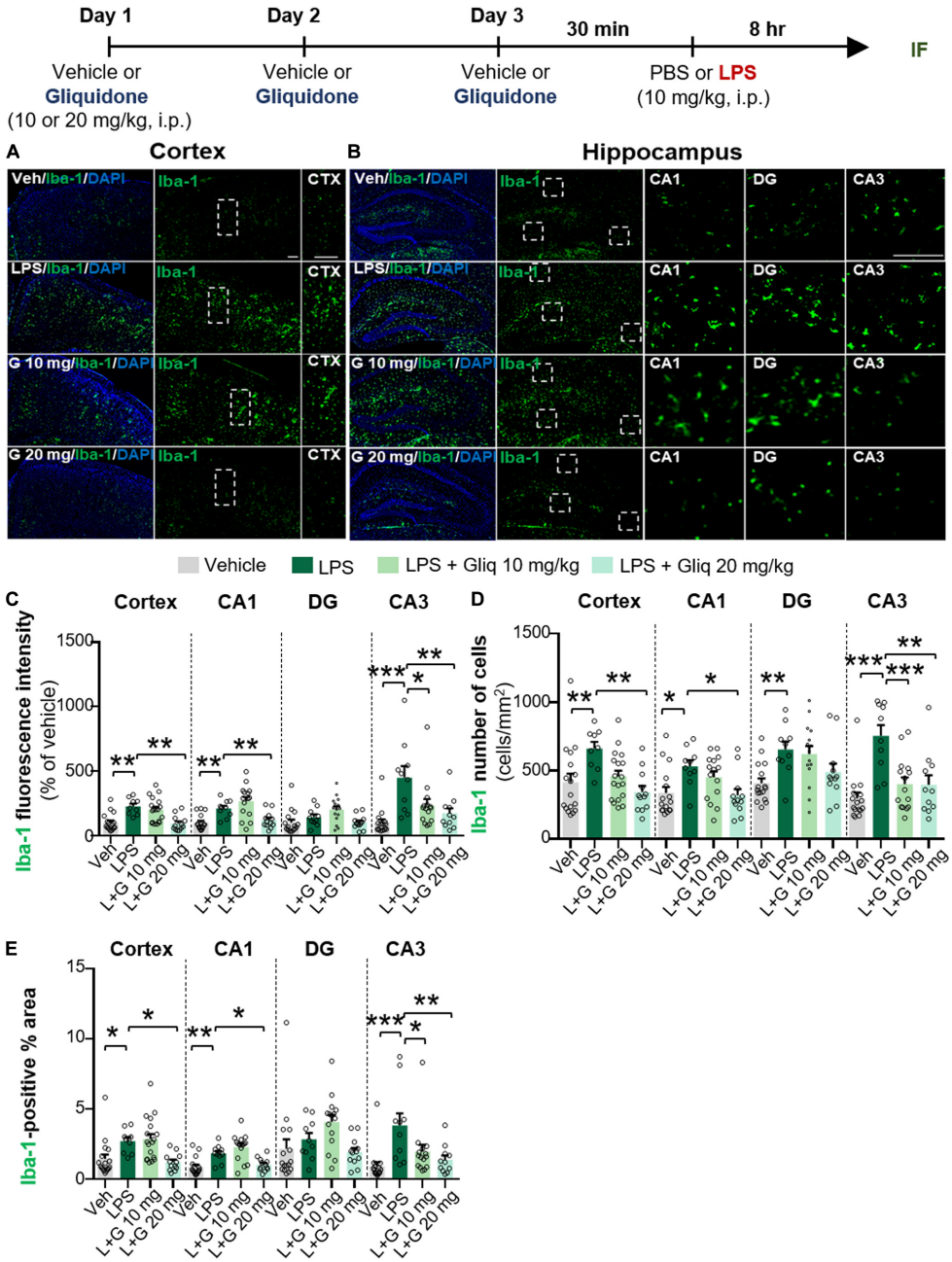


**FIGURE 1 |** Gliquidone decreases LPS-stimulated microgliosis and hypertrophy in the cortex and hippocampus in C57BL6/N mice. The *in vivo* experimental procedure for gliquidone and LPS injection is shown at the top of the figure. **(A,B)** Immunofluorescence staining of Iba-1 expression in brain slices from mice treated as described in the experimental procedure. **(C–E)** Quantification of the results in **(A,B)** ($n$ = 10–18 brain slices from 4 mice/group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bar = 100 μM.

were treated as described above, followed by measurement of proinflammatory cytokine levels in peripheral blood by ELISA. Treatment of wild-type mice with 10 or 20 mg/kg gliquidone significantly abolished the LPS-induced increases in serum IL-1β and IL-6 levels but not TNF-α (**Supplementary Figures 1A–C**). These data indicate that gliquidone selectively alters peripheral proinflammatory cytokine levels in LPS-treated wild-type mice.

## Post- and Pretreatment of BV2 Microglial Cells With Gliquidone Abolishes Lipopolysaccharide-Induced Proinflammatory Cytokine mRNA Levels

Since gliquidone regulates micro/astroglial activation, proinflammatory cytokine levels, and NLRP3 inflammasome

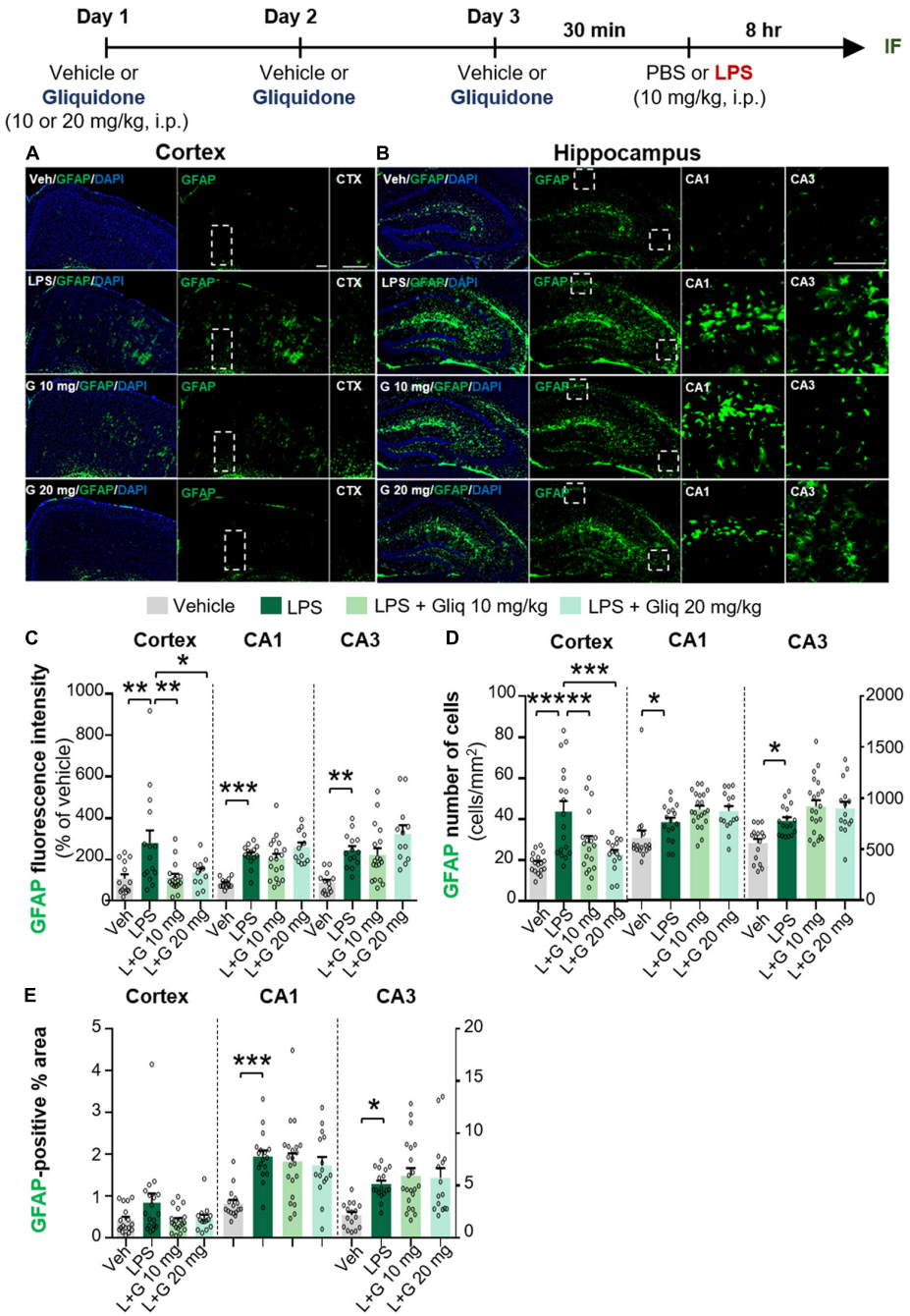


**FIGURE 2 |** Gliquidone decreases LPS-stimulated astrogliosis and the number of GFAP-positive cells in the cortex in C57BL6/N mice. **(A,B)** Immunofluorescence staining of GFAP expression in brain slices from mice treated as shown at the top of the figure. **(C–E)** Quantification of the results in **(A,B)** ($n$ = 12–18 brain slices from 4 mice/group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale bar = 100 μM.

activation in LPS-treated wild-type mice, we next verified the effects of gliquidone on LPS-mediated proinflammatory cytokine levels *in vitro*. We first assessed gliquidone cytotoxicity and induction of mitochondrial arrest using CCK or MTT assays in BV2 microglial cells. No cytotoxicity was observed in BV2 microglial cells after treatment with up to 50 μM gliquidone for 6 h (**Figures 6A,B**). In BV2 microglial cells treated for 24 h, gliquidone had no cytotoxicity up to 25 μM, but 50 μM gliquidone significantly diminished cell viability (**Figure 6C**). Based on these data, a gliquidone concentration of 5 μM, 10-fold lower than the concentration producing mitochondrial dysfunction, was used in subsequent experiments.

Gliquidone is a ligand of SUR1, which is part of the Kir6 channel (Wu et al., 2018), and thus we assessed

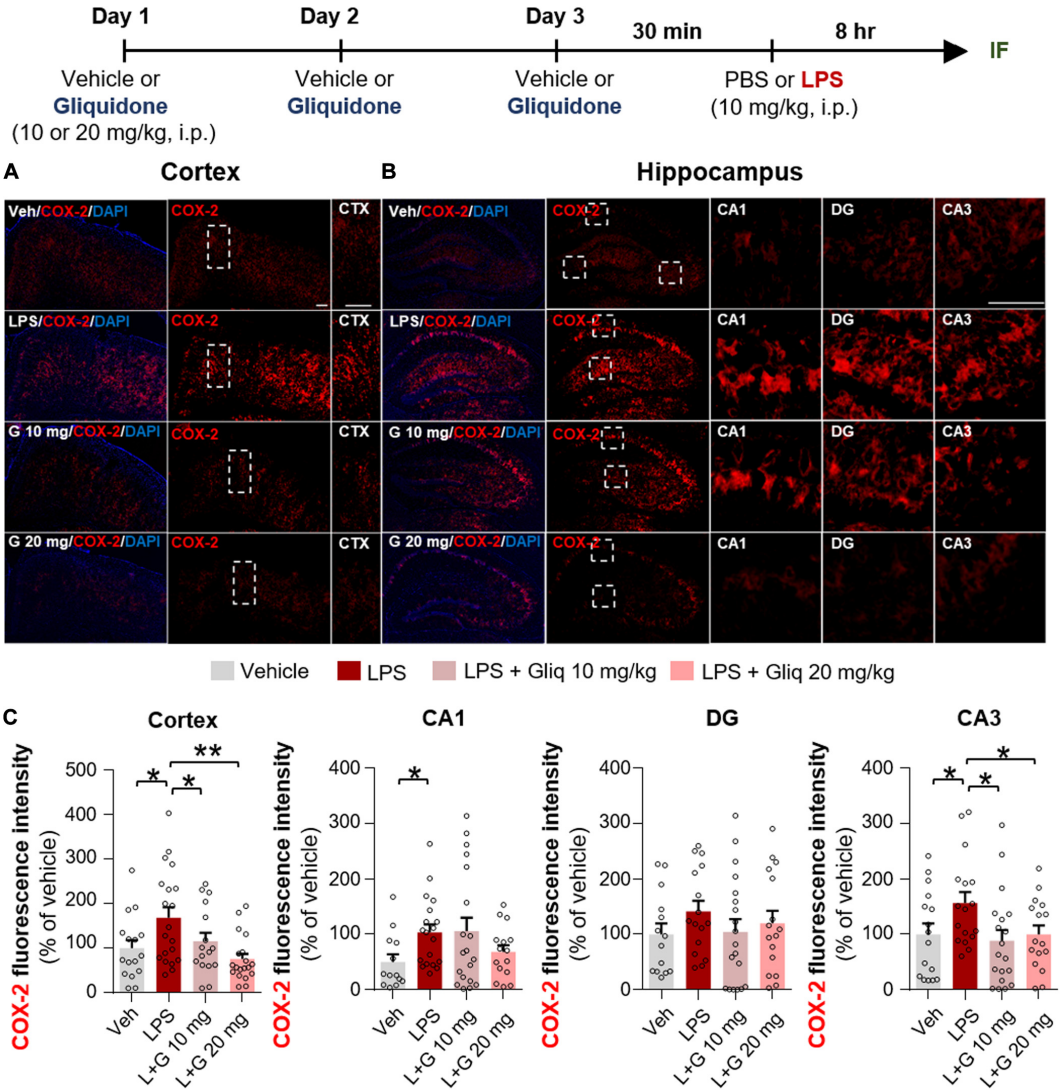


**FIGURE 3 |** Gliquidone diminishes the LPS-induced elevation of COX-2 levels in the brain in C57BL6/N mice. **(A,B)** Immunofluorescence staining of COX-2 expression in brain slices from mice treated as shown at the top of the figure. **(C)** Quantification of the results in **(A,B)** ($n$ = 11–18 brain slices from 4 mice/group). $^{*}p < 0.05$, $^{**}p < 0.01$, Scale bar = 100 μM.

the effects of gliquidone on SUR1 mRNA levels as well as proinflammatory cytokine levels by q-PCR. Treatment of BV2 microglial cells with gliquidone alone for 6 or 24 h did not alter SUR1 mRNA levels (**Figure 6D**). In addition, gliquidone alone reduced COX-2 mRNA levels but did not affect IL-1β, iNOS, and IL-6 mRNA levels in BV2 microglial cells (**Figure 6E**). These results indicate that 5 μM gliquidone itself selectively affects mRNA levels of the proinflammatory cytokine COX-2.

We then examined the effects of gliquidone on LPS-stimulated microglial SUR1 and proinflammatory cytokine mRNA levels in BV2 microglial cells. Cells were pretreated for 30 min with 200 ng/ml LPS or PBS and treated for 5.5 or 23.5 h with 5 μM gliquidone or vehicle (1% DMSO). Treatment with 5 μM gliquidone for 23.5 h but not 5.5 h significantly abolished the LPS-induced increase in SUR1 mRNA levels in BV2 microglial cells (**Figure 6F**). In addition, RT-PCR analysis showed that gliquidone post-treatment significantly reduced the LPS-induced increases in IL-1β, IL-6, iNOS, and COX-2 mRNA levels (**Figures 6G,H**). Confirming these results, q-PCR showed significant reductions in LPS-mediated IL-1β, IL-6, and COX-2 mRNA levels, with a trend toward decreased iNOS mRNA levels (**Figure 6I**). Moreover, we found that pretreatment with 5 μM gliquidone for 30 min significantly reduced the increases in IL-1β, IL-6, iNOS, and COX-2 mRNA levels in BV2 microglial cells induced by treatment with 200 ng/ml LPS for 5.5 h (**Supplementary Figures 2A–C**). Taken together, these results show that post- and pretreatment with gliquidone modulates LPS-induced microglial proinflammatory responses *in vitro*.

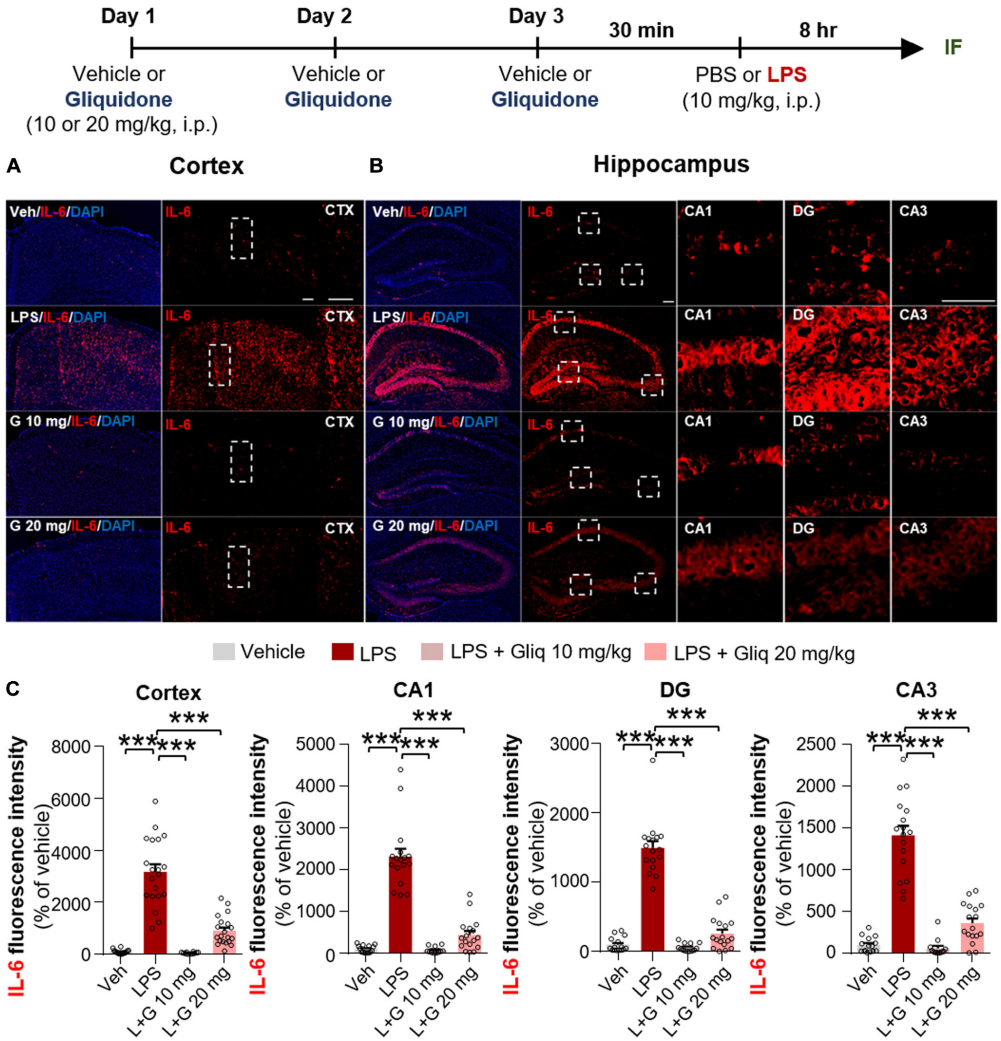


**FIGURE 4 |** Gliquidone eliminates the LPS-induced elevation of IL-6 levels in the brain in C57BL6/N mice. **(A,B)** Immunofluorescence staining of IL-6 expression in brain slices from mice treated as shown at the top of the figure. **(C)** Quantification of the results in **(A,B)** ($n$ = 12–20 brain slices from 4 mice/group). ***$p < 0.001$. Scale bar = 100 μM.

## Gliquidone Suppresses Lipopolysaccharide-Mediated Microglial ERK and STAT3/NF-κB Phosphorylation

In microglial cells, LPS stimulates TLR4 and ATP-sensitive $K^+$ channels via ERK/STAT3 or NF-κB signaling (Rodriguez-Gomez et al., 2020). To examine whether gliquidone alone regulates ERK signaling mediated by ATP-sensitive $K^+$ channels, BV2 microglial cells were treated sequentially with PBS for 45 min and 5 μM gliquidone or vehicle (1% DMSO) for 45 min. Subsequently, Western blotting was conducted with anti-p-ERK, anti-ERK, and anti-β-actin antibodies. We found that gliquidone alone did not alter p-ERK and total ERK levels in BV2 microglial cells (**Figures 7A,B**).

To examine whether gliquidone affects LPS-stimulated microglial ERK signaling, BV2 microglial cells treated with 200 ng/ml LPS or PBS for 45 min were post-treated with 5 μM gliquidone or vehicle (1% DMSO) for 45 min. Subsequently, Western blotting was conducted with anti-p-ERK, anti-ERK, and anti-β-actin antibodies. Gliquidone significantly decreased the LPS-induced increase in p-ERK levels but did not alter total ERK or β-actin levels (**Figures 7C,D**).

To assess the effects of gliquidone itself on nuclear STAT3 or NF-κB phosphorylation, BV2 microglial cells were sequentially treated with PBS for 30 min and 5 μM gliquidone or vehicle (1% DMSO) for 5.5 h, and ICC was performed with anti- p-$STAT3^{s727}$ or anti-p-NF-κB antibodies. We found that gliquidone alone did not alter nuclear p-STAT3 and p-NF-κB levels in BV2 microglial cells (**Figures 7E–H**).

We then tested whether gliquidone alters LPS-mediated nuclear STAT3/NF-κB phosphorylation, BV2 microglial cells were sequentially treated with 200 ng/ml LPS or PBS for 30 min and 5 μM gliquidone or vehicle (1% DMSO) for 5.5 h. ICC assay demonstrated that gliquidone significantly

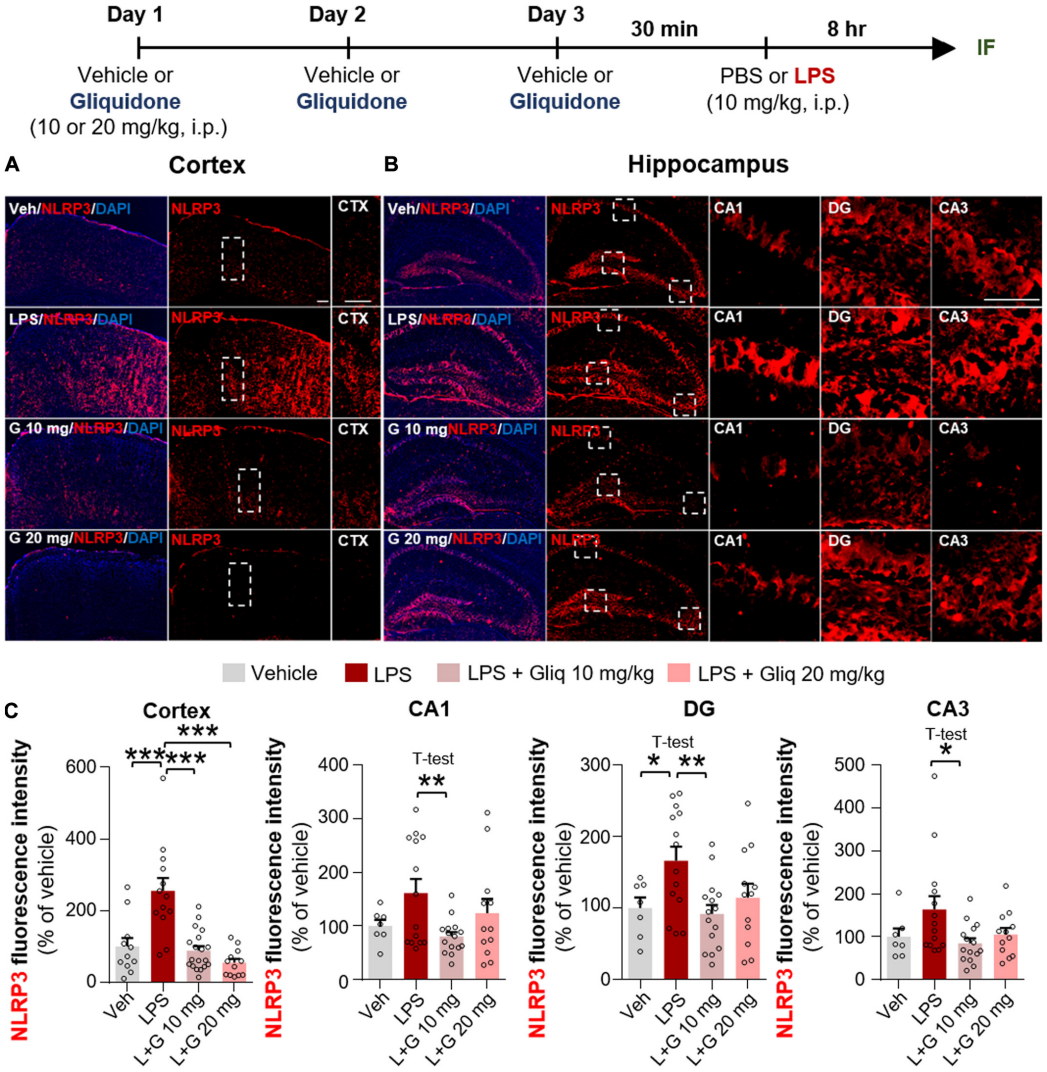


**FIGURE 5 |** Gliquidone eliminates the LPS-mediated increase in NLRP3 inflammasome levels in the brain in C57BL6/N mice. **(A,B)** Immunofluorescence staining of NLRP3 in brain slices from mice treated as shown at the top of the figure. **(C)** Quantification of the results in **(A,B)** ($n$ = 7–19 brain slices from 4 mice/group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bar = 100 μM.

reduced the LPS-mediated increases in nuclear STAT3 and NF-κB phosphorylation in BV2 microglial cells (**Figures 7I–L**). These results indicate that gliquidone modulates downstream ERK/STAT3 or NF-κB signaling regulated by TLR4 and ATP-sensitive $K^{+}$ channels in microglial cells in response to neuroinflammation.

## Gliquidone Abolishes Lipopolysaccharide-Stimulated NLRP3 Inflammasome Activation in BV2 Microglial Cells

Since gliquidone downregulated LPS-evoked NLRP3 inflammasome formation in wild-type mice, we investigated whether gliquidone itself affects NLRP3 inflammasome activation. Treatment of BV2 microglial cells with PBS for 30 min followed by 5 μM gliquidone for 5.5 or 23.5 h did not alter NLRP3 and pro-IL-1β mRNA levels (**Figures 8A–C**).

We then investigated the effects of gliquidone on LPS-mediated microglial NLRP3 inflammasome activation. After treatment for 30 min with 200 ng/ml LPS or PBS, BV2 microglial cells were treated with 5 μM gliquidone or vehicle (1% DMSO) for 5.5 h or 23.5 h, and NLRP3/pro-IL-1β mRNA levels were detected by q-PCR. Post-treatment with gliquidone for 5.5 h significantly reduced LPS-enhanced pro-IL-1β mRNA levels, and a trend toward decreased NLRP3 mRNA levels was observed (**Figures 8D,E**). In addition, LPS-induced NLRP3 mRNA levels were markedly decreased by post-treatment with gliquidone for 23.5 h (**Figure 8F**). ICC analysis showed that gliquidone significantly downregulated LPS-induced microglial NLRP3 protein levels (**Figures 8G,H**). These data imply that gliquidone regulates the LPS-induced neuroinflammatory

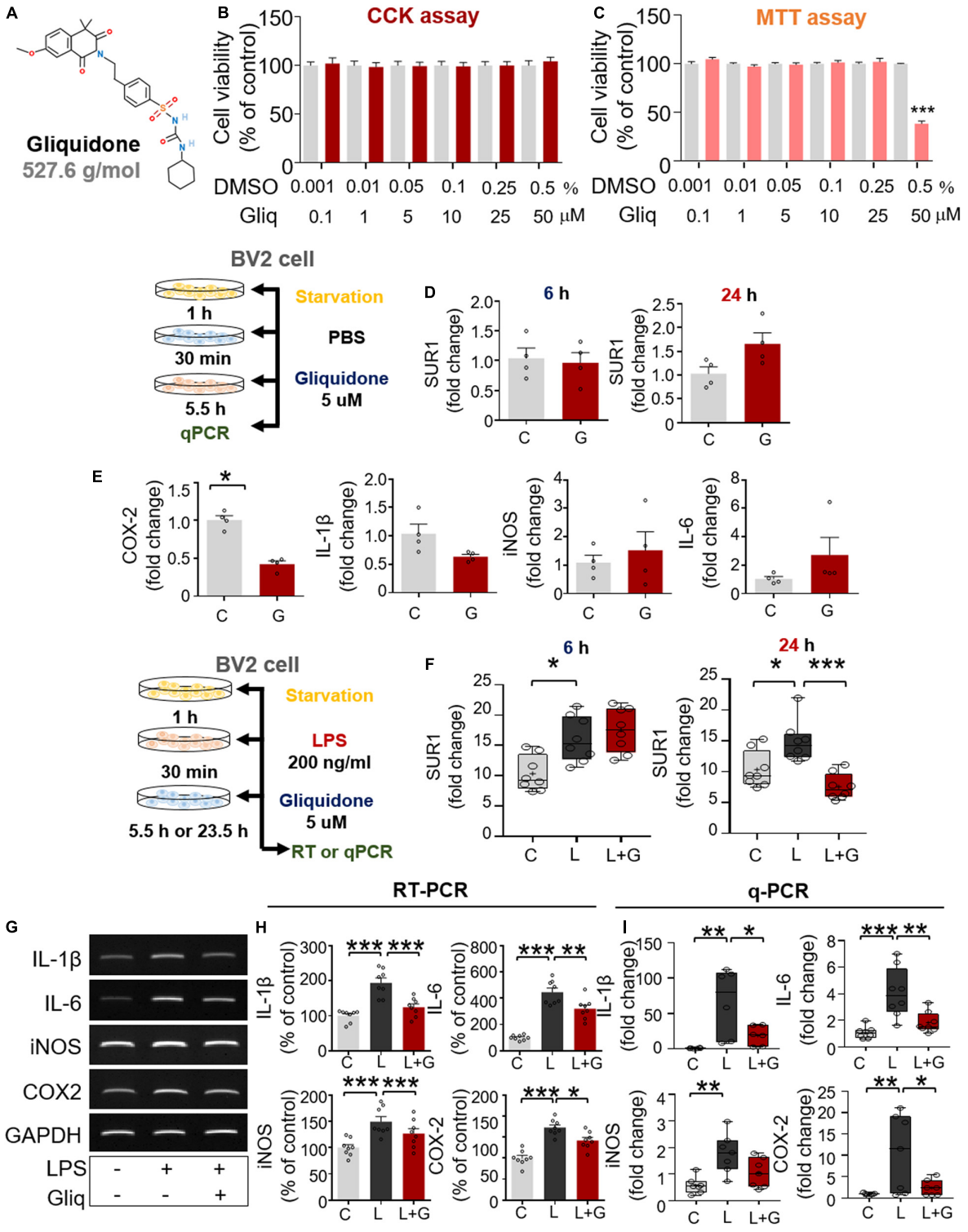


**FIGURE 6 |** Post-treatment with gliquidone reduces LPS-stimulated proinflammatory cytokine mRNA levels in BV2 microglial cells. **(A)** Structure of gliquidone. **(B,C)** CCK and MTT assays were conducted after treating cells with gliquidone at the indicated doses or with vehicle for 6 or 24 h (CCK: 6 h, $n$ = 16/dose; MTT: 24 h, $n$ = 17/dose). **(D)** SUR1 mRNA levels in cells treated for 6 or 24 h with gliquidone alone or vehicle, as determined by q-PCR ($n$ = 4/group). **(E)** Proinflammatory cytokine mRNA levels in cells treated with gliquidone alone or vehicle for 6 h, as determined by q-PCR ($n$ = 4/group). **(F)** Real time-PCR (q-PCR) analysis of SUR1 mRNA levels in cells treated sequentially with LPS for 30 min and gliquidone for 5.5 or 23.5 h ($n$ = 8/group). **(G,H)** RT-PCR analysis of proinflammatory cytokine levels in cells treated sequentially with LPS and gliquidone ($n$ = 8/group). **(I)** Real time-PCR (q-PCR) analysis of proinflammatory cytokine levels in cells treated sequentially with LPS and gliquidone (IL-1β, $n$ = 6/group; IL-6, $n$ = 8/group; iNOS, $n$ = 7/group; COX-2, $n$ = 7/group). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

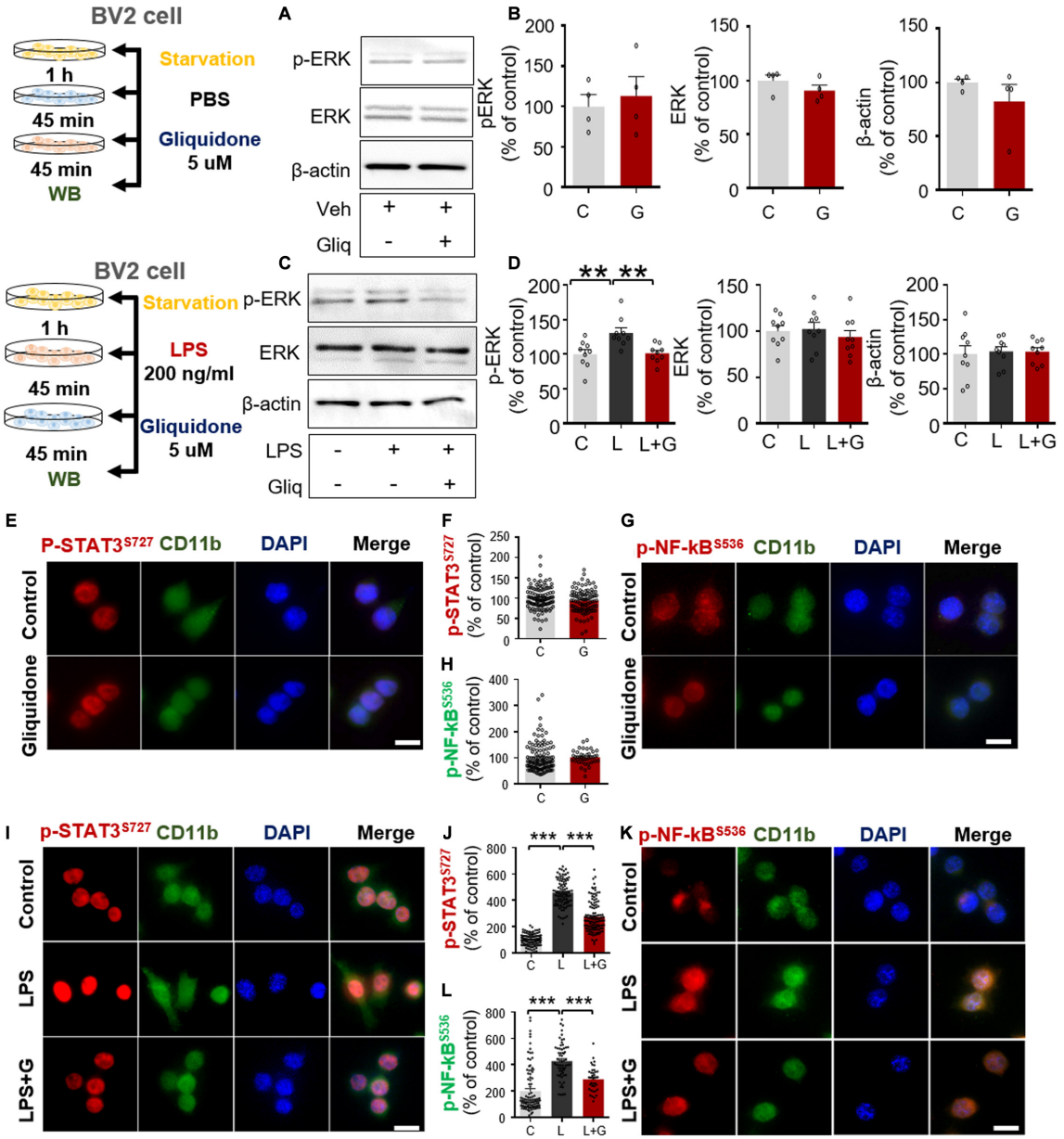


**FIGURE 7 |** Gliquidone suppresses LPS-mediated ERK and STAT3/NF-κB phosphorylation in BV2 microglial cells. **(A,B)** Western blot analysis of p-ERK, ERK, and β-actin levels in cells treated with gliquidone alone or vehicle as shown ($n$ = 4/group). **(C,D)** Western blot analysis of p-ERK, ERK, and β-actin levels in cells treated sequentially with LPS and gliquidone as indicated ($n$ = 9/group). **(E–H)** Immunocytochemistry analysis of CD11b and p-STAT3$^{S727}$ levels or CD11b and NF-κB$^{S536}$ levels in cells treated with gliquidone or vehicle (p-STAT3$^{S727}$: C, $n$ = 112; gliquidone, $n$ = 98; p-NK-kB$^{S536}$: C, $n$ = 149; gliquidone, $n$ = 70). **(I–L)** Immunocytochemistry analysis of CD11b and p-STAT3$^{S727}$ levels or CD11b and NF-κB$^{S536}$ levels in cells treated sequentially with LPS and gliquidone (p-STAT3$^{S727}$: C, $n$ = 119; L, $n$ = 100; LPS + gliquidone, $n$ = 116; p-NK-kB$^{S536}$: C, $n$ = 101; L, $n$ = 61; LPS + gliquidone, $n$ = 37). $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bar = 20 μM.

responses by decreasing NLRP3 inflammasome formation in BV2 microglial cells.

## Gliquidone Reduces Lipopolysaccharide-Evoked IL-1β and IL-6 mRNA Levels in Primary Astrocytes

Since gliquidone downregulates LPS-mediated microglial neuroinflammatory responses in BV2 microglial cells, we next examined the effects of gliquidone on LPS-induced astrocytic neuroinflammatory responses. Mouse primary astrocytes were treated with 200 ng/ml LPS or PBS for 0.5 h and post-treated with 5 μM gliquidone or vehicle (1% DMSO) for 5.5 or 23.5 h. q-PCR analysis showed that gliquidone post-treatment for 5.5 or 23.5 h did not alter SUR1 mRNA levels (**Figures 9A,B**). In addition, gliquidone post-treatment significantly decreased LPS-stimulated IL-1β and IL-6 mRNA levels but did not alter

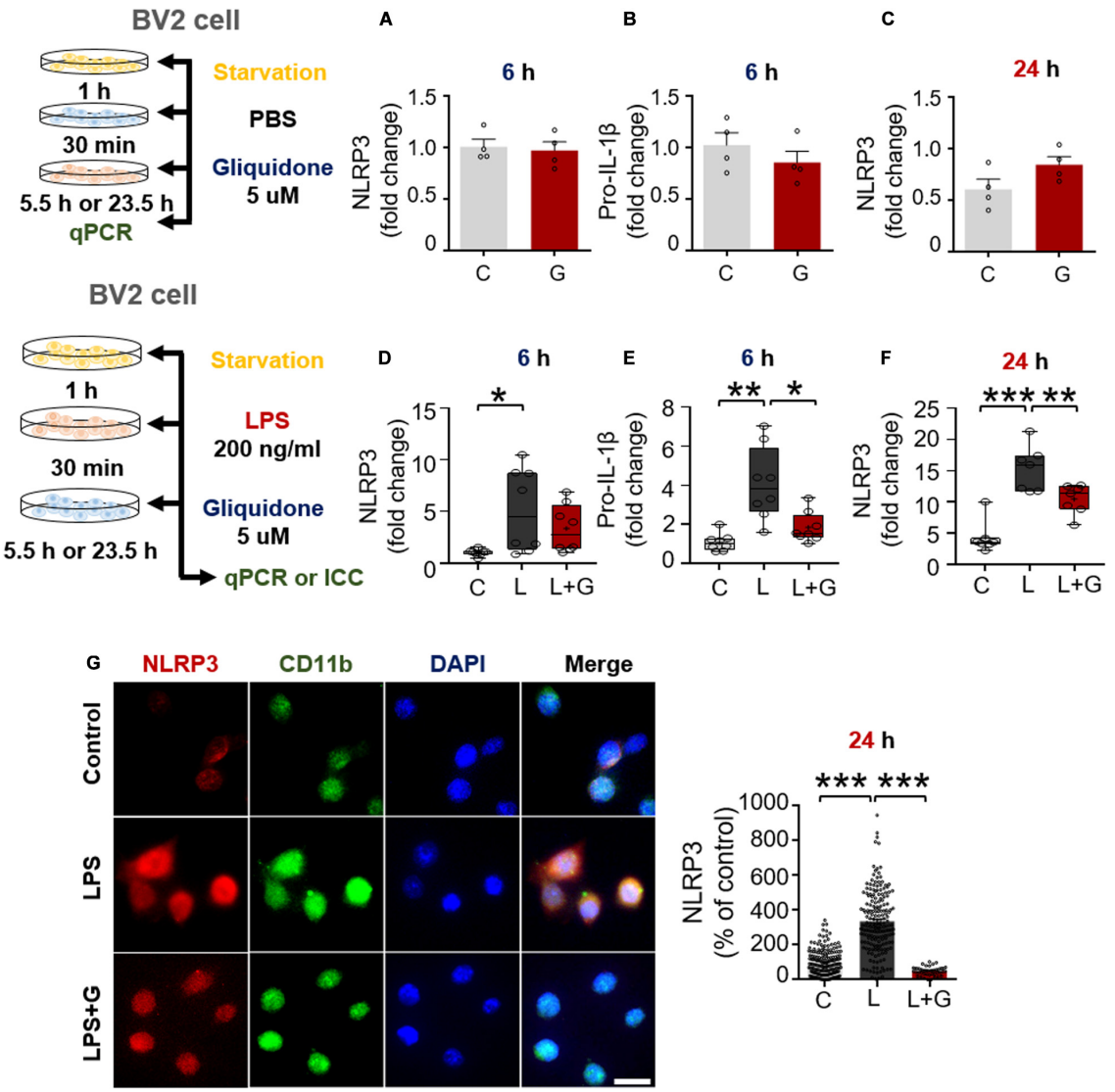


**FIGURE 8 |** Gliquidone inhibits LPS-stimulated NLRP3 and pro-IL-1β in BV2 microglial cells. **(A–C)** Real time-PCR (q-PCR) analysis of NLRP3 and pro-IL-1β mRNA levels in cells treated with gliquidone alone or vehicle for 5.5 or 23.5 h ($n$ = 4/group). **(D–F)** q-PCR analysis of NLRP3 and pro-IL-1β mRNA levels in cells treated sequentially with LPS and gliquidone for 6 or 24 h (6 h NLRP3 and pro-IL-1β, $n$ = 8/group; 24 h NLRP3, $n$ = 7/group). **(G,H)** Immunocytochemistry analysis of CD11b and p-NLRP3 levels in cells treated sequentially with LPS and gliquidone (C, $n$ = 189; L, $n$ = 198; LPS + gliquidone, $n$ = 145). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bar = 20 μM.

iNOS and COX-2 mRNA levels (**Figures 9C–F**). These results suggest that gliquidone modulates proinflammatory cytokines less effectively in astrocytes than in microglia.

## Gliquidone Downregulates Lipopolysaccharide-Stimulated Nuclear STAT3 and NF-κB Phosphorylation in Primary Astrocytes

Given the significant reduction of downstream ERK signaling in BV2 microglial cells treated with gliquidone (**Figure 7**), we then investigated the effects of gliquidone on LPS-mediated astrocytic ERK signaling. Initially, we evaluated the purity of the primary astrocytes culture by measuring the GFAP/DAPI ratio, indicating an astrocyte purity of greater than 80% (**Supplementary Figures 3A,B**). Subsequently, mouse primary astrocytes were treated with 200 ng/ml LPS or PBS for 0.5 h followed by 5 μM gliquidone or vehicle (1% DMSO) for 5.5 h, and ICC was performed with an anti-p-ERK antibody. Gliquidone treatment did not affect LPS-evoked ERK phosphorylation in primary astrocytes (**Supplementary Figures 3C,D**).

Next, we examined the effects of gliquidone on LPS-induced astrocytic STAT3 or NF-κB phosphorylation in the nucleus. Again, we first confirmed that our primary astrocytes had a purity of greater than 80% (**Figures 10A,B**). Subsequent

ICC analysis of cells treated as described above with anti-CD11b and anti-p-STAT3$^{S727}$ or anti-CD11b and p-NF-κB$^{S536}$ antibodies indicated that gliquidone significantly reduced the LPS-stimulated increases in nuclear STAT3 and NF-κB phosphorylation in primary astrocytes (**Figures 10C–F**). These results indicate that gliquidone modulates LPS-mediated astrocytic STAT3 or NF-κB signaling in a p-ERK-independent manner in response to neuroinflammation.

## Gliquidone Does Not Modulate Lipopolysaccharide-Induced NLRP3 Inflammasome Activation in Primary Astrocytes

Since gliquidone downregulated LPS-evoked proinflammatory cytokine levels and nuclear STAT3 and NF-κB phosphorylation in primary astrocytes, we further examined the effects of gliquidone on LPS-induced astrocytic NLRP3 inflammasome activation. Mouse primary astrocytes were treated with 200 ng/ml LPS or PBS for 0.5 h and post-treated with 5 μM gliquidone or vehicle (1% DMSO) for 5.5 h or 23.5 h, and NLRP3 and pro-IL-1β were detected by q-PCR or ICC. Gliquidone post-treatment for 5.5 h significantly diminished the LPS-induced increase in pro-IL-1β mRNA levels, whereas no changes in NLRP3 mRNA and protein levels were observed after gliquidone post-treatment for 5.5 or 23.5 h (**Supplementary Figures 4A–D**). These results indicate that gliquidone regulates LPS-mediated astrocytic proinflammatory cytokine levels by inhibiting STAT3/NF-κB signaling but does not modulate ERK signaling and NLRP3 inflammasome activation in primary astrocytes *in vitro*.

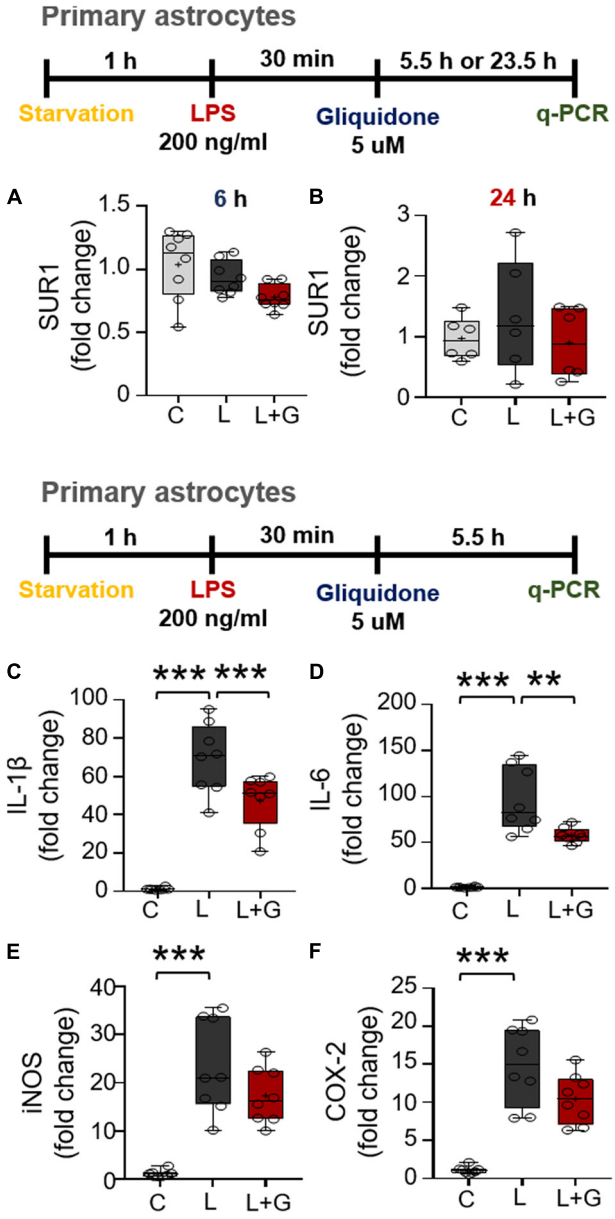


**FIGURE 9 |** Post-treatment with gliquidone reduces LPS-stimulated IL-1β and IL-6 mRNA levels in primary astrocytes. **(A,B)** Real time-PCR (q-PCR) analysis of SUR1 mRNA levels in primary astrocytes treated sequentially with LPS and gliquidone as indicated (6 h, $n$ = 8/group; 24 h, $n$ = 6/group). **(C–F)** Real time-PCR (q-PCR) analysis of proinflammatory cytokine levels in primary astrocytes treated sequentially with LPS and gliquidone as indicated ($n$ = 8/group). $^{**}p < 0.01$, $^{***}p < 0.001$.

## DISCUSSION

In this study, we demonstrated that gliquidone, a sulfonylurea drug that selectively binds sulfonylurea receptor subunit 1 (SUR1), significantly reduced LPS-induced microglial activation and COX-2 and IL-6 proinflammatory cytokine levels by inhibiting NLRP3 inflammasome activation in a model of neuroinflammatory disease, with smaller effects on astrogliosis. In LPS-treated BV2 microglial cells, gliquidone diminished LPS-induced microglial proinflammatory cytokine levels and downstream ERK/STAT3/NF-κB phosphorylation by suppressing NLRP3 inflammasome formation. By contrast, in primary astrocytes, gliquidone selectively abolished LPS-stimulated astrocytic proinflammatory cytokine levels by diminishing STAT3/NF-κB activation via NLRP3-independent mechanisms. The ability of gliquidone to alter LPS-mediated micro- and astroglial neuroinflammation *in vitro* and *in vivo* suggests that this drug could be useful for the prevention and/or treatment of neuroinflammation-associated diseases.

Gliquidone targets SUR1, an inhibitory and regulatory subunit of ATP-dependent $K^+$ (K-ATP) channels (Ashcroft, 2005; Martin et al., 2017), leading to K-ATP channel closure. K-ATP channels are ubiquitously expressed in neurons and glia in several brain regions, including the cortex and hippocampus (McLarnon et al., 2001; Sun and Feng, 2013), and have been linked to inflammatory responses *in vitro* and *in vivo* (Zhou et al., 2008; Rodriguez et al., 2013; Du et al., 2018). For instance, levels of K-ATP channel components [e.g., inward-rectifier potassium subunit 6.1 (Kir 6.1), Kir 6.2, SUR1 and SUR2B] are increased in reactive microglia in neuroinflammation-linked brain pathologies (Ramonet et al., 2004; Ortega et al., 2012, 2013). Membrane and mitochondrial

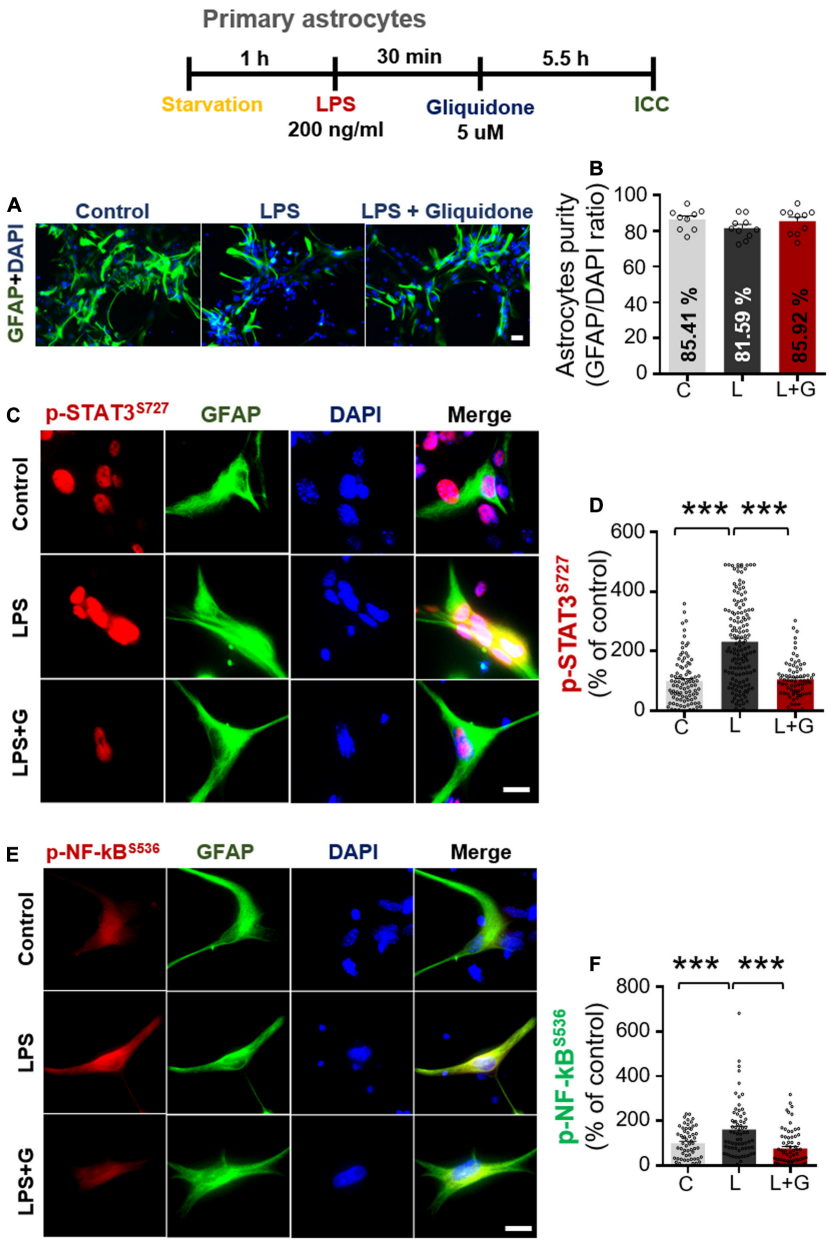


**FIGURE 10 |** Gliquidone abolishes LPS-induced STAT3 and NF-κB phosphorylation in primary astrocytes. **(A,B)** Primary astrocytes were treated sequentially with LPS or PBS and with gliquidone (5 μM) or vehicle (1% DMSO) as indicated, and immunocytochemistry was performed with an anti-GFAP antibody. The purity of the primary astrocytes was measured by the GFAP/DAPI ratio (C, $n = 1726$; L, $n = 876$; LPS + gliquidone, $n = 1527$). **(C–F)** Immunocytochemistry analysis of GFAP and p-STAT3$^{S727}$ or GFAP and p-NF-κB$^{S536}$ expression in primary astrocytes treated sequentially with LPS and gliquidone (p-STAT3$^{S727}$: C, $n = 94$; L, $n = 145$; LPS + gliquidone, $n = 76$; p-NF-κB$^{S536}$: C, $n = 59$; L, $n = 68$; LPS + gliquidone, $n = 75$). ***$p < 0.001$, Scale bar = 20 μM.

K-ATP channels regulate microglial and astroglia activation by controlling membrane electrical changes and mitochondrial ATP metabolism (Rodriguez et al., 2013). In cultured microglia derived from wild-type mice, LPS and interferon-γ (IFN- γ) co-treatment upregulates Kir 6.1 and Kir 6.2 (Virgili et al., 2011), and the K-ATP channel blocker glimepiride significantly reduces the expression of CD14 in BV2 microglial cells and primary microglia (Ingham et al., 2014). In addition, glibenclamide, a K-ATP channel blocker, alleviates neuroinflammation by inhibiting NLRP3 inflammasome activation in BV2 microglial cells and significantly reduces morphine-mediated Iba-1 fluorescence intensity in the spinal cord in mice (Qu et al., 2017). Moreover, K-ATP channels in astrocytes have been closely linked to neurodegeneration via mitophagy in a mouse model of PD, which increases NLRP3 inflammasome formation (Du et al., 2018). In the present study, we found that exposure to the K-ATP channel

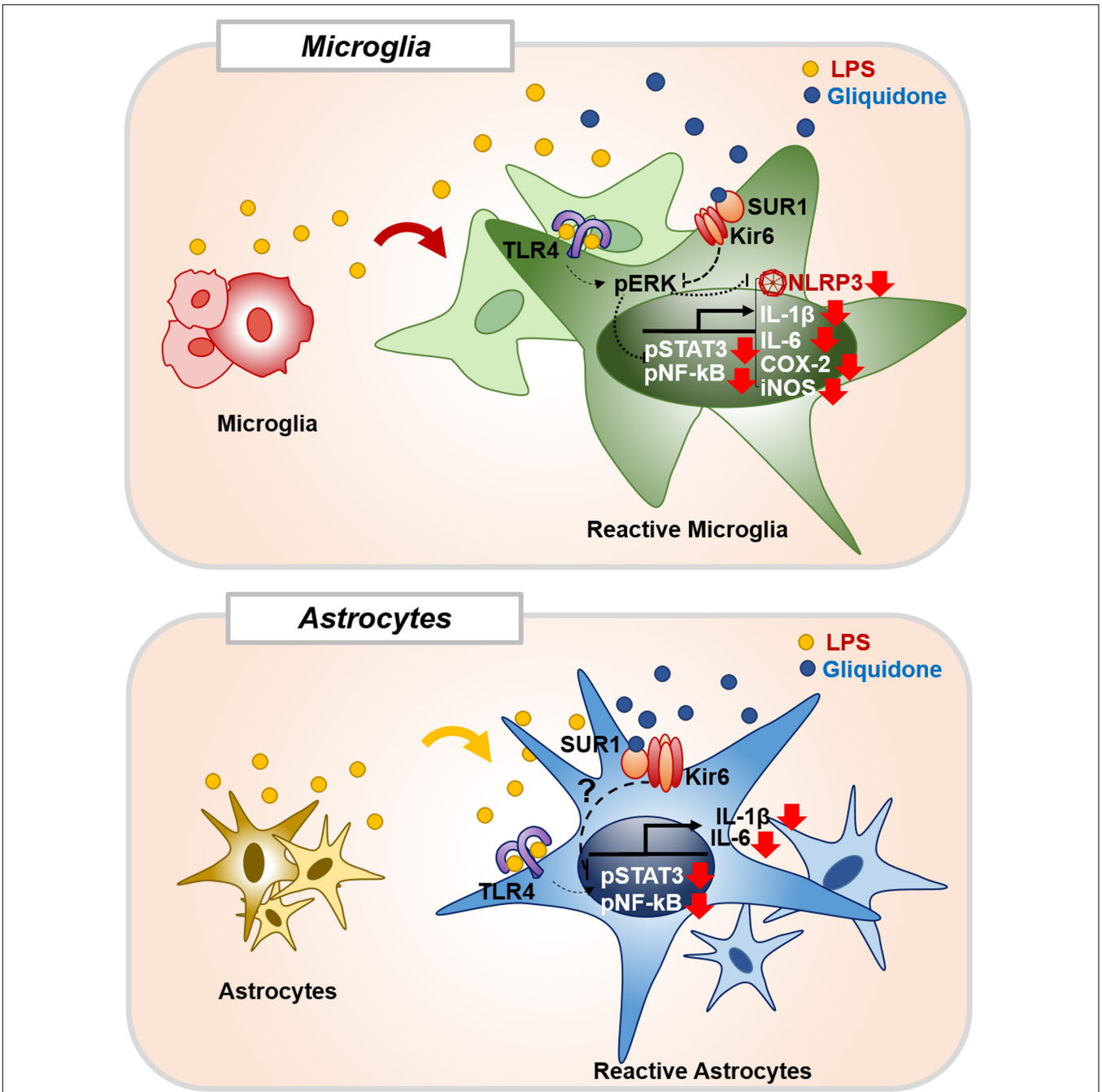


**FIGURE 11 |** Gliquidone affects LPS-stimulated neuroinflammatory responses *in vitro* and *in vivo*. In BV2 microglial cells and wild-type mice, gliquidone reduces LPS-stimulated microglial proinflammatory cytokine expression by modulating ERK-associated STAT3/NF-κB signaling in an NLRP3-dependent manner. In primary astrocytes, gliquidone selectively downregulates LPS-induced astrocytic proinflammatory cytokine expression by decreasing STAT3/NF-κB phosphorylation via an NLRP3-independent pathway. Accordingly, gliquidone may have therapeutic potential for neuroinflammation-related neurodegenerative diseases.

antagonist gliquidone significantly decreased LPS-mediated microglial activation and hypertrophy in the brains of wild-type mice (**Figure 1**). In addition, gliquidone significantly decreased LPS-mediated astrocyte activation and the number of GFAP-positive cells in the cortex but not the hippocampus (**Figure 2**). Taken together with the literature, our findings suggest that gliquidone specifically modulates microglial activation rather than astrogliosis by regulating K-ATP channel signaling in this LPS-induced model of neuroinflammatory disease.

K-ATP channel blockers influence proinflammatory cytokine mRNA and/or protein levels and release (Nakamura et al., 2015; Qu et al., 2017; Du et al., 2019). For example, glibenclamide

significantly suppresses LPS-induced plasma IL-6 and TNF-α levels in *ex vivo* endotoxemia mice (Schmid et al., 2011). Daily administration of another sulfonylurea K-ATP channel blocker, gliclazide, significantly decreases serum TNF-α levels in albino LACA mice (Mourya et al., 2018). In the present study, gliquidone significantly suppressed LPS-induced COX-2 and IL-6 proinflammatory cytokine levels and peripheral inflammation in wild-type mice (**Figures 3**, **4** and **Supplementary Figure 1**). Moreover, gliquidone significantly downregulated LPS-mediated NLRP3 levels in wild-type mice (**Figure 5**). These data indicate that gliquidone and other K-ATP channel inhibitors can modulate proinflammatory cytokine expression as well as peripheral inflammation by altering NLRP3 inflammasome formation *in vivo*.

An important finding of this study is that gliquidone treatment significantly reduced LPS-mediated Iba-1, COX-2, IL-6, and NLRP3 levels in the cortex and/or hippocampal CA1/CA3 regions in wild-type mice but did not alter LPS-stimulated Iba-1 and COX-2 levels in the DG region (**Figures 1**, **3**). However, this raises the question as to how does gliquidone differentially modulate the induction of Iba-1, COX-2, IL-6, and NLRP3 by LPS according to brain region? It is possible that the receptor subunit (i.e., SUR1 or Kir 6) and/or K-ATP channel targeted by gliquidone is differentially expressed in a brain region-specific manner (cortex, hippocampus CA1 vs. DG regions), resulting in differences in the effects of gliquidone on LPS-induced Iba-1, COX-2, IL-6, and NLRP3 levels between the cortex and hippocampus in wild-type mice. Another possibility is that a 3-day duration of daily injections was not sufficient to influence LPS-stimulated Iba-1, COX-2, IL-6, and NLRP3 levels in the hippocampus (CA1 vs. DG region). Future work, will need to examine whether a differential expression of the target receptor subunit (SUR1 and Kir6) and/or K-ATP channel exists between the cortex and hippocampal CA1 vs. DG region and if this alters LPS-induced proinflammatory responses in the presence and absence of gliquidone. Furthermore, the effect of increasing the duration of gliquidone treatment (i.e., daily for 7 or 14 days) upon LPS-evoked neuroinflammation in the cortex and hippocampus should be examined and compared with 3 days injection protocol.

K-ATP channel blockers have been previously shown to modulate proinflammatory cytokine expression *in vitro*. For instance, the sulfonylurea K-ATP channel blocker glimepiride significantly reduced the LPS-induced increase in TNF-α expression in RAW 264 macrophage cells (Ingham et al., 2014). In BV2 microglial cells, glibenclamide significantly suppressed morphine- and LPS-induced proinflammatory cytokine levels (Qu et al., 2017; Xu et al., 2017). Gliquidone is a second-generation K-ATP blocker with few side effects, but its ability to modulate LPS-induced neuroinflammatory responses and its mechanism of action have not been investigated. Here, we found that either pre- or post-treatment with gliquidone significantly reduced LPS-induced proinflammatory cytokine expression in BV2 microglial cells, which express the K-ATP channel subunits Kir 6.1 and Kir 6.2 (Rodriguez et al., 2013; Du et al., 2018) (**Figure 6** and **Supplementary Figure 2**). In primary astrocytes, gliquidone significantly diminished LPS-induced proinflammatory cytokine levels (**Figure 9**), suggesting that gliquidone modulates the target receptor itself and that blockade of K-ATPs is critical for proinflammatory cytokine production in microglia and astrocytes. In future work, we will perform epigenetic knockdown or inhibit individual K-ATP channel subunits (i.e., SUR1, SUR2B, Kir 6.1, Kir 6.2) to examine whether gliquidone alters LPS-mediated proinflammatory cytokine release by regulating SUR1 specifically or all four K-ATP subunits. Overall, our findings suggest that K-ATP channel modulation by gliquidone reduces LPS-induced proinflammatory cytokine release from glial cells.

Lipopolysaccharide binds TLR4 to stimulate TLR4-linked proinflammatory cytokine production and transcription factor activation in microglia and astrocytes (Park and Lee, 2013; Molteni et al., 2016). Under normal conditions, K-ATP channels regulate $Ca^{2+}$ influx, and increased $Ca^{2+}$ conductance activates TLR4-associated MAPK signaling in microglia (Katz et al., 2006; Sharma and Ping, 2014). Indeed, several studies have demonstrated that TLR4- and K-ATP-linked MAPK/ERK signaling modulates proinflammatory cytokine expression (Xiao et al., 2002; Lin and Chai, 2008; Cargnello and Roux, 2011; Plociennikowska et al., 2015). For example, the first-generation K-ATP channel antagonist tolbutamide significantly inhibits ERK phosphorylation in U87 glioma cells, and this decrease is rescued by the K-ATP channel agonist diazoxide (Huang et al., 2009). In BV2 microglial cells, the K-ATP channel antagonist and SUR1 agonist glibenclamide significantly reduces LPS-induced MAPK signaling (e.g., p-ERK, p-JNK, and p-P38) in a dose-dependent manner (Xu et al., 2017). Here, we found that the anti-diabetic drug and second-generation K-ATP channel blocker gliquidone significantly reduced LPS-stimulated p-ERK levels in BV2 microglial cells but not in primary astrocytes (**Figure 7** and **Supplementary Figure 3**), indicating that gliquidone affects TLR4- and K-ATP-associated ERK signaling in microglia. However, it is possible that gliquidone inhibits other MAPK signaling pathways to modulate LPS-induced proinflammatory responses; future experiments will address this possibility by using specific inhibitors and/or regulating gene expression levels (e.g., siRNA).

Given the effects of gliquidone on LPS-linked ERK phosphorylation in microglia, we investigated whether gliquidone modulates the transcription factor STAT3 and NF-κB, a downstream molecule that is crucial for promoting proinflammatory cytokine expression (Nam et al., 2018; Ryu et al., 2019). Recent studies have demonstrated links of K-ATP channels with STAT3 and NF-κB in the response to inflammation in the central nervous system (CNS) and peripheral nervous system (PNS). For instance, glibenclamide abolishes K-ATP channel opening-induced increases in rat hepatic p-STAT3 levels (Fouad et al., 2020). In human cerebrospinal fluid and cardiomyocytes, the K-ATP channel opener diazoxide and the mitochondrial K-ATP channel agonist rapamycin significantly increase STAT3 phosphorylation (Kishore et al., 2011; Das et al., 2012). Furthermore, morphine-induced impairment of the ATP/ADP ratio stimulates K-ATP channel-associated NF-κB activation by inducing NLRP3 inflammasome formation in microglia (Qu et al., 2017). In the present study, gliquidone significantly reduced LPS-mediated nuclear p-STAT3 and

p-NF-κB levels in BV2 microglial cells and primary astrocytes (**Figures 7**, **10**). Therefore, our findings and previous work suggest that gliquidone suppresses LPS-evoked STAT3/NF-κB activation by inhibiting K-ATP channels in microglia and primary astrocytes. It is possible that gliquidone affects LPS-mediated proinflammatory cytokine production in microglia and/or astrocytes by inhibiting another transcription factor, such as NFATc-1 (nuclear factor of activated T-cells, cytoplasmic 1), which is known to be involved in TLR4-mediated downstream signaling. Thus, future studies will examine whether gliquidone affects other LPS-associated MAPK signaling pathways and transcription factors linked to K-ATP channels.

Excessive activation of the NLRP3 inflammasome is a key contributor to pathogenesis in various neuroinflammatory responses. In addition, associations of NLRP3 with intracellular K efflux/concentration in microglia and astrocytes have recently been reported. For example, numerous NLRP3 inflammasome activators [i.e., danger-associated molecular patterns (DAMPs) such as silica and uric acid crystals and pathogen-associated molecular patterns (PAMPs) such as LPS and morphine] are known to induce potassium efflux (Qu et al., 2017; Yang et al., 2019). The K-ATP channel agonist LLME (lysosomotropic agent Leu-Leu-*O*-methyl ester) significantly increases intracellular potassium efflux in C57BL/6 mice and *Nlrp3*$^{-/-}$ mice, which induces NLRP3 inflammasome activation independent of calcium signaling (Katsnelson et al., 2015). In addition, K efflux and NLRP3 inflammasome activation are significantly increased in LLME-treated bone marrow-derived dendritic cells, suggesting that NLRP3 is closely linked to K-ATP channel activation (Katsnelson et al., 2016). Reducing the intracellular $K^+$ concentration is sufficient to activate NLRP3 inflammasome formation in nlrp3$^{-/-}$ macrophages (Munoz-Planillo et al., 2013). Interestingly, the classic K-ATP channel blocker glibenclamide reduces morphine-induced neuroinflammation by inhibiting NLRP3 inflammasome activation in CD-1 mice (Qu et al., 2017). Consistent with these observations, we found that the second-generation K-ATP channel blocker gliquidone significantly inhibited LPS-mediated NLRP3 inflammasome activation in wild-type mice and BV2 microglial cells but not primary astrocytes (**Figures 5**, **8** and **Supplementary Figure 4**). These data indicate that gliquidone differentially regulates LPS-mediated NLRP3 inflammasome activation to alter neuroinflammatory responses in microglia and astrocytes. The underlying mechanism by which gliquidone influences signaling factors involved in NLRP3 inflammasome formation requires further investigation *in vivo* and *in vitro*.

## CONCLUSION

In summary, gliquidone downregulates LPS-stimulated increases in microglial morphology and activation as well as proinflammatory cytokine IL-6 and COX-2 levels by decreasing NLRP3 inflammasome formation in wild-type mice. Compared with its effects on microglia, gliquidone has smaller effects on astrogliosis and astroglial hypertrophy in LPS-treated wild-type mice. In BV2 microglial cells, gliquidone suppresses LPS-induced proinflammatory cytokine levels by modulating ERK/STAT3/NF-κB phosphorylation through the inhibition of NLRP3 inflammasome activation. In primary astrocytes, gliquidone selectively affects LPS-mediated proinflammatory cytokine levels and reduces STAT3/NK-kB phosphorylation in an NLRP3-independent manner (**Figure 11**). These results suggest that gliquidone has anti-inflammatory effects by modulating LPS-induced micro- and astroglial neuroinflammation and could be a novel therapy for neuroinflammation-related neurodegenerative diseases.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by Korea Brain Research Institute (KBRI, approval no. IACUC-19-00042).

## AUTHOR CONTRIBUTIONS

JK and H-SH: study conception and design. J-HP and JK: acquisition of data. JK: preparation of figures and tables. JK, KS, SJM, KC, and H-SH: writing and editing of manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.754123/full#supplementary-material

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REVIEW
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# Alpha-Synuclein Induced Immune Cells Activation and Associated Therapy in Parkinson's Disease

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Parkinson's disease (PD) is a neurodegenerative disorder closely related to immunity. An important aspect of the pathogenesis of PD is the interaction between α-synuclein and a series of immune cells. Studies have shown that accumulation of α-synuclein can induce an autoimmune response that accelerates the progression of PD. This study discusses the mechanisms underlying the interaction between α-synuclein and the immune system. During the development of PD, abnormally accumulated α-synuclein becomes an autoimmune antigen that binds to Toll-like receptors (TLRs) that activate microglia, which differentiate into the microglia type 1 (M1) subtype. The microglia activate intracellular inflammatory pathways, induce the release of proinflammatory cytokines, and promote the differentiation of cluster of differentiation 4 + (CD4 +) T cells into proinflammatory T helper type 1 (Th1) and T helper type 17 (Th17) subtypes. Given the important role of α-synuclein in the immune system of the patients with PD, identifying potential targets of immunotherapy related to α-synuclein is critical for slowing disease progression. An enhanced understanding of immune-associated mechanisms in PD can guide the development of associated therapeutic strategies in the future.

**Keywords: Parkinson's disease (PD), α-synuclein, microglia, T cells, therapeutics**

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## INTRODUCTION

Parkinson's disease (PD) affects more than 3% of the population aged 65 years and older and is the second most common neurodegenerative disease globally (Ascherio and Schwarzschild, 2016; Bonam and Muller, 2020). PD is an enormous economic burden for the patients and society. The cost incurred by a single patient with PD in the United States (US) is estimated more than $10,000 (Kowal et al., 2013; Yang W. et al., 2020). The main manifestations of PD are motor symptoms such as tremor, rigidity, bradykinesia, and dyskinesia and non-motor symptoms such as autonomic dysfunction, cognitive abnormalities, and emotional problems (Jankovic, 2008; Palmeri et al., 2017). Epidemiology studies have shown that genetics along with environmental factors such as gut microbiota, pesticides, and heavy metal contribute to the pathogenesis of PD (Scheperjans et al., 2015; Pietrucci et al., 2019). The major pathological event in PD is the death of dopaminergic neurons in the substantia nigra pars compacta and the accumulation of α-synuclein (Surmeier, 2018), which is predominantly observed at the presynaptic terminals. Previous study has reported that α-synuclein can be released from the neurons in human model system (El-Agnaf et al., 2003; Henderson et al., 2019). Autophagy, metabolic disorder, and neuroinflammation have been proposed as the mechanisms linking α-synuclein deposition to neuronal death. For example, defects

in autophagy result in abnormal α-synuclein clearance (Park et al., 2020) and recent studies have reported that the dysregulation of interactions among endoplasmic reticulum stress, unfold protein response, and autophagy are observed in the pathogenesis of PD (Ren et al., 2021). Besides, many convinced results also reported that mutations within autophagy-related genes such as PTEN induced putative kinase 1 (*PINK1*), *PARKIN*, *PARK7* (*DJ-1*) play an essential role in mitochondrial dysfunction and the dysfunction of mitochondria can successively induce a series of metabolic disorders (Kasten et al., 2018). However, the production of autophagy and mitochondrial dysfunction such as inducible nitric oxide (iNO), reactive oxygen species (ROS), and abnormal accumulation proteins can all involve the activation of central nervous system (CNS) immune system and chronic neuroinflammation that result in PD (Marogianni et al., 2020).

In CNS, microglial cells mainly act as macrophages in the brain parenchyma; other minor peripheral infiltration immune cells such as T cells, dendritic cells, and B cells are located in protective covers of the brain and most of them are generally in the resting state (Engelhardt, 2008). However, abnormal immune system activation is found in PD (Baird et al., 2019; Thomas Broome et al., 2020). Typically, researchers found that the inflammatory process in PD is mainly induced by microglia that helps in the release of cytokines. Besides, it was also reported that in the pathogenesis of PD, both the innate and adaptive immune responses are essential for the inflammation of neuronal cells (Kannarkat et al., 2013; Chen et al., 2018; Schonhoff et al., 2020). For instance, cluster of differentiation 8 + (CD8 +) and cluster of differentiation 4 + (CD4 +) T cells are detected in both the postmortem brain tissue from the patients with PD and mouse models of PD (Brochard et al., 2009).

Simultaneously, α-synuclein accumulation and the formation of Lewy bodies (LBs) in dopaminergic neurons contribute to the inflammatory mechanism in PD (Baba et al., 1998; Wang et al., 2020). PD-associated α-synuclein accumulation is reported to increase in the peripheral plasma and cerebrospinal fluid (CSF). Abnormally increased α-synuclein might be closely related to the abnormal activation of the central and peripheral immune system that is also revealed to affect the pathological mechanisms of the microglia and T cells (Olesen et al., 2018). Above all, clarifying the relationships among these factors is critical for understanding the role of neuroinflammation in the development and pathogenesis of PD.

In this study, we discuss the contribution of neuroinflammation to PD including the effect of α-synuclein on the microglia and T cells (**Figure 1**). We will also discuss some potential immune-based therapeutic strategies that target α-synuclein.

# EFFECT OF α-SYNUCLEIN ON MICROGLIA

## Overview of Microglia

Since the first report of activated microglia in postmortem brain tissue samples from the patients with PD, microglia have been implicated in the neurodegenerative diseases such as PD and Alzheimer's disease (AD) (McGeer et al., 1988; Streit et al., 2004; Gerhard et al., 2006). Microglia can act as the macrophage in the CNS to protect against pathogens and regulate the homeostasis in the brain (Casano and Peri, 2015; De Schepper et al., 2020). The subtype of microglia consists of M1, which mainly functions in the first line to clear the pathogens and M2, which is generally considered to inhibit the proinflammatory responses with an increase to repair in gene expression (Tang and Le, 2016). In the resting state, a small number of microglia can be activated to clear the pathogens or abnormal proteins and, thereby, maintain homeostasis. However, abnormal protein deposits and increase in ROS can induce the activation of M1 microglia. During the progression of PD, abnormal α-synuclein aggregated is phagocytosed, stimulating the inflammation response. A previous study demonstrated that microglia-mediated neuroinflammation affects dopamine neurons (DNs) survival in the patients with PD, implying that it plays an important role in the pathogenesis of PD (Ouchi et al., 2005). A recent study also revealed that microglia exert this effect via the regulation of DJ-1 (Lin et al., 2021). Besides, a recent comment also introduced the mechanisms of α-synuclein handling by microglia (Song et al., 2021). Consequently, the interaction between the microglia and α-synuclein is a critical aspect of the pathogenesis of PD.

## Inflammatory Pathway of Microglia Induced by α-Synuclein

Toll-like receptors (TLRs) and the NOD-, LRR- and pyrin domain-containing protein3 (NLRP3) inflammasome are involved in the activation of microglia. Other factors such as senescence and gut microbiota can also influence the function of microglia.

One essential component that facilitates the activation and interaction of microglia with α-synuclein is the TLR. As reported, TLR4 in microglia contributes to the generation of ROS induced by α-synuclein and proinflammatory cytokines (Qin et al., 2005; Fellner et al., 2013). Simultaneously, as a type of pattern recognition receptor (PRR), α-synuclein can bind to TLR2 to active microglia, which was described as α-synuclein induced non-cell autonomous neurotoxic effects of microglia (Kim et al., 2013, 2016). There are also other studies indicate that the proinflammatory cytokines could be directly activated by higher-order oligomeric α-synuclein by binding to TLR1/2 and it was achieved by nuclear factor-kappa B (NF-κB) translocation and tumor necrosis factor-α (TNF-α) induction via a Myeloid differentiation primary response 88 (MYD88)-dependent pathway (Daniele et al., 2015).

Activation of NLRP3 inflammasome is also considered an important pathogenic mechanism in microglia (Swanson et al., 2019). It was revealed that α-synuclein could act as a signal to promote the NLRP3 inflammasome assembly in microglia (Lu and Hu, 2012). Furthermore, activated NLRP3 inflammasome will also increase the accumulation of α-synuclein. Another study also demonstrated that the downregulation of the NLRP3 inflammasome components reduces the expression of

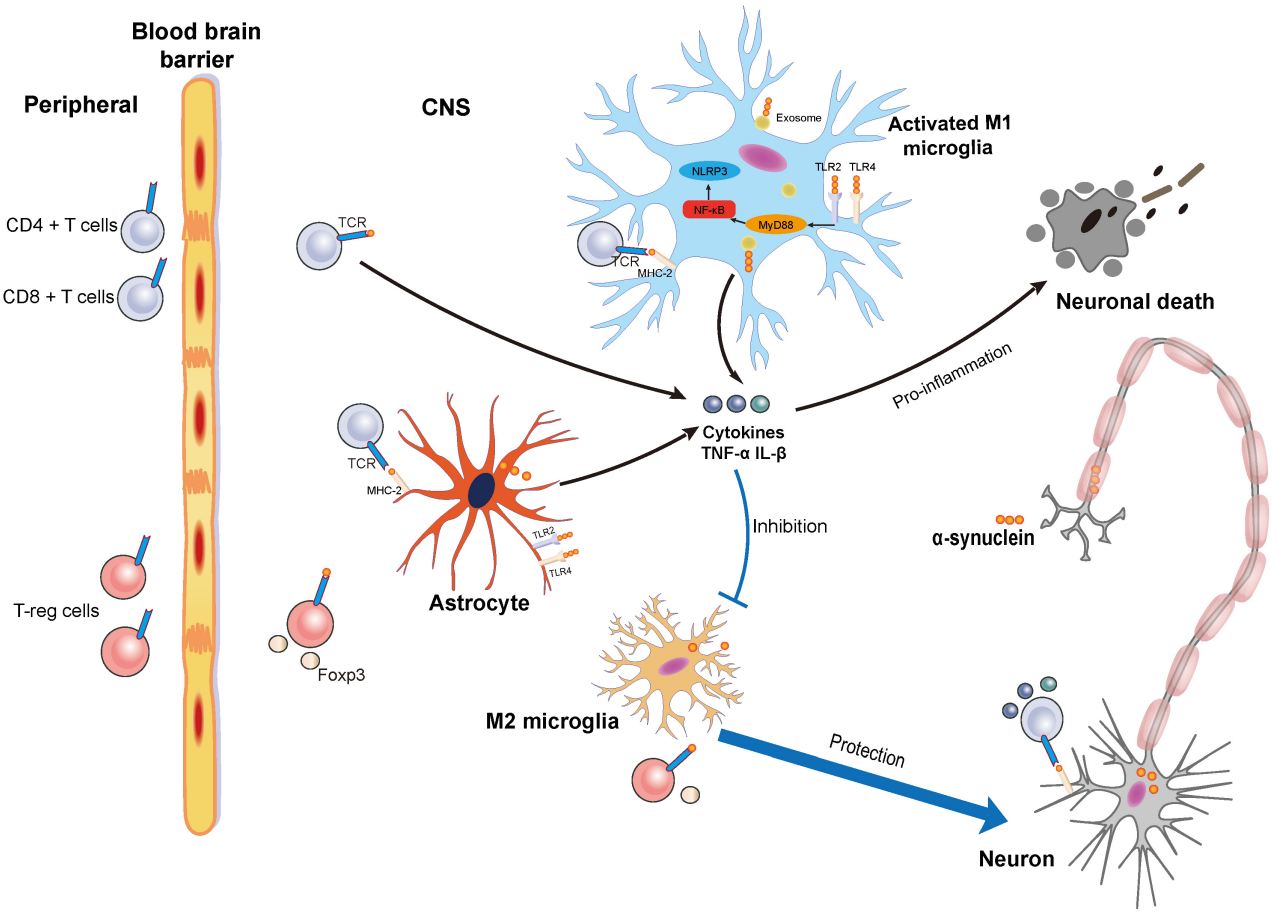


**FIGURE 1 |** Schematic illustration of neuronal death and protection induced by the activation of microglia via α-synuclein and the relationship between α-synuclein and T cells. Accumulated α-synuclein released from neurons binds the Toll-like receptor 2 (TLR2) or Toll-like receptor 4 (TLR4) to induce a proinflammation cascade. At the same time, nuclear factor-kappa B (NF-κB) is activated to induce the MyD88 pathway for M1 microglia activation. α-synuclein can also be transferred into microglia by exosome. Proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) can be released from activated M1 microglia to result in neuronal death. Damage to the blood–brain barrier (BBB) in Parkinson's disease (PD) results in the infiltration of T cells including cluster of differentiation 4 + (CD4 +) T cells, cluster of differentiation 4 + (CD8 +) T cells, and regulatory T cells from the peripheral blood that can infiltrate into central nervous system (CNS). In the CNS, microglia and astrocyte act as the antigen-presenting cells (APCs) to present the α-synuclein to the T cells. Activated CD4 + and CD8 + T cells release cytokines that promote neuronal death, while Tregs exert a protective effect on neurons.

the cytokines interleukin (IL)-1β and IL-18, implying that it contributes to cytotoxic microglia regulation (Javed et al., 2020).

## How Can α-Synuclein Activate Microglia?

Gene mutation is an essential factor affecting the activation of microglia. On the molecular level, a series of complex signal pathways and gene expressions are involved in the interaction between α-synuclein and microglia functions related to PD. For example, Fyn is a Src family tyrosine kinase that can be expressed in the brain. It is revealed as a critical signal mediator in the process of microglial-induced neuroinflammation in PD. This Fyn kinase-mediated pathway will activate NLRP3 inflammasome and consequently induce IL-1β secretion by microglia (Panicker et al., 2015, 2019; Nalls et al., 2018; Kam et al., 2020). Besides, the lack of PINK1 will increase the stimulation of α-synuclein by microglia to produce more NO to provoke immune response and death of neurons (Sun et al., 2018). To further understand the molecular mechanisms of α-synuclein associated immune-mediated neurotoxicity, Sarkar et al. analyzed the proteomics of α-synuclein-mediated microglial activation in microglia of mouse. It was confirmed that the expression of 864 genes such as *Irg1*, *Ifit1*, and *Pyhin* increased during the activation of microglia. In addition, they also observed that some critical proteins such as Cdc123, Sod1, and Grn, which play an important role in the different processes, including metabolic and lysosomal, were decreased (Sarkar et al., 2020).

Recently, researchers have observed a series of metabolic changes combined with the activation of microglia to satisfy the energy requirement of activation of inflammatory cells. A mounting of studies had revealed that metabolic reprogram provides critical power to activation of microglia. In this process, increased glycolysis and decreased fat metabolism were shown

to be closely associated with the regulation of α-synuclein (Ganeshan and Chawla, 2014; Yang et al., 2021). A recent study has revealed that α-synuclein can bind pyruvate kinase M2 (PKM2) directly to promote the glycolysis and the migration of microglia. In this case, microglia will be converted to M1 proinflammatory type to increase the inflammatory reaction (Qiao et al., 2019).

The above studies demonstrate that besides the direct activation of the inflammation pathway, α-synuclein can also regulate the activation of microglia via the abnormal expression of proteins caused by gene mutation or through metabolic regulation. These lines of evidence confirm the association between α-synuclein and activation of microglia in PD.

## Relationship Between Activation of Microglia and Deposition and Transmission of α-Synuclein

### Senescence of Microglia and Deposition of α-Synuclein

Given that PD is strongly related to aging, the senescence of microglia is an important factor in the pathogenesis of PD. A previous study has demonstrated that senescent microglia release TNF-α to promote the deposition of α-synuclein (Sierra et al., 2007). Besides, Angelova and Brown found a method to establish a senescent microglia phenotype module *in vitro* based on iron overload. They observed that the secretion of TNF-α by microglia was increased, which could affect the function of α-synuclein and increased its expression and aggregation levels (Angelova and Brown, 2018). Thus, the senescence of microglia may affect the deposition of α-synuclein in PD.

### Alpha-Synuclein Transmission Through Internalization of α-Synuclein-Containing Exosomes by Microglia

It has been hypothesized that the prion-like spread of α-synuclein in the brain promotes the progression of PD (Stopschinski and Diamond, 2017; Steiner et al., 2018). To investigate how this process is regulated by microglia, George et al. developed mouse models to deplete or activate the cells of microglia to evaluate the effect on the transfer of hu-α-syn. This resulted in the accumulation of huα-syn in grafted dopaminergic neurons with fewer cells of microglia. These results demonstrated that active state regulation might influence the transfer of α-synuclein, which indicates the possibility of controlling the neuropathology expansion of PD (George et al., 2019). Besides, the state of microglia can be regulated by exosomes and affect α-synuclein. This pathway can be observed both in the central and peripheral nervous systems. To confirm the conclusion, Yun Xia et al. injected plasma exosomes from the patients with PD into the striatum of the brains of mice to evaluate exosome function in the transmission of α-synuclein. They demonstrated that microglia could uptake plasma exosomes effectively. The immunofluorescent costaining showed that the microglia released exosomal α-synuclein, which could promote exosomal α-synuclein to transfer to neurons (Xia et al., 2019). This result suggested that microglial cells can also play a critical role in the transmission of α-synuclein through the exosomal pathway. In addition, it was also considered that the progression of PD could be altered by the regulation of exosome secretion and microglial activation state. Therefore, senescence can activate microglia, which promote the transmission of α-synuclein via exosomes in the progression of PD.

Taken together, the evidence to date demonstrates that the interactions between microglia and α-synuclein in PD are related to the factors including recognition of PRR, multiple signal transduction pathways, metabolic regulation, senescence of microglia, and exosome secretion. These factors play an essential role in the neurotoxic effect of microglia in PD and are potential biomarkers of the progression and therapeutic targets of PD.

## Neuroprotective Effect of Microglia and α-Synuclein

Although the neurotoxic mechanism of microglia has been demonstrated, there is also evidence for their neuroprotective function. A study observed that removing microglia can increase the accumulation of α-synuclein, which suggested that the important function of microglia might clear the α-synuclein from the extracellular space and indicated the potential function of neuroprotective by microglia (George et al., 2019). One of the interesting hypotheses of the neuroprotective mechanism of microglia is removing extracellular α-synuclein and protecting neurons through selective autophagy. This process is regarded as a possible mechanism of synucleinphagy. Choi et al. identified that microglia activated by α-synuclein could engulf α-synuclein into autophagosomes to be degraded by synucleinphagy, a type of autophagy. They also demonstrated that TLR4-NF-κB-p62 mediated synucleinphagy that could clear the α-synuclein to realize the neuroprotective function of microglia (Choi et al., 2020a,b). The study focused on human genomics has revealed that the autophagy-lysosome system was critical in the pathogenesis of PD (Chang et al., 2017). Therefore, strategies targeting the regulation of α-synuclein homeostasis in CNS can promote the neuroprotective function of microglia in the patients with PD.

# EFFECT OF α-SYNUCLEIN ON T CELLS

## Overview of T Cells

T cells play a critical role in the adaptive immune system by clearing pathogens such as bacteria and viruses. During infection, naïve T cells are activated, expanded, and differentiated into effector T cells that have an anti-infection function. The phenotype of differentiated T cells consists of CD8 + T cells, which is cytotoxic "killer cells" to kill the infected cells directly; CD4 + T cells, which act as "helper cells" to regulate the death of cells indirectly; and regulatory T cells (Treg), which is important in immune tolerance. Usually, T cells are not likely to infiltrate into CNS because of the immune privilege of the blood–brain barrier (BBB). However, T cells can infiltrate CNS and regulate neurons when BBB is damaged (Mundt et al., 2019). In the recent decades, evidence shows the significant relationship

between activation of T cells and neurodegenerative diseases, especially AD and PD (Ethell et al., 2006; Yang Q. et al., 2020). Consequently, the mechanism of different subtypes of T cells is an important problem that can help to clarify further immunological mechanisms of PD and build targets for PD therapy. There are few reports about the mechanism of T helper type 2 (Th2) cells related to PD and the details of Th2 will not be included in this study. In the following sections, we discuss the role of T lymphocytes in the pathogenesis of PD.

### CD8 + T Cells

As a "killer T cell," CD8 + T cells can be divided into CD8 + effector T cells and CD8 + memory T cells that can directly bind antigens. Previous reports observe that the percentage of CD8 + T cells is predominantly increased among the peripheral blood in PD (Baba et al., 2005). This alternation reflects that CD8 + T cells may participate in the pathogenesis of PD. To identify the conclusion, Jordi et al. exploited immunohistochemistry and immunofluorescence to investigate the state of CD8 + T cells infiltration in the progression of PD. The results showed that in the earliest stage, a robust CD8 + T cells infiltration with no dopaminergic neuronal death in the absence of α-synuclein. However, it was observed that CD8 + T cells cause infiltration with an accumulation of α-synuclein and neuronal death throughout the later stages. Therefore, these evidence indicated that the accumulation of α-synuclein and neuronal death are associated with the activation of CD8 + T cells (Galiano-Landeira et al., 2020).

### CD4 + T Cells

CD4 + T cells will differentiate into activated subtypes including proinflammatory T helper type 1 (Th1) and T helper type 17 (Th17) cells and anti-inflammatory Th2 and Treg upon interaction with the antigen-presenting cells (APCs). These activated subtypes will migrate in the brain to achieve their function. Kustrimovic et al. investigated the level of CD4 + T cell subtypes in peripheral blood of the patients with PD without therapy (Kustrimovic et al., 2018). The results show decreased circulating naïve CD4 + T cells with the increased percentage of Th1, IFN-γ, and TNF-α cells. Additionally, the percentage of Th2 and Treg cells decreases due to Th1 bias (Kustrimovic et al., 2018). Inferring the mechanisms underlying these changes is a promising strategy for treating PD.

## Effect of CD4 + T Cells on Neuron

### Proinflammatory Cells Th1 and Th17

As reported, the proportion of Th1 cells is increased in the peripheral blood of the patients with PD and cytokines such as TNF-α secreted by the activation of Th1 might affect neurons (Pennock et al., 2013; MacMahon Copas et al., 2021). Th1 cells enhance inflammation by promoting the secretion of cytokines. However, it remains unclear how Th1 cells contribute to the pathogenesis of PD. More studies about it are necessary in the future.

The number of circulating Th17 was found to be elevated in the early stage of PD (Chen et al., 2017). Proinflammation Th17 cells are thought to mediate the pathology of PD by inducing the upregulation of cytokines such as IL-6, IL-23, and IL-1β (Manocha et al., 2017). Th17 cells can also directly induce the apoptosis of dopaminergic neurons by binding to some molecular proteins in the membrane such as leukocyte function-associated antigen (LFA)-1 and intercellular adhesion molecule (ICAM)-1 interaction that are activated to induce dopaminergic neuronal death to promote PD (Liu et al., 2017). In addition, compared to the normal cells, neurons of midbrain derived from PD-induced pluripotent stem cells (iPSCs) demonstrate the mechanism of pathogenesis via the increase of IL-17–IL-17R signaling and NF-κB expression associated with the activation of Th17 (Sommer et al., 2018). This evidence approved the Th17 proinflammatory role and its related signal pathway that make the critical effects on dopaminergic neuronal death. Therefore, inhibiting Th17 or its related function may have a neuroprotective effect in PD.

### Protective Effect of the Treg

Regulatory T cells is CD4 + CD25 + T cell that can express Forkhead box (FOX)P3 to maintain self-tolerance and prevent autoimmunity (Sakaguchi, 2004). In PD, Treg is indicated to play an important role in suppressing the function of effector T cells to prevent neuronal death. Jessica A. et al. evaluated the association between the progression of PD and the state of Treg and demonstrated that the immune suppression and neuroprotective function of Treg cells were impaired in the patients with PD (Saunders et al., 2012). This result suggests the neuroprotection of Treg in PD. Further research focused on the mechanism of protection of Treg. Yan et al. built mice models with the preparation of Treg in ventral mesencephalic (VM) neurons before treating 1-methyl-4-phenylpyridinum (MPP +) to investigate the neuroprotective pathway of Treg. The results revealed the dopaminergic neurons could be protected from MPP + toxic through CD47-signal regulatory protein alpha (SIRPA) interaction and Ras-related C3 botulinum toxin substrate 1 (Rac1)/Akt (also known as protein kinase B) pathway controlled by Treg (Huang et al., 2017). This observation provides mechanistic insight into the role of Treg in PD and potential therapeutic targets.

In conclusion, CD4 + T cells play a central role in the pathogenesis of PD. Th1 and Th17 exploit multiple pathways to mediate neuronal death and promote the progression of PD. On the other hand, this neurotoxic effect can be abrogated by increasing the number of Tregs. Taken together, a more detailed exploration of the regulation of CD4 + T cells in PD is warranted.

## Alpha-Synuclein in the Relationship Between Autoimmune Response and T Cells

Alpha-synuclein acts as the neuron-encoded protein mainly involved in the transport of vesicles and neurotransmitters across the neurons. During the PD, the overaccumulation of α-synuclein can make α-synuclein become the autoantigens recognized by the immune cells. A previous study has demonstrated that α-synuclein in the hematopoietic cells is associated with the abnormal activation of the adaptive immune response in PD (Shameli et al., 2016) and α-synuclein can be presented by

microglia, which acted as APCs to stimulate T cell response. Besides, a recent study demonstrates that astrocytes with accumulated α-synuclein were also shown as APCs to induce the activation of T cells and spread the inflammation (Rostami et al., 2020). Both α-synuclein and modified α-synuclein can affect the function of T cells. For example, nitrated (N) α-synuclein is revealed to induce the response of T cells and contribute to T cell-mediated neurotoxic process. This process of PD is considered to be achieved by new antigenic epitopes creation via the oxidative modification of protein, which can regulate nigrostriatal degeneration to promote the progression of pathology in PD (Benner et al., 2008; Reynolds et al., 2009). It was also reported that N-α-synuclein can modulate the neuroprotective effect of Tregs.

Stimulation of the peripheral blood samples from the patients with PD by using human-α-synuclein-derived peptide and homology herpes simplex virus1 (HSV1) peptides activated both the CD8 + T cells and CD4 + T cells compared to the healthy group (Caggiu et al., 2017). A specific type of TNF-α-secreting cell was also observed in the above stimulated blood samples. These results imply that α-synuclein affects the secretion of cytokines by T cells in the pathogenesis of PD and that infection with HSV can also promote the progression of PD. In addition to full-length α-synuclein that can induce the increase of CD8 + T and CD4 + T cells, modified α-synuclein was shown to increase its antigenicity and further stimulate the immune cell responses.

Bone marrow-derived dendritic cells (BMDCs) exert a neuroprotective effect in PD by inducing Treg. In this study, the continued costimulation with N-α-synuclein and granulocyte-macrophage colony-stimulating factor (GM-CSF) decreased the number of Tregs in a model of PD induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The mild costimulation on DC and Treg is demonstrated to achieve the protective effect on MPTP-PD models (Schutt et al., 2018). Consequently, we can suggest that the full-length or modified α-synuclein can affect different types of T cells to promote the progression of PD.

Major histocompatibility complex (MHC) is an essential component of adaptive immunity. In PD, overexpressed α-synuclein is an antigen recognized by MHC. Microglia MHC was shown to participate in the activation of both the innate and adaptive immune responses (Harms et al., 2013). With respect to the antigenic specificity of the adaptive immune response in PD, current evidence suggests that α-synuclein is an important target of the T-cell response and autoimmunity (González et al., 2015). Usually, α-synuclein is tolerated by T cells. However, α-synuclein will break this tolerance and can be presented to T cells to induce autoimmune response during PD. Sulzer et al. indicated the association between α-synuclein epitopes presented by specific MHC alleles and T-cell-related PD, demonstrating that the Y39 epitope of α-synuclein plays an important role in inducing T-cell response and secretion of cytokine. This research also suggests that PD results from the autoimmunity response mediated by α-synuclein (Sulzer et al., 2017). Thus, activation of autoimmunity by an aberrant accumulation of α-synuclein is an important mechanism underlying the pathogenesis of PD, although the detailed mechanism remains to be determined.

# ALPHA-SYNUCLEIN AS AN IMMUNOTHERAPEUTIC TARGET IN THE PATIENTS

At present, L-3,4-dihydroxyphenylanine (L-DOPA) and deep brain stimulation are still the gold standard for the therapy of PD to relieve the motor and non-motor symptoms. However, long-term treatment with L-DOPA is not sufficient to control the symptoms that consider the newly available approach to alleviate the symptoms of PD such as immunotherapy, which exploits the specific antigen–antibody binding to regulate neuroinflammation. Given that the interactions between α-synuclein and immune cells underlie the pathogenesis of PD, immunotherapy targeting on α-synuclein is a promising therapeutic strategy. Potential immunotherapies target on α-synuclein for PD are discussed in the following sections.

## Passive and Active Immunization

Reducing the expression and aggregation of α-synuclein is an effective strategy for the treatment of PD. Previous evidence has shown that the active immunization can utilize lysosomal pathways to degenerate aggregated α-synuclein (Masliah et al., 2005). Meanwhile, anti-α-synuclein antibodies are revealed to clear the α-synuclein accumulation by activating autophagy and microglia through passive immunization (Masliah et al., 2011; Bae et al., 2012).

To confer an effective target for passive immunization antibodies, a mounting of preclinical experiments had been established. For example, Games et al. (2014) investigate the effect of monoclonal antibodies 1H7, 5C1, and 5D12 on α-synuclein in the mThy1-α-syn mouse model. The results demonstrate that the C-terminal (CT) of α-synuclein is the target of antibodies and the antibodies reduce CT-truncated α-synuclein aggregation in axons to relieve the symptoms (Games et al., 2014). This is also considered as a method to limit the transmission of α-synuclein via the sequester of extracellular protein. Besides, Weihofen et al. (2019) evaluate the characteristics and efficacy of human-derived α-synuclein antibody BIIB054 in mice of PD and report that BIIB054 plays an effective role in preventing the transmission of α-synuclein. To evaluate the safety and efficacy of the antibodies for humans, the phase 1 clinical trial of BIIB054 is established to prove the safety and tolerance in volunteers with favorable pharmacokinetics (Brys et al., 2019). With the mounting of clinical trials about passive immunization on α-synuclein is processed, monoclonal antibodies can be an effective approach for future treatment. However, because passive immunization does not induce immune memory, multiple treatments would be required. A long-lasting effect may be achieved by active immunotherapy.

PD01A and PD03A are two active vaccine candidates that induce the production of specific antibodies in CSF and plasma and can target aggregated α-synuclein. Compared with passive immunization, these two active vaccines are expected to generate long term and more specific therapy of PD. The preclinical trial has reported that these vaccines achieve their neuroprotective function, which is probably mediated by the

activation of microglia and the antigen–antibody internalization to reduce the accumulation of α-synuclein (Mandler et al., 2014). A recent phase 1 clinical trial has also demonstrated that the administration of PD01A was well tolerated (Volc et al., 2020).

The above preclinical and clinical trials have demonstrated the safety and efficacy of both the passive and active immunization in the management of PD; their combination with humoral immunization is also being a potential therapeutic strategy. Rockenstein et al. (2018) designed a novo vaccination method that includes an APC-targeting glucan particle (GP) vaccine delivery system, encapsulated α-synuclein antigen, and rapamycin (RAP). This new vaccination combines cellular immunization and an active humoral system to induce both the Treg and anti-α-synuclein antibodies to boost the neuroprotective effect. The preclinical results have shown that the combination vaccine can achieve an enhanced efficacy compared to single cellular immunization (Rockenstein et al., 2018).

### Immune Modulator

Another strategy for mitigating abnormal α-synuclein accumulation in PD is to restore the balance between pro- and anti-inflammatory factors with an immune modulator. Sargramostim is an important human recombination GM-CSF approved by the US Food and Drug Administration (FDA) for bone marrow-related diseases that acts by promoting the recovery of Treg (Smith et al., 2006). In a phase 1 clinical trial, sargramostim was shown to effectively regulate Tregs to relieve the neuroinflammation in PD (Gendelman et al., 2017). Similarly, LBT-3627, a vasoactive intestinal peptide receptor 2 (VIPR2) peptide agonist, functions as an immune modulator to restore Treg function in a α-synuclein overexpressed model of PD (Mosley et al., 2019).

Recently, REXO-C/ANP/S hybrid nanoparticle system is demonstrated to clear the accumulation of α-synuclein and abnormal immune activation in PD (Liu et al., 2020). The full name of this engineered system is called rabies virus glycoprotein (RVG) peptide–modified exosome (EXO) curcumin/phenylboronic acid-poly [2-(dimethylamino) ethyl acrylate] nanoparticle/small interfering RNA targeting alpha-synuclein (*SNCA*). In this system, exosomes derived from immature dendritic cells act as a coat of the full system to promote the immune suppression. Liu et al. (2020) have shown that REXO-C/ANP/S can achieve immune activation clearing by inhibiting Th17 and enhancing Treg to regulate the immune system in mice with PD.

In summary, there is accumulating evidence that immunotherapies targeting α-synuclein are effective treatments for the patients with PD. However, more clinical trials are still needed in the future to confirm their long-term efficacy and safety.

## CONCLUSION

Immune mechanisms are important in the development and progression of PD, especially immune cell activation regulated by α-synuclein. This study presented the current state of knowledge regarding α-synuclein-induced microglia activation and their interactions and autoimmune responses induced by full-length proteins or modified peptides of α-synuclein. The existing evidence indicates that the interaction between α-synuclein and microglia or T cells is a "double-edged sword." α-synuclein can induce the activation of microglia or effector T cells, leading to the production of neurotoxic cytokines and promote selective autophagy of microglia to protect neurons. How to achieve a balance of the "double-edged sword" to protect neurons is an important future direction in the research of PD.

Generally, α-synuclein is a normal functional protein that is mainly responsible for transport of vesicle and secretion of neurotransmitters. In PD, accumulation of α-synuclein makes it an autoantigen and causes an autoimmune response in immune cells. Like experimental autoimmune encephalomyelitis (EAE), an autoimmune disease with predominant activation of Th17 and immune-related therapy, it can significantly relieve this disease (Shin et al., 2018). These pieces of evidence show the close connection between the pathology and immunology of PD and PD might be a type of autoimmunity reaction-mediated disease.

Despite progress in the therapeutic strategies, PD remains an incurable disease. Therefore, we suggest that immunotherapy may also be effective in PD. As one of the biomarkers of PD, a mounting of studies reported that α-synuclein is related to immune therapy such as vaccines of PD, monoclonal antibodies, and immune modulators. The relation between α-synuclein and the immune system is expected to become a key for future treatment of PD. More detailed studies on the immune mechanism regulating α-synuclein can provide new directions for the development of treatments to slow or halt the progression of PD.

## AUTHOR CONTRIBUTIONS

Both authors selected the related literature, conceptualized, designed, wrote, edited, and revised the manuscript.

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REVIEW
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# Microglia Polarization in Alzheimer's Disease: Mechanisms and a Potential Therapeutic Target

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Neuroinflammation regulated by microglia is one of the important factors involved in the pathogenesis of Alzheimer's disease (AD). Activated microglia exhibited phenotypes termed as M1 and M2 phenotypes separately. M1 microglia contribute to the development of inflammation *via* upregulating pro-inflammatory cytokines, while M2 microglia exert anti-inflammation effects through enhancing the expression of anti-inflammation factors. Moreover, M1 and M2 microglia could be mutually transformed under various conditions. Both M1 and M2 microglia are implicated in AD. Amyloid-β (Aβ) and hyperphosphorylated tau are two major components of AD pathological hallmarks, neuritic plaques, and neurofibrillary tangles. Both Aβ and hyperphosphorylated tau were involved in microglial activation and subsequent inflammation, which further contribute to neuronal and synaptic loss in AD. In this review, we summarized the roles of M1 and M2 microglia in AD and underlying mechanisms, which will provide an insight into the role of microglia in the pathogenesis of AD and highlight the therapeutic potential of modulating microglia.

**Keywords: neuroinflammation, microglia activation, M1 microglia, M2 microglia, Alzheimer's disease**

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## INTRODUCTION

As a devastating age-related brain disorder, Alzheimer's disease (AD) is characterized by progressive memory loss and cognitive deficits. Extracellular neuritic plaques, mainly consisting of amyloid-β (Aβ) and intracellular neurofibrillary tangles (NFTs) containing hyperphosphorylated tau, are the major pathological hallmarks of AD (Palomer et al., 2019; Wang Z. et al., 2019; Frolich, 2020). Although genetic factors, abnormal cholesterol metabolism, and protein homeostasis deficiency have been reported to contribute to AD pathogenesis (Karch and Goate, 2015; Liebsch et al., 2019; van der Kant et al., 2019; Wang and Davis, 2021), molecular mechanisms of AD remain elusive.

Emerging evidence suggested that neuroinflammation mediated by microglia was an important feature of AD (Griciuc et al., 2019; Paouri and Georgopoulos, 2019). Microglia were activated through a classic pathway or an alternative pathway, termed M1 or M2 microglia (Wang Y. et al., 2019). M1 and M2 microglia had different properties and functions

(Wang Y. et al., 2019), which were differentially involved in the pathogenesis of AD (Colton et al., 2006; Paouri and Georgopoulos, 2019). Advanced understanding of the roles of M1 and M2 microglia in AD will provide new clues about the pathological mechanisms and therapeutic targets for AD. In this study, we summarized the recent progress about the functions of M1 and M2 microglia in AD pathogenesis and discussed underlying mechanisms.

# CHARACTERISTICS OF MICROGLIA

## The Origin and Proliferation of Microglia

Microglial cells play vital roles in regulating the homeostasis of the central nervous system (CNS; Subhramanyam et al., 2019). However, the origin of microglia is still controversial. Earlier studies indicated that microglial cells descended from meningeal macrophages, which were invaded into the brain during the late stage of embryonic development (Alliot et al., 1999). Later, it was found that microglia in the parenchyma of the CNS displayed macrophage markers and originated from the hemopoietic system (Perry et al., 1985; Lawson et al., 1990; Gordon et al., 1992; Alliot et al., 1999). Alliot et al. (1999) reported that microglia were derived from its progenitors originating from the hemopoietic cells as early as embryonic day 8 (E8) in the yolk sac. The microglial progenitors migrated into the brain subsequently and increased the number fast (Alliot et al., 1999). Ginhoux et al. (2010) showed that adult microglial cells were originated from the original myeloid progenitors before E8. Kierdorf et al. (2013) also reported that microglia were originated from the c-Kit positive erythro myeloid progenitors as early as E8 in the yolk sac. Early studies have demonstrated that there were three waves of hematopoiesis in the yolk sac during the embryonic development of mice (Yoder, 2014). Except for the classical microglial progenitors mainly produced during the first wave of hematopoiesis in the yolk sac, there also existed a microglial subpopulation originated from the second wave of hematopoiesis in the yolk sac (De et al., 2018). Taken together, most of these studies indicated that microglia were originated from the yolk sac during development. However, further investigation is necessary to clarify which wave of hematopoiesis and which type of cells in the yolk sac were mainly responsible for the microglial origination.

The microglia isolated from adult postmortem cerebral cortex exhibited proliferative ability *in vitro* and maintained its phenotype (Guo et al., 2016). The colony-stimulating factor-1 receptor (CSF1R) was vital for the development of microglia (Ginhoux et al., 2010; Erblich et al., 2011). Microglia could be repopulated after the withdrawal of CSF1R antagonism PLX5622 in both adult and aged mice (O'Neil et al., 2018). Moreover, CSF1R inhibitor PLX3397 treatment resulted in a dramatic decrease in microglia number, while microglia were restored rapidly after the removal of PLX3397 in mice (Elmore et al., 2014). Elmore et al. (2014) revealed that the rapidly repopulated microglia were from the nestin-positive non-microglial progenitor cells, indicating that the newborn microglia were derived from the nestin-positive progenitors but not the remaining microglia. However, Huang et al. (2018) found that the repopulated microglial cells were not derived from the blood cells or astrocytes, neurons, and NG2 cells but from CX3CR1-positive microglia, demonstrating that the adult microglia could maintain its population through self-renewal. The inconformity of these two studies might result from the different protocols of drug treatment and different types of transgenic mice.

## Microglia Polarization

Growing evidence indicated that two types of activated microglia existed, namely, M1 phenotype and M2 phenotype (Wang Y. et al., 2019), although there are different opinions about the existence of different phenotypes of microglia activation (Ransohoff, 2016). M1 phenotype microglia were the classical form of microglial activation and contributed to the development of inflammatory responses especially in neurodegeneration (Tang and Le, 2016; Kwon and Koh, 2020). M1 microglia could be induced by lipopolysaccharide (LPS)/interferon-γ (IFN-γ; Tang and Le, 2016; **Figure 1**). M2 microglia were the alternative form of microglia activation and considered as the anti-inflammatory response phenotype, contributing to tissue repair and neuroprotection (**Figure 1**). Moreover, M2 microglia could be induced by interleukin-4 (IL-4)/IL-13 (Tang and Le, 2016; Kwon and Koh, 2020; **Figure 1**). In addition, the expression levels of pro-inflammatory markers were enhanced significantly in M1 microglia, while the expression levels of anti-inflammatory factors increased in M2 microglia (Lam et al., 2017; Kwon and Koh, 2020). As the pro-inflammatory factors were significantly upregulated in M1 microglia, the inflammation-associated factors such as IL-1β, IL-6, and inducible nitric oxide synthase (iNOS) were taken as the markers of M1 microglia (Jin et al., 2018; **Figure 1**). The expression of some relatively specific markers was upregulated in M2 microglia such as arginase 1 (ARG1), chitinase-3-like-3 (YM1, also called CHI3L3), and found in inflammatory zone 1 (FIZZ1; Jin et al., 2018; Veremeyko et al., 2018; Wu et al., 2019; **Figure 1**). Due to the diversity of diseases and different stages of diseases, it is essential to identify specific markers of microglial activation in various pathological conditions.

## Regulation of Microglia Polarization

Accumulated evidence showed that microglial polarization could be regulated. For example, PGC-1-related coactivator (PRC) was significantly increased in M2 microglia induced by IL-4 (Mou et al., 2015). Consistently, the overexpression of PRC increased the mRNA expression of M2 microglial markers such as ARG1, FIZZ1, and YM1, promoting microglia activation toward the M2 phenotype (**Figure 1**; Mou et al., 2015). Trehalose-6,6′-dibehenate (TDB) could decrease the expression of M1 microglial markers such as the proIL-1β and IL-6 induced by LPS (Mohanraj et al., 2019), while treatment with TDB also led to the significant increase of M2 microglia markers such as ARG1 and YM1/2 (Mohanraj et al., 2019; **Figure 1**). In addition, the dehydrocorydaline administration promoted the microglia toward the M2 phenotype in the spinal cord of a mouse model of bone cancer pain (Huo et al., 2018). Furthermore, TOPK, a mitogen-activated protein kinase, promoted the M2

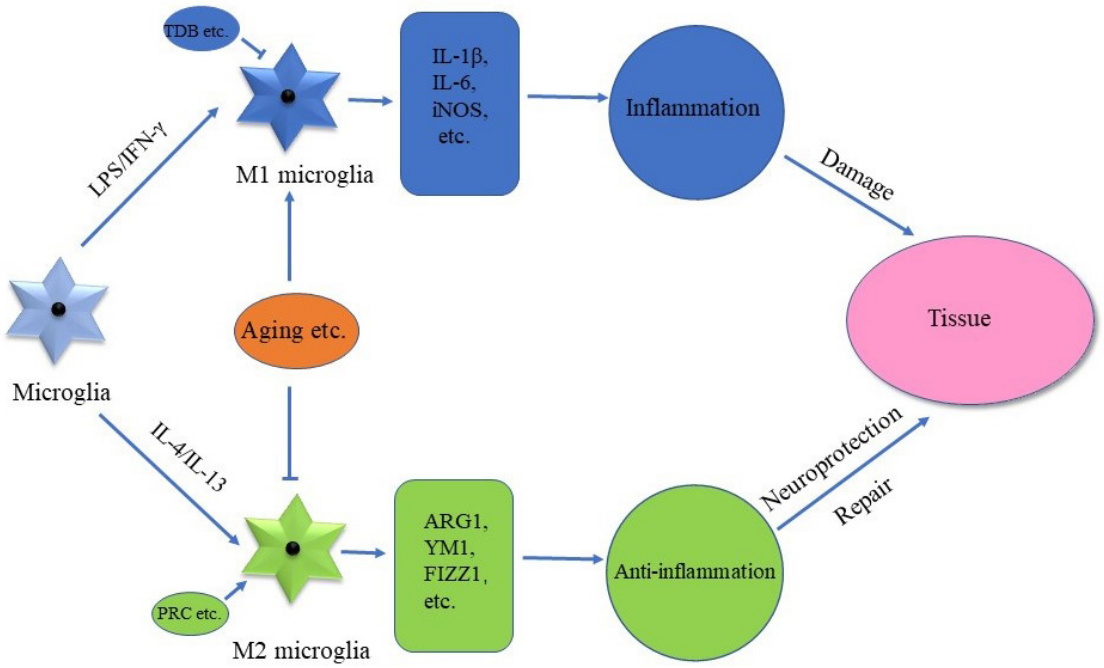


**FIGURE 1 |** A paradigm of M1 and M2 phenotypes of microglial cells. Microglia activation is categorized into two phenotypes, namely, M1 phenotype and M2 phenotype. M1 microglia could be induced by lipopolysaccharide/interferon-γ (LPS/IFN-γ), leading to the increase of pro-inflammatory factors. M2 microglia could be induced by IL-4/IL-13, which leads to the increase of anti-inflammatory factors. Thus, M1 microglia contribute to the inflammatory response, while M2 microglia contribute to the neuroprotection and repair processes of tissues.

microglial phenotype possibly through inhibiting the activity of histone deacetylases HDAC1 and HDAC2 (Han et al., 2018), while inhibiting H3K27me3 demethylase Jmjd3 enhanced the pro-inflammatory responses and suppressed the microglial M2 phenotype (Tang et al., 2014). It indicated that microglial polarization could be regulated by many signaling pathways.

Importantly, evidence indicated that aging plays a pivotal role in the balance of M1 and M2 phenotypes. Aging promoted the M1 phenotype with higher levels of pro-inflammatory factors such as IL-1β and tumor necrosis factor-α (TNF-α), while it decreased the activation of M2 microglia with a reduction of M2 markers such as ARG1, following peripheral surgery (Zhang Z. J. et al., 2019; **Figure 1**). When compared to 6-month-old mice, the expression of M1-related transcripts, such as S100A9 and CXCL13, tended to increase in 24-month-old mice treated with IL-1β, IL-12, and TNF-α, while an overall reduction of M2 microglia-associated markers such as ARG1, CHI3L3, and FIZZ1 was observed in 24-month-old mice treated with IL-4 and IL-13 cocktail. It indicated that aging facilitates the M1 phenotype but inhibits the M2 phenotype (Lee et al., 2013).

## DYSREGULATION OF MICROGLIA POLARIZATION IN ALZHEIMER'S DISEASE

Increased mRNA of TNF-α was observed in the frontal lobe cortex of patients with AD (Colton et al., 2006). The mRNA of CHI3L1 and CHI3L2, which are the two YM1 closely related genes, was robustly increased in the cortex of the patients with AD (Colton et al., 2006). It indicated that both classically activated microglial cells and alternatively activated microglia existed in AD brains (**Figure 2**). However, cell population-specific alteration of these markers was undefined. For example, the expression of CD40 was upregulated in microglia of AD brains (Togo et al., 2000), while CD40 ligand deficiency resulted in a significant reduction of TNF-α expression in cultured microglia of AD model mice (Tan et al., 1999). It indicated that the CD40-mediated pathway may play an important role in the M1 phenotype (Tan et al., 1999; Walker and Lue, 2015; **Figure 2**). Recent studies showed that activated microglia around amyloid plaques consisted of CD11c-positive and CD11c-negative subgroups in AD mice (Kamphuis et al., 2016). More interestingly, the transcriptional profiling analysis implied that CD11c-positive cells displayed increased immunosuppressive features counteracting the inflammatory response (Kamphuis et al., 2016). Hence, it was deduced that CD11c might be a marker of M2 microglia in AD (**Figure 2**).

Many susceptibility genes preferentially expressed in microglia of the aged human brain (e.g., *TREM2*, *CD33*, *SORL1*, and *INPP5D)* were associated with AD (Olah et al., 2018), while genetic analysis also indicated that various immune-related genes were associated with AD risk (Karch and Goate, 2015). It is highly supported that microglia may play a key role in AD pathogenesis (Karch and Goate, 2015;

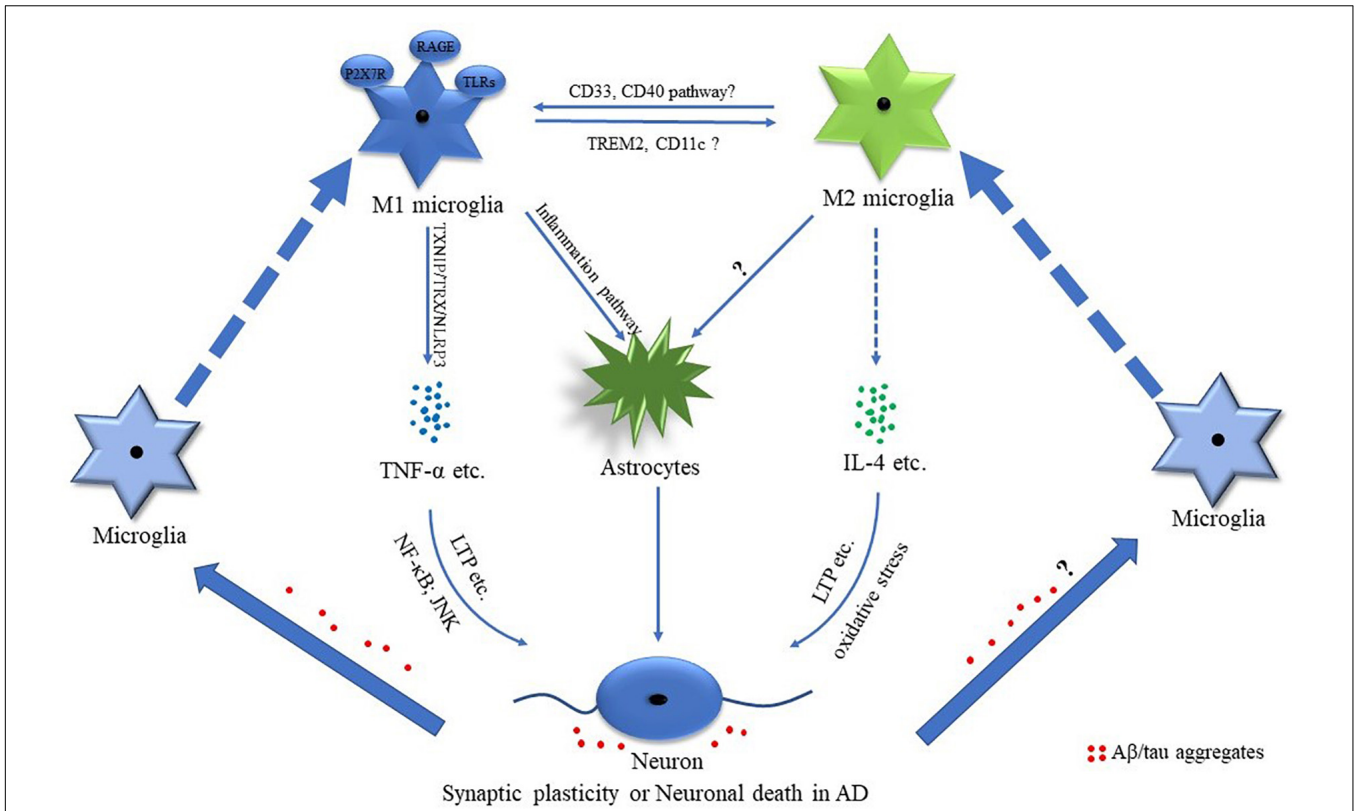


**FIGURE 2 |** Potential mechanisms of M1 and M2 phenotypes of microglial cells in AD. Mounting evidence suggests that M1 and M2 microglia are involved in the pathological processes of Alzheimer's disease (AD). The AD-associated pro-inflammatory and anti-inflammatory factors such as tumor necrosis factor-α (TNF-α) and interleukin-4 (IL-4) play important roles in regulating synaptic plasticity and neuronal death. Amyloid-β (Aβ)/tau aggregates are involved in regulating microglia-related inflammation.

Katsumoto et al., 2018). For example, soluble triggering receptor expressed on myeloid cells 2 (sTREM2), a marker of microglia activation, was enhanced in cerebrospinal fluid at the stages of preclinical subjective cognitive decline, mild cognitive impairment (MCI), and AD dementia compared with healthy controls (Nordengen et al., 2019). In addition, TREM2 rare variants increased the risk of AD in Asian and European populations (Jonsson et al., 2013; Kleinberger et al., 2014; Jiang et al., 2016; Efthymiou and Goate, 2017). *TREM2* mRNA was enhanced in patients with AD. Increased TREM2 promoted ARG1 expression and reduced the nitric oxide (NO) production (Zhang et al., 2018; **Figure 2**). In addition, TREM2 promoted microglial phagocytosis (Kawabori et al., 2015; Zhang et al., 2018). Moreover, TREM2 deficiency did accelerate AD progress (Wang et al., 2015; Ma et al., 2016; Efthymiou and Goate, 2017), which might be associated with TREM2 deficiency-mediated downregulation of ARG1 in microglial cells (Zhang et al., 2018).

CD33 was mainly expressed in microglia and macrophages in the brain (Zhao, 2019). It was reduced in peripheral mononuclear cells of patients with AD, while it was increased in the frontal cortex of patients with AD (Griciuc et al., 2013; Hu et al., 2014). Consistently, the number of CD33-positive microglia markedly increased in the cortex of AD cases (Griciuc et al., 2013). CD33 knockout led to the increase of inflammasome genes and anti-inflammatory gene expression in AD mice (Griciuc et al., 2019), while a recent study further demonstrated that CD33 knockdown significantly decreased the pro-inflammatory-related transcripts in AD mice (Griciuc et al., 2020; **Figure 2**).

P2X7 receptor (P2X7R), which is a purinergic receptor, was significantly increased in microglial cells of the patients with AD, which was associated with the pathology of AD. The expression of P2X7R was higher in microglia of AD mice at both the advanced and the late stages, while no significant difference was detected at the early stage (Lee et al., 2011; Martinez-Frailes et al., 2019). The activation of P2X7R promoted microglia migration toward the senile plaques, while the inhibition of P2X7R promoted the phagocytosis of microglia (Martinez-Frailes et al., 2019). Moreover, P2X7R was involved in the degenerative neuron-induced microglial activation contributing to the activation of astrocytes resulting in the amplification of the neuroinflammation and neuronal damage (Yiangou et al., 2006; Glass et al., 2010). Consistently, P2X7R inhibition significantly reduced the expression of IL-1β in spinal cord microglia of a rat pain model (Zhou et al., 2019). It indicated that P2X7R may be a key player of M1 microglia (**Figure 2**).

Notably, microglia not only contributed to neuronal death by releasing pro-inflammatory cytokines but also exerted neuroprotective function *via* phagocytosis in AD (Pourbadie et al., 2018; Rangaraju et al., 2018). Specifically, the proportions of $CD44^{-}CXCR4^{+}$ and $CD44^{+}CXCR4^{-}$ microglia were altered with age in AD mice. It suggested that aging may affect the profiles of M1 and M2 microglia in AD as CD44 and CXCR4 contributed to the pro-inflammatory and anti-inflammatory processes, respectively (Rangaraju et al., 2018). Moreover, it was indicated that M2 microglia switched into the M1 phenotype at the advanced stage of AD (Jimenez et al., 2008). However, the regulation of M1/M2 microglia in AD remains elusive.

# THE ROLE OF MICROGLIA POLARIZATION IN THE PATHOGENESIS OF ALZHEIMER'S DISEASE

## Mutual Regulation Between Aβ Aggregates and Microglial Polarization

Amyloid-β was associated with the microglia activation in AD (Glass et al., 2010). The pathological analysis showed that activated microglia were accumulated around the plaques (Yin et al., 2017). Aggregated Aβ-induced M1 microglia were partially mediated by the receptor for advanced glycoxidation end-products (RAGE) and toll-like receptors (TLRs) (**Figure 2**), while the pro-inflammatory cytokines of M1 microglia further led to the activation of astrocytes, contributing to neuronal loss in AD (Glass et al., 2010; Desale and Chinnathambi, 2020; **Figure 2**). Early studies demonstrated that concentrated fibrillar Aβ induced the microglia/monocyte activation with the increased expression of iNOS, which was TNF-α dependent, contributing to neuronal death (Combs et al., 2001; Chen et al., 2005). Recent studies showed that Aβ-induced pro-inflammatory factors such as IL-6 and TNF-α were possibly medicated by thioredoxin-interacting protein (TXNIP)/thioredoxin (TRX)/NOD-like receptor pyrin domain-containing protein 3 (NLRP3) pathway in microglial cells (Feng and Zhang, 2019; **Figure 2**). Moreover, Aβ could induce the activation of NLRP3 inflammasome in microglia, contributing to the activation of microglia toward M1 polarization (Halle et al., 2008; Liang et al., 2020; Zhang et al., 2020). Another study showed that Aβ led to the enhancement of pro-inflammatory cytokines such as IL-1β and IL-6 *via* regulating the homeostasis of ornithine decarboxylase and antizyme (Cheng et al., 2019). In addition, Cui et al. (2019) reported that enhanced NF-E2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) signaling significantly attenuated the pro-inflammatory responses and oxidative stress induced by Aβ. These results indicated that Aβ could directly induce or potentiate the M1 activation of microglia.

As the immune cells maintain the tissue homeostasis in the CNS, microglia cleared the debris and misfolded proteins by phagocytosis in the brain (Kettenmann et al., 2011; Zhou et al., 2020). It was reported that microglia cleared the Aβ *via* phagocytosis and proteases-mediated degradation (Lopez-Valdes and Martinez-Coria, 2016). Chronically, the activation of M1 microglia released the cytotoxic factors and might have deficits in the clearance of Aβ aggregates, accelerating the progress of AD (Glass et al., 2010; Lian et al., 2016; Zhang X. et al., 2019). The inactivation of CD33 promoted microglial uptake of Aβ, while the enhancement of CD33 significantly inhibited the uptake of Aβ by microglia (Griciuc et al., 2013). CD33 promoted the development of Aβ aggregates, contributing to AD pathology (Griciuc et al., 2013). In addition, TLR2 deficiency led to the enhancement of Aβ phagocytosis (Liu et al., 2012), while TLR2 knockout promoted M1 microglia to M2 microglia in bone marrow chimeric APP transgenic mice (Liu et al., 2012). Deferoxamine promoted the M2 activation of microglia and significantly reduced the Aβ deposits in AD mice (Zhang and He, 2017). Sarsasapogenin-AA13 significantly alleviated Aβ-induced cognitive deficiency possibly through reducing the M1 activation, increasing the M2 activation, and enhancing Aβ clearance in mice (Huang et al., 2017). It was suggested that treadmill exercise enhanced the M2 activation and decreased the M1 activation of microglia, contributing to the decrease of Aβ deposition and improvement of cognition in AD mice (Zhang X. et al., 2019). These results indicated that microglial polarization has a significant effect on Aβ deposition, i.e., M2 microglia might have a strong ability to clear Aβ. However, further investigation is needed to elucidate the association between microglial activation and Aβ clearance.

## Mutual Regulation Between Tau Aggregates and Microglial Polarization

Increasing evidence indicated that tau was closely associated with microglia activation. The tau-induced activation of microglia was medicated by the NLRP3 inflammasome, contributing to the release of IL-1β (Ising et al., 2019). NLRP3 knockout greatly inhibited tau hyperphosphorylation induced by brain homogenates of APP/PS1 mice, indicating the vital roles of NLRP3 in tau pathology (Ising et al., 2019). Ionized calcium-binding adaptor molecule-1 (Iba1) was often taken as the marker of microglia activation following inflammatory stimulation (Hoogland et al., 2015). Tau aggregates could spread the localization of Iba1 to the cell membrane, and tau oligomer also induced the increase of cytosolic Iba1 levels in microglia, indicating that tau could drive the pro-inflammatory activation of microglia in AD (Das et al., 2020; **Figure 2**). On the other hand, the pro-inflammatory cytokines released from the activated microglia also contributed to the regulation of tau phosphorylation and the formation of tau aggregates (Li et al., 2003; Barron et al., 2017; Leyns and Holtzman, 2017). For example, IL-1β secreted by microglia triggered tau phosphorylation through the p38 mitogen-activated protein kinase (MAPK) pathway (Li et al., 2003; Barron et al., 2017; Desale and Chinnathambi, 2020). TNF-α induced the hyperphosphorylation of tau while the long-term treatment of TNF-α led to a marked decrease of tau hyperphosphorylation in AD mice (Janelsins et al., 2008). However, the immunohistochemical analysis showed that another pro-inflammatory factor IFN-γ significantly

decreased the levels of hyperphosphorylated tau in the brain of AD mice, indicating the potential roles of IFN-γ in tau hyperphosphorylation (Mastrangelo et al., 2009). Moreover, it was suggested that microglia exerted the cleaning function to clear tau oligomers by phagocytosis in AD (Das et al., 2020). It indicated that tau aggregates and M1 microglial activation are possibly mutually regulated.

### Synaptic Dysfunction and Neuronal Death Mediated by Microglia Polarization

Emerging evidence demonstrated that the AD-associated pro-inflammatory factors such as IL-1β and TNF-α played important roles in synaptic dysfunction and neuronal death (Gaur and Agnihotri, 2015; Pettigrew et al., 2016; Rincon-Lopez et al., 2017; Rizzo et al., 2018; Xiao et al., 2020). For example, increased IL-1β was observed during the long-term potentiation (LTP), while IL-1 receptor blockade led to the impairment of LTP in the hippocampus, indicating that IL-1β is implicated in the regulation of synaptic plasticity (Schneider et al., 1998; Coogan et al., 1999; Avital et al., 2003). Moreover, IL-1β promoted neuronal death in the hippocampus of developing rats with status epilepticus (Rincon-Lopez et al., 2017). In addition, TNF-α mediated polyinosinic-polycytidylic acid [Poly(I:C)]-induced elimination and formation of the dendritic spine in wild-type mice (Garre et al., 2017). Consistently, dominant-negative TNF-α significantly inhibited dendritic spine elimination and formation in the somatosensory cortex of experimental autoimmune encephalomyelitis (EAE) mice (Yang et al., 2013). TNF-α significantly increased the LTP levels of the hippocampus (Pettigrew et al., 2016; **Figure 2**). Furthermore, TNF-α induced neuronal apoptosis possibly through the upregulation of c-Jun N-terminal kinase (JNK) activation and increased nuclear factor kappa B (NF-κB) p65 and iNOS (Yang et al., 2002; Kraft et al., 2009; Xiao et al., 2020). The aforementioned evidence indicated the important role of TNF-α in the regulation of synaptic plasticity and neuronal death (**Figure 2**).

Anti-inflammatory cytokines were implicated in synaptic plasticity and neuronal death. A significant increase of IL-4 was observed in patients with AD (King et al., 2018), which played important roles in the protection of synaptic plasticity and neuronal death (Maher et al., 2005; Bhattarai et al., 2016; Hernandez-Espinosa et al., 2019; Jeong et al., 2019; Taipa et al., 2019). Maher et al. (2005) reported that the decrease of IL-4-related signaling contributed to the LTP deficits in the aged rat (**Figure 2**), while IL-4 and IL-10 administration significantly decreased the neuronal injury induced by excitotoxic damage in wild-type animals (Hernandez-Espinosa et al., 2019). However, IL-4 aggravated the neuronal death induced by prothrombin kringle-2 (pKr-2) possibly through regulating the oxidative stress (Jeong et al., 2019; **Figure 2**). All these results implicated that the AD-related pro-inflammatory and anti-inflammatory cytokines were involved in modulating synaptic plasticity and neuronal death.

Taken together, besides involving in the regulation of the synaptic dysfunction and neuronal death by the inflammatory-related factors, the activated microglia could also affect the pathological process of AD through regulating Aβ and tau deposition. It is noteworthy that different pro-inflammatory cytokines or anti-inflammatory factors might have their unique function in Aβ and tau pathology in AD.

## THE THERAPEUTIC POTENTIAL OF TARGETING MICROGLIA POLARIZATION

The Aβ and tau aggregates mainly induced M1 microglia (Glass et al., 2010; Das et al., 2020), while the inflammation mediated by microglia was involved in the formation of Aβ and tau aggregates, as well as the synaptic and neuronal loss contributing to the neurodegeneration (Jimenez et al., 2008; Glass et al., 2010; Gaur and Agnihotri, 2015; Lian et al., 2016; Pettigrew et al., 2016; Rincon-Lopez et al., 2017; Rizzo et al., 2018; Zhang X. et al., 2019; Xiao et al., 2020). Thus, it was crucial to clarify the molecular mechanisms of microglial activation in AD, which will help to find the efficient drugs that targeted the microglial activation such as the inhibition of pro-inflammation or the promotion of anti-inflammation. For example, CD33 inactivation promoted the uptake of Aβ by microglia (Griciuc et al., 2013). Consistently, CD33 knockdown significantly decreased the pro-inflammatory-related transcripts and Aβ plaque in AD mice at an early age (Griciuc et al., 2020). Thus, CD33 might be a potential target for AD treatment. In addition, P2X7R was indicated as a key player in M1 microglia (Zhou et al., 2019). Targeting P2X7R might be beneficial for AD treatment by inhibiting M1 microglia. Moreover, increased TREM2 promoted M2 phenotype, indicating that increasing the TREM2 activity may be a potential approach for AD treatment by promoting M2 microglia (Zhang et al., 2018). Furthermore, TLR2 knockout contributed to the transition from M1 to M2 microglia (Liu et al., 2012). It suggested that targeting TLR2 might be a potential approach for AD treatment by promoting M1 to M2 shift. Therefore, efficiently targeting the abovementioned or more candidates facilitating the shift from M1 to M2 may have therapeutic potential for AD.

## CONCLUSION

Mounting evidence has shown that the microglia-associated neuroinflammation was one of the major hallmarks of AD, while the microglial activation was correlated with the progress of AD. M1 and M2 microglia, i.e., pro-inflammation and anti-inflammation phenotypes, played differential roles in AD pathogenesis although precise characteristics and regulation of them still need to be fully elucidated. Balancing M1 and M2 microglia or promoting the shift from M1 to M2 might have therapeutic potential for AD treatment.

## AUTHOR CONTRIBUTIONS

QW and HY wrote the manuscript. WL, BY, HC, and ZX contributed to the revision of the manuscript. YW conducted the editing of the manuscript. All authors approved the final manuscript.

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REVIEW
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# Role of G Protein-Coupled Receptors in Microglial Activation: Implication in Parkinson's Disease

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Parkinson's disease (PD) is one of the prevalent neurodegenerative diseases associated with preferential loss of dopaminergic (DA) neurons in the substantia nigra compacta (SNc) and accumulation of α-synuclein in DA neurons. Even though the precise pathogenesis of PD is not clear, a large number of studies have shown that microglia-mediated neuroinflammation plays a vital role in the process of PD development. G protein-coupled receptors (GPCRs) are widely expressed in microglia and several of them act as regulators of microglial activation upon corresponding ligands stimulations. Upon α-synuclein insults, microglia would become excessively activated through some innate immune receptors. Presently, as lack of ideal drugs for treating PD, certain GPCR which is highly expressed in microglia of PD brain and mediates neuroinflammation effectively could be a prospective source for PD therapeutic intervention. Here, six kinds of GPCRs and two types of innate immune receptors were introduced, containing adenosine receptors, purinergic receptors, metabotropic glutamate receptors, adrenergic receptors, cannabinoid receptors, and melatonin receptors and their roles in neuroinflammation; we highlighted the relationship between these six GPCRs and microglial activation in PD. Based on the existing findings, we tried to expound the implication of microglial GPCRs-regulated neuroinflammation to the pathophysiology of PD and their potential to become a new expectation for clinical therapeutics.

**Keywords: G protein-coupled receptor (GPCR), microglial activation, Parkinson's disease, neuroinflammation, dopaminergic (DA) neuronal loss**

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## INTRODUCTION

Microglia are the main resident macrophages in the central nervous system (CNS), which originate from the yolk sac and merge into CNS at the early stage development (McGeer et al., 1988; Colonna and Butovsky, 2017; Prinz et al., 2019). Under physiological conditions, microglia make great contributions to the CNS homeostasis; however, in face of inflammatory stimuli, microglia rapidly transform their status into activated (Colonna and Butovsky, 2017; Wolf et al., 2017; Li and Barres, 2018). The link between microglia and PD was first reported 33 years ago and they found a mass of reactive microglia in PD brain (McGeer et al., 1988). Whereafter, a number of studies indicated

that microglia-regulated neuroinflammation plays vital roles in PD pathogenesis (Jankovic, 2008; De Virgilio et al., 2016). Reactive microglia could be simply classified into two categories, including M1 phenotype and M2 phenotype (Ransohoff, 2016; Tang and Le, 2016), although this classification is not precise. M1 phenotype makes microglia detrimental with exaggerated pro-inflammatory factors, while M2 phenotype is neuroprotective and produces anti-inflammatory mediators like Arg1, IL-10, and CD206 (Jha et al., 2016; Tang and Le, 2016). In PD brain, excessive pro-inflammatory like interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) lead to severe damage to DA neurons (Sawada et al., 2006). Damaged DA neurons in turn stimulate microglial activation, resulting in a positive feedback loop of microglial activation and DA neuronal death (Glass et al., 2010). Although it is still undetermined whether neuroinflammation is the cause of PD pathogenesis or a downstream response of neuronal damage, some studies reported that inhibiting microglial action or clear activated microglia is neuroprotective and could relieve some motor symptoms in PD animal models (Gu et al., 2018), suggesting targeting neuroinflammation might show new lights on the research of PD pathogenesis.

Mammalian β-adrenergic receptor, as the first G protein-coupled receptor (GPCR), was identified in 1986 (Dixon et al., 1986). Nearly 800 members GPCRs are found and become the largest superfamily of cell surface receptors in mammals (Fredriksson et al., 2003; Nieto Gutierrez and McDonald, 2018). Almost 90% of the GPCRs can be bound by a wide range of neurotransmitters and neuromodulators, like norepinephrine, dopamine, and serotonin in brain (Doze and Perez, 2012). It is well documented that some microglial innate immune receptors, including Toll-like receptors (TLRs) and NOD-like receptors (NLRs) regulate microglial activation through the recognition of their ligands (Lan et al., 2017; Salvi et al., 2017; Ifuku et al., 2020; Ma et al., 2020). Recently, it has been reported that microglial TLR4 could mediate neuron-released α-synuclein clearance through selective autophagy (Choi et al., 2020). Interestingly, in the PD animal model, α-synuclein accumulation in microglia has been demonstrated to induce its activation by binding with several microglial innate immune receptors, such as CXCR4 (Li Y. et al., 2019). Numbers of endogenous or exogenous stimuli and perturbation could act on GPCRs, changing a wide variety of cell signaling, affecting the pathogenesis of many neurodegenerative disorders, including PD (Guixa-Gonzalez et al., 2012; Gandía et al., 2013; Azam et al., 2020). Moreover, G protein-coupled receptor kinases (GRKs) can act as the regulators of dopamine receptor functions, further influencing PD development (Gurevich et al., 2016). Currently, GPCRs have already been the targets of 34% Food and Drug Administration (FDA)-approved drugs available and some GPCRs have potential for clinical treatment in neurodegenerative disorders (Lütjens and Rocher, 2017; Hauser et al., 2018). Whether GPCRs also play roles in the regulation of microglia activation is worth further study.

In this review, we attempted to assess the relationship between GPCRs and neuroinflammation and the mechanisms underlying this phenomenon, as well as its importance in PD. We would like to discuss the role of GPCRs in microglial activation and we will focus on adenosine receptors, purinergic receptors, metabotropic glutamate receptors, adrenergic receptors, cannabinoid receptors, and melatonin receptors. We intended to focus on the therapeutic perspective of GPCRs as emerging drug targets for the development of novel therapeutic agents to PD treatment.

## ROLE OF ADENOSINE RECEPTORS IN MICROGLIAL ACTIVATION OF PARKINSON'S DISEASE

Adenosine, as a neurotransmitter, is widely expressed in various cell types of CNS, including glial cells and neurons (Liu et al., 2019). Adenosine exerts physiological effects through four subtypes of adenosine receptors: $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$ receptors, which belong to the purinergic GPCR family (Peleli et al., 2017). It is well known that adenosine acts as a vital role in neuronal excitability and synaptic transmission (Boison and Aronica, 2015); however, the adenosine-mediated role in glial function remains unclear. The dysfunction of adenosine receptors is reported to be closely related to PD pathogenesis (Cunha, 2016). Caffeine, as an adenosine antagonist, is neuroprotective against PD (Brothers et al., 2010; Bagga et al., 2016). In the MPTP-induced mouse model of PD, adenosine deaminase inhibition remarkably ameliorated DA neuronal death and improved motor disabilities (Huang et al., 2019).

$A_1$ receptors are highly expressed in the cortex and hippocampus (Soliman et al., 2018). Even though there is no significant change of $A_1$ receptors in the early stages of PD detected by $^{11}$C-MPDX PET (Mishina et al., 2017), an obvious increase of $A_1$ receptors in the cerebellum was detected in virus-infected encephalitis rat model (Paul et al., 2014), suggesting the possible role of $A_1$ receptors in some inflammatory-mediated pathological conditions. $A_1$ receptors activation in microglia could suppress glioblastoma growth acting (Synowitz et al., 2006). Stimulation of $A_1$ receptors inhibits microglial morphological activation through suppressing ATP-induced $Ca^{2+}$ influx (Luongo et al., 2014), indicating the possible beneficial role of $A_1$ receptors in microglia-mediated neuroinflammation. One study showed that paeoniflorin inhibits neuroinflammation via activating microglial $A_1$ receptors in the MPTP mouse model (Liu et al., 2006), implying the protective role of $A_1$ receptors in PD. Unlike $A_1$ receptors, more research based on the relationship between $A_{2A}$ receptors and microglial activation was reported. In MPTP-treated mice, there is a significant increase in $A_{2A}$ receptor expression both in the substantia nigra (SN) and in the striatum (Gyoneva et al., 2014). Moreover, In lipopolysaccharide (LPS)-stimulated primary microglia, increased $A_{2A}$ receptors expression was also detected (Orr et al., 2009). Also, $A_{2A}$ receptors inhibition could suppress microglia activation in different neurodegenerative disorders (Gyoneva et al., 2014; Madeira et al., 2016), while $A_{2A}$ receptor activation could counteract the anti-inflammatory effects mediated by dopamine and further strengthen neuroinflammation (Meng et al., 2019). The combination of adenosine $A_{1A}$ receptor agonist and

adenosine $A_{2A}$ receptor antagonist has a stronger inhibitory effect on neuroinflammation; among them, $A_{2A}$ antagonists have been used in clinical evaluation for the treatment of PD (Marucci et al., 2021). Interestingly, CD73-derived adenosine was reported to regulate inflammation and neurodegeneration (Meng et al., 2019).

Even though there is no definitive research of $A_{2B}$ and $A_3$ receptors in PD pathogenesis, in LPS-treated microglia, $A_{2B}$ receptors activation has been reported to aggravate pro-inflammatory factors production through phosphorylation of cAMP response element binding (CREB) and promoting the p38 mitogen-activated protein kinase (MAPK) pathway (Koscsó et al., 2012; Merighi et al., 2017). The role of $A_3$ receptor in microglia has also been reported. Activation of the microglial $A_3$ receptors by adenosine or Cl-IB-MECA, a selective adenosine $A_3$ receptor agonist, is capable of inhibiting TNF-α production through suppressing Akt and NF-κB activation in BV-2 microglia cells, while MRS1523, a selective $A_3$ receptor antagonist, reverses this neuroprotective effect (Hammarberg et al., 2003; Lee et al., 2006).

## ROLE OF PURINERGIC RECEPTORS IN MICROGLIAL ACTIVATION OF PARKINSON'S DISEASE

Purinergic receptors are categorized into two kinds of receptor families, containing ATP-gated ion channels (P2X) and GPCRs (P2Y) (Burnstock, 2017). P2X receptors are further subdivided into seven subtypes: P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, and P2X7 receptors. GPCRs (P2Y) receptors are further subdivided into eight subtypes, including five Gq-coupled receptors: P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 and three Gi-coupled receptors: P2Y12, P2Y13, and P2Y14 (Burnstock, 2017). It is well reported that purinergic signaling is involved in the pathophysiology of CNS, including PD (Puchałowicz et al., 2014; Tóth et al., 2019). A recent study showed that purinergic receptor modulation could reverse aberrant $Ca^{2+}$ signaling, might appear novel therapeutic potential for HD and PD (Glaser et al., 2020). P2X7 receptors are preferentially expressed on microglia compared to that on astrocytes and oligodendrocytes (Illes, 2020). P2X7 receptors are the amplifier of CNS damage in neurodegenerative diseases (Illes, 2020). In LPS-primed microglia, extracellular ATP generated from damaged tissue and astrocytes could bind to P2X7 receptors, leading to the inflammasome assembly and the releases of IL-1β and IL-18, which further make neurons damage (Heneka et al., 2018). In addition, P2X7 receptors in DA neurons are responsible for α-synuclein-induced oxidative stress and mitophagy impairment (Wilkaniec et al., 2020), implying its damage exacerbation in PD. P2X7 receptor antagonist can inhibit neuroinflammation and may be a potential drug target for the treatment of movement disorders (Fonteles et al., 2020), such as PD. Besides P2X7, P2X4 in the P2X receptor family is reported to regulate microglial function (Suurväli et al., 2017). In the experimental autoimmune encephalomyelitis (EAE) model, P2X4 receptors signaling inhibition or P2X4 knockout increases pro-inflammatory gene expression in microglia (Zabala et al., 2018). Moreover, P2X4 receptor protein is increased in the SN of the PD rat model and P2X4 receptor activation is involved in DA neuronal autophagy inhibition (Zhang X. et al., 2021), indicating that P2X4 receptor might serve as a potential way for the treatment of PD.

P2Y receptors, as G-coupled receptors are also well reported to tend to regulate microglial function (Von Kügelgen and Hoffmann, 2016). One study indicated that P2Y1 receptors might be the modulator of microglia–astrocyte interaction. It showed that microglia-derived cytokines could act on astrocytes, resulting in the downregulation of P2Y1 receptor and exerting neuroprotective effects (Shinozaki et al., 2017). Exogenous ATP triggered P2Y1 receptors activation, resulting in $Ca^{2+}$ release from intracellular stores, making microglia to be pro-inflammatory (Orellana et al., 2013). In addition, both P2Y1 and P2Y12 are involved in ADP-induced migration of microglia mediated by transforming growth factor (TGF-β) (De Simone et al., 2010). There are very few studies about P2Y(2/4) receptors in neuroinflammation. Both P2Y2 and P2Y4 receptors are linked to amyloid beta (Aβ)-induced self-uptake by microglial pinocytosis, showing their more important role in AD (Kim et al., 2012; Li et al., 2013).

It seems that P2Y6 receptors play a more significant role than other P2Y receptors in PD.

P2Y6 receptors were markedly upregulated both in LPS-induced microglia and in the peripheral blood mononuclear cells (PBMCs) of PD patients (Yang et al., 2017). UDP, as the ligand of P2Y6, could promote microglial activation via the ERK1/2 pathway (Yang et al., 2017). Blocking UDP/P2Y6 receptor signaling could reverse these PD pathological processes (Yang et al., 2017; Anwar et al., 2020). It is also reported that a combination of P2Y6 and P2X7 receptor antagonists can be more protective in the 6-OHDA-induced PD rat model (Oliveira-Giacomelli et al., 2019). Moreover, P2Y6 receptors contribute to MPTP-induced neuronal SHSY5Y cell death (Qian et al., 2018). Therefore, P2Y6 receptor might be a potential clinical biomarker of PD. The role of P2Y12 and P2Y13 mediating microglial function has also been reported (Haynes et al., 2006; Zeng et al., 2014; Tatsumi et al., 2015). During neuropathic pain, P2Y12 receptor-dependent GTP-RhoA/ROCK2 signaling pathway upregulate excitatory synaptic transmission and microglial activation (Yu et al., 2019). A very recent study indicated that IL-1β release is increased remarkably in the P2Y13 knockout microglia while release evoked by LPS and ATP was not affected (Kyrargyri et al., 2020).

## ROLE OF METABOTROPIC GLUTAMATE RECEPTORS IN MICROGLIAL ACTIVATION OF PARKINSON'S DISEASE

It has been well reported that there is abundant glutamate in the CNS. Glutamate is an excitatory neurotransmitter maintaining communication and balance between glial cells and neurons (Ribeiro et al., 2017). Glutamate receptors are categorized into two types, namely, metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors. mGluRs are further divided

into three subgroups: Group I including mGlu1 and mGlu5 receptors; Group II including mGlu2 and mGlu3 receptors; and Group III consists of mGlu4, mGlu6, mGlu7, and mGlu8 receptors on the basis of sequence homology (Perfilova and Tyurenkov, 2016). mGluRs have been reported to be the therapeutic targets in PD (Litim et al., 2017). Among these mGluRs, Group III mGluRs activation could suppress glutamate release from presynaptic terminals of microglia, preventing neurons from excitotoxicity (Williams and Dexter, 2014). mGlu5 receptor seems to be closely linked to neuroinflammation. mGlu5 receptor is a potential regulator of microglial function and was first found in glial cells in 1999 (Biber et al., 1999). One study reported that microvesicles (MVs) released from microglia BV2 cells contribute to the communication between microglia and neurons and this interaction was mediated by mGlu5 receptor, resulting in the increased rotenone-induced neurotoxicity (Beneventano et al., 2017). This finding is really interesting. In the inflammatory-induced PD mouse model, the traditional Chinese medicine triptolide inhibits microglial activation via increasing mGlu5 receptor expression (Huang et al., 2018). Moreover, in the recent study of this group, they found that mGlu5 agonists inhibit α-synuclein-mediated neuroinflammation by regulating the binding of mGlu5 to α-synuclein (Zhang Y.N. et al., 2021), further indicating the significant role of mGlu5 in PD. Importantly, in mGlu5 receptor knockout ($mGluR5^{-/-}$) mice, the increasing number of both microglia and astrocytes was found (Carvalho et al., 2019), implying that mGlu5 is involved in neuroinflammation.

## ROLE OF ADRENERGIC RECEPTORS IN MICROGLIAL ACTIVATION OF PARKINSON'S DISEASE

Adrenergic receptors are divided into two groups, including α and β adrenergic receptors, and further subdivided into several kinds of subtypes, containing $\alpha_1$, $\alpha_2$ (subtypes $\alpha_{2A}$, $\alpha_{2B}$, and $\alpha_{2C}$), $\beta_1$, $\beta_2$, and $\beta_3$ (Strosberg, 1993). These adrenergic receptors exert physiological effects mainly through their ligand binding named noradrenaline (Strosberg, 1993). Dysfunction of the locus coeruleus noradrenergic system has been reported to be closely related to PD progression (O'Neill and Harkin, 2018; Sommerauer et al., 2018). Noradrenergic impairment contributes to the high prevalence of the non-motor symptoms in PD (O'Neill and Harkin, 2018). There are a few studies on the relationship between α-adrenergic receptors and microglial function. One study reported that dexmedetomidine, as a selective $\alpha_2$-adrenoceptor agonist, suppresses LPS-induced release of pro-inflammatory cytokines from hippocampal microglia specifically through $\alpha_2$-adrenergic receptor activation (Yamanaka et al., 2017). In another research, dexmedetomidine could regulate microglial polarization induced by 6-OHDA. Upon the pretreatment of dexmedetomidine, the inhibitory effects of 6-OHDA on IL-4-mediated induction of the anti-inflammatory factors, like IL-10 and IL-13, were remarkably alleviated, while the release of pro-inflammatory factors, such as IL-6 and IL-1β, were inhibited (Zhang et al., 2017).

Rather than α-adrenergic receptors, β-adrenergic receptors, especially $\beta_2$-adrenergic receptors seem to be more closely linked to neuroinflammation in PD pathogenesis. The relationship between microglia and β-adrenergic receptors was first reported in 1988 (Fujita et al., 1998). They found that a $\beta_2$-selective agonist terbutaline but not a $\beta_1$-selective agonist restrains the microglial proliferation via enhancing intracellular cAMP level (Fujita et al., 1998). A number of studies reported that $\beta_2$-adrenergic receptors inhibit microglia-mediated neuroinflammation via multiple signaling pathways to protect DA neurons from damage (Qian et al., 2009, 2011; Peterson et al., 2014; O'Neill et al., 2020). One of the studies showed that transformation from an M1- to M2-like phenotype in LPS-activated microglia by $\beta_2$-adrenergic receptors agonists involves activation of the classical cAMP/PKA/CREB as well as the PI3K and p38 MAPK signaling pathways (Sharma et al., 2019). Another study reported that low doses of salmeterol inhibit microglial activation through a $\beta_2$AR/β-arrestin2-dependent but cAMP/protein kinase A-independent pathway (Qian et al., 2011). A recent study indicated that $\beta_2$-adrenergic receptors activation using different agonists dramatically alleviates the progression of dopaminergic neurodegeneration via inhibiting microglial activation in the LPS-challenged inflammatory PD mouse model (O'Neill et al., 2020).

## ROLE OF CANNABINOID RECEPTORS IN MICROGLIAL ACTIVATION OF PARKINSON'S DISEASE

Cannabinoid (CB) receptors as classical G-protein coupled receptors are widely expressed throughout CNS, as well as certain parts of the body. Cannabinoid receptors mainly contain two subtypes, namely, CB1 and CB2 receptors (Zou and Kumar, 2018). A recent study showed that GPR55, an orphan receptor that can be activated by many classical cannabinoids, has been added as a third cannabinoid receptor (Moriconi et al., 2010). It is well documented that endocannabinoid as the endogenous ligand of cannabinoid receptors mediates large numbers of physiological effects through ligand-receptor binding (Zou and Kumar, 2018). Both Gi and Gs proteins were regulated by cannabinoid receptors activation (Howlett et al., 1986; Glass and Felder, 1997). A mass of studies regard cannabinoid receptors as the potential therapeutic target for PD (Baul et al., 2019; Cristino et al., 2020). CB receptors are equipped to play protective roles in different cellular stress in PD. Here, we focused on the roles of microglial CB1 and CB2 receptors, as well as GPR55 in neuroinflammation.

In MPTP-induced PD mouse model, pretreatment with non-selective CB receptors agonist WIN55212-2 or HU210 inhibited NADPH oxidase reactive oxygen species (ROS) production and reduced pro-inflammatory cytokines production from activated microglia, leading to increased DA neurons survival in the SN and improved the mouse motor function (Chung et al., 2011). The anti-inflammatory and neuroprotective effects were reversed upon treatment with CB1 receptor selective antagonist AM251 or SR14716A, confirming the importance of the CB1 receptor (Chung et al., 2011). It has been reported that the

treatment of selective CB1 receptor antagonist SR141716A significantly increases pro-inflammatory factors (TNF-α, IL-1β, and IL-6) and chemokines (MCP-1 and CX3CL1) production in BV2 microglial cells through upregulating TLR4 and NF-κB/p65 expressions, further accelerating the clinical onset and development of EAE (Lou et al., 2018). However, one study showed that the anti-inflammatory effects of CB1 receptor might not be such important. In CB1 receptor knockout (*Cnr1*$^{-/-}$) mice, less noradrenergic neurons in the locus coeruleus were found compared to their age-matched wild-type controls. Nevertheless, there was no enhanced pro-inflammatory profile in *Cnr1*$^{-/-}$ mice even the density of microglia was increased (Gargano et al., 2020). Very interestingly, CB1 receptor was found to be localized on neuronal mitochondria, the G protein-coupled mitochondrial CB1 (mtCB1) receptors modulate memory processes through regulating mitochondrial energy metabolism (Hebert-Chatelain et al., 2016). This effect was mediated by mtCB1/mtGαi/PKA-dependent phosphorylation of complex I subunit NDUFS2 (Hebert-Chatelain et al., 2016). In their recent research, mtCB1 was also found in astrocytes, regulating glucose metabolism via phosphorylation of the mitochondrial complex I subunit NDUFS4 (Jimenez-Blasco et al., 2020). These two studies are indeed striking, as regulation of mitochondrial energy metabolism on microglial activation is currently a research hotspot. Whether microglial mtCB1 exists, if exists, will microglial mtCB1 modulate microglial activation through regulating mitochondrial energy metabolism mediated by certain molecules? This is really worthy of further study.

More research about CB2 receptors has been reported than CB1 receptors on microglia. One study reported that CB1 receptor gene expression was unchanged, while CB2Ar (A isoform, CB2Ar) gene expression was significantly increased (fourfold) in the SN of patients with PD (Navarrete et al., 2018). Another study also found the upregulation of CB2 receptors in microglia of SN in postmortem tissues of PD patients (Gómez-Gálvez et al., 2016). Moreover, in the rotenone or 6-OHDA-induced PD rat model, obvious upregulation of CB2 receptors expression was detected (Concannon et al., 2015, 2016). Interestingly, in the inflammatory rat PD model induced by Poly (I:C) or LPS, a more remarkable CB2 receptors increase was found (Concannon et al., 2015, 2016), implying that CB2 receptors were more influenced by inflammatory stimulation. These studies indicated targeting the CB2 receptor might represent a viable way for neuroinflammation modification in PD. CB2 receptor activation has been reported to inhibit neurotoxin-mediated neuroinflammation through regulating multiple molecules, including NRF2, ERK1/2, cPLA2, and NF-κB (Ribeiro et al., 2013; Galán-Ganga et al., 2020). Importantly, a much more intense deterioration of tyrosine hydroxylase (TH)-containing nigral neurons in CB2 receptor-deficient mice compared to wild-type animals (Gómez-Gálvez et al., 2016), indicating a potential neuroprotective role for CB2 receptor. The role of microglial GPR55 has also been reported recently. They found that KIT77, as an inverse agonist on GPR55 independent of the endocannabinoid system, significantly inhibited the release of PGE2 in primary microglia partially relying on the reduction of protein synthesis of mPGES-1 and COX-2 (Saliba et al., 2018). Recently, as the crystal structure of CB1 and CB2 receptors was analyzed (Krishna Kumar et al., 2019; Li X. et al., 2019), it becomes the guiding light for further research on CB receptors and really contributes to the drug discovery targeting CB receptors in PD.

## ROLE OF MELATONIN RECEPTORS IN MICROGLIAL ACTIVATION OF PARKINSON'S DISEASE

Melatonin is an amine hormone secreted mainly through the pineal gland in mammals (Hardeland et al., 2011). Circadian rhythm modulation and anti-oxidation effects have been thought to be the central physiological functions of melatonin (Reiter et al., 2000; Sanchez et al., 2015). G-protein coupled receptors (GPCR), namely, MT1 (encoded by *MTNR1A*) and MT2 (*MTNR1B*) regulate the majority of the biological functions of melatonin (Zlotos et al., 2014). Both MT1 and MT2 receptors are widely expressed in brain and play important roles in neurodegenerative diseases (Liu et al., 2016). It has been reported that MT1 receptors are reduced in AD patients (Wu et al., 2007). Silence of *MTNR1A* increases the amyloidogenic processing of amyloid precursor protein (APP) and exacerbates mutant huntingtin-mediated toxicity (Wang et al., 2011; Sulkava et al., 2018), indicating that MT1 plays a protective role in neurons. MT2 receptor is also closed related to AD pathogenesis. There was impaired hippocampal MT2 receptor signaling and MT2 activation ameliorated dendritic abnormalities through the cAMP-C/EBPα/miR-125b/GluN2A signaling pathway in AD (Tang et al., 2019). However, the role of MT receptors in PD pathogenesis.

It is well known that the relationship between melatonin and PD is mainly dependent on the anti-inflammatory and antioxidant effects. The majority of these neuroprotective effects on DA neurons in PD is receptor-independent and relies on antioxidant properties of the melatonin drug structure (Reiter et al., 2000, 2016; Pandi-Perumal et al., 2013). The role of MT1 and MT2 receptor in regulating PD pathogenesis, especially neuroinflammation remains unclear. Importantly, one study reported that there is a significant reduction of both MT1 and MT2 receptors in amygdaloid nucleus and SN of PD patients (Adi et al., 2010), possibly implying the significance of MT receptor in PD progression. Sleep disorder caused by circadian rhythm dysfunction is regarded as one of the vital non-motor symptoms of PD and often occurs in the early stage of PD progression (Fifel, 2017). Some researchers agree that dysfunction of circadian rhythm might be involved in PD pathogenesis (Lauretti et al., 2017). Both MT1 and MT2 receptors play vital roles in the regulation of circadian rhythm (Pandi-Perumal et al., 2007; Comai and Gobbi, 2014). Dysfunction of MT1 or MT2 might have the potential to participate in PD progression. The role in microglial MT1 on neuroinflammation is poorly understood. Our latest study indicated that microglial MT1 regulates microglial activation through PDHA1-mediated enhancement of oxidative phosphorylation (Gu et al., 2021). Others also reported that non-selective agonists such as

**TABLE 1 |** Reported microglial GPCRs and their potential roles in PD.

| Microglial GPCRs | Sub-types | Ligands | Reported mechanisms | References |
|---|---|---|---|---|
| Adenosine receptors | $A_1$ | Adenosine, paeoniflorin (agonist) | Promoting ATP-induced $Ca^{2+}$ influx, neuroinflammation inhibition | Liu et al. (2006), Brothers et al. (2010), Luongo et al. (2014), Bagga et al. (2016) |
| | $A_{2A}$ | Preladenant SCH58261 Caffeine (antagonist) | Microglial activation inhibition, neuroprotective effects in MPTP model, increase of $A_{2A}$ in MPTP or LPS-treated model | Orr et al. (2009), Brothers et al. (2010), Gyoneva et al. (2014), Bagga et al. (2016), Madeira et al. (2016) |
| | $A_{2B}$ | Adenosine, BAY60-6583 (agonist) | Promoting p-CREB and p-p38 | Koscsó et al. (2012), Merighi et al. (2017) |
| | $A_3$ | Adenosine, Cl-IB-MECA (agonist) | Suppressing Akt and NF-κB activation | Hammarberg et al. (2003), Lee et al. (2006) |
| Purinergic receptors | P2Y1 | ATP and ADP (agonist) | Increasing $Ca^{2+}$ release from intracellular stores, promoting pro-inflammatory factors production | De Simone et al. (2010), Orellana et al. (2013) |
| | P2Y12 | ADP (agonist) | TGF-β-induced microglial migration | De Simone et al. (2010) |
| | P2Y6 | UDP (agonist) | P2Y6 upregulation in LPS-induced microglia, ERK1/2 activation, promoting MPTP-induced neuronal cell death | Yang et al. (2017), Qian et al. (2018), Anwar et al. (2020) |
| | P2Y12 | $P2Y12^{-/-}$mice | GTP-RhoA/ROCK2 signaling activation in $P2Y12^{-/-}$ mice | Yu et al. (2019) |
| | P2Y13 | $P2Y13^{-/-}$microglia | IL-1β release is increased remarkably in $P2Y13^{-/-}$ microglia | Kyrargyri et al. (2020) |
| Metabotropic glutamate receptors | mGlu5 | CHPG (agonist) | Inhibiting α-synuclein-mediated neuroinflammation | Zhang Y.N. et al. (2021) |
| | | Triptolide | Inhibiting microglial activation via increasing mGlu5 receptor expression | Huang et al. (2018) |
| | | $mGluR5^{-/-}$mice | Increasing number of both microglia and astrocytes | Carvalho et al. (2019) |
| Adrenergic receptors | α2 | Dexmedetomidine (agonist) | Suppressing LPS-induced release of pro-inflammatory cytokines, regulating microglial polarization induced by 6-OHDA | Yamanaka et al. (2017), Zhang et al. (2017) |
| | $\beta_2$ | Terbutaline, Salmeterol, Clenbuterol, Formoterol (agonist) | Enhancing intracellular cAMP level, inhibit microglia-mediated neuroinflammation, activating classical cAMP/PKA/CREB as well as the PI3K and p38 MAPK signaling pathways, β2AR/β-arrestin2-dependent pathway, inhibiting microglial activation in LPS-challenged inflammatory PD mouse model | Fujita et al. (1998), Qian et al. (2011), Peterson et al. (2014), Sharma et al. (2019), O'Neill et al. (2020) |
| Cannabinoid receptors | CB1 | Endocannabinoid, WIN55212-2, HU210 (agonist), SR141716A (antagonist) | Inhibiting NADPH and ROS, increasing pro-inflammatory factors (TNF-α, IL-1β, and IL-6) and chemokines (MCP-1 and CX3CL1) | Chung et al. (2011), Lou et al. (2018) |
| | CB2 | | Upregulation of microglial CB2 receptors in PD model | Concannon et al. (2015), Concannon et al. (2016), Gómez-Gálvez et al. (2016), Navarrete et al. (2018) |
| | | WIN55212-2, Cannabinoids, CP55940 (agonist) | NRF2, ERK1/2, cPLA2, and NF-κB inhibition, neurotoxin-mediated neuroinflammation inhibition | Ribeiro et al. (2013), Galán-Ganga et al. (2020) |
| | GPR55 | KIT77 (agonist) | Reduce protein synthesis of mPGES-1 and COX-2 | Saliba et al. (2018) |
| Melatonin receptors | MT1 | Ramelteon (agonist) | Enhancing PDHA1-mediated enhancement of oxidative phosphorylation | Gu et al. (2021) |
| | MT1/MT2 | Agomelatine, Ramelteon, Melatonin, (agonist) | Suppressing NF-κB nuclear translocation, promoting Nrf2 nuclear accumulation | Molteni et al. (2013), Wang et al. (2019) |

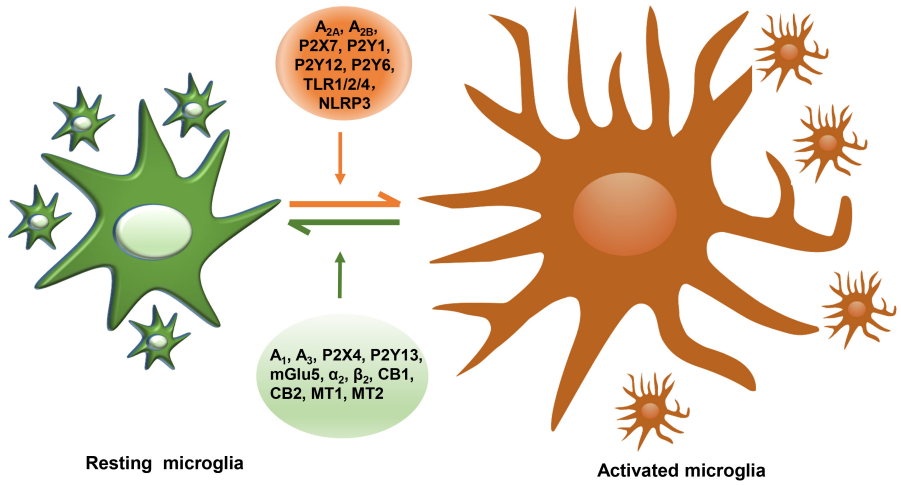


**FIGURE 1 |** The summary of reported microglial GPCRs in microglial activation of PD. GPCRs expressed in microglia containing $A_{2A}$, $A_{2B}$, P2X7, P2Y1, P2Y12, P2Y6, TLR1/2/4, and NLRP3 act as detrimental roles, promoting microglial activation, damaging DA neurons, while microglial $A_1$, $A_3$, P2X4, P2Y13, mGlu5, $\alpha_2$, $\beta_2$, CB1, CB2, MT1, and MT2 play protective roles in neuroinflammation.

ramelteon or agomelatine exert powerful anti-inflammatory effects (Molteni et al., 2013; Wang et al., 2019). Agomelatine, as a novel antidepressant, inhibits LPS-induced upregulation of pro-inflammatory factors via suppressing NF-κB nuclear translocation (Molteni et al., 2013). Ramelteon, which is used for treating insomnia clinically, protects neuronal degeneration in traumatic brain injury (TBI) through promoting nuclear factor erythroid 2-related factor 2 (Nrf2) nuclear accumulation, leading to the increasing of downstream factors of NRF2, including SOD-1, heme oxygenase-1, and NQO1 (Wang et al., 2019). It is very interesting that MT1 receptor is also located on neuronal mitochondria, like CB1 receptor. They found that melatonin is also synthesized in the mitochondrial matrix and released by the organelle to activate the mitochondrial MT1 signal-transduction pathway, thus inhibiting stress-mediated cytochrome c release and caspase activation, protecting neurons from death (Suofu et al., 2017). As microglial MT1 is closely linked to mitochondrial metabolic programming, there is the possibility that microglial mitochondrial MT1 exists and regulates mitochondrial-mediated microglial activation.

## ROLE OF INNATE IMMUNE RECEPTORS IN MICROGLIAL ACTIVATION OF PARKINSON'S DISEASE

Except for the GPCRs mentioned above, innate immune receptors, also named pattern recognition receptors (PRRs), were widely expressed in innate immune cells, including microglia (De Nardo, 2015; Rodríguez-Gómez et al., 2020). It is well known that innate immune receptors are critical for modulating inflammatory responses (Rodríguez-Gómez et al., 2020). In regard to neurodegenerative diseases, TLRs and NOD-like receptors (NLRs), as best-characterized PRRs, play vital roles in neuroinflammation (Block et al., 2007; Kigerl et al., 2014; Kumar, 2019). Upon the binding of engaging ligands, microglial TLRs are activated, further causing internalization and conformational changes, leading to the recruitment of the adaptor proteins MyD88 or TRIF (TIR-domain-containing adaptor-inducing interferon-β) (De Nardo, 2015). Some transcription factors, including nuclear factor κB (NF-κB) are activated by downstream signaling cascades, resulting in subsequent production of pro-inflammatory cytokines (De Nardo, 2015). The relationship between TLRs and neuroinflammation is well clarified. For the pathogenesis of PD, TLRs are closely associated with α-synuclein. Misfolded or fibrillar α-synuclein released from neurons could be bound by the microglial TLR1/2 heterodimer, triggering NF-κB-mediated TNFα production (Kim et al., 2013). Another study also showed that fibrillar α-synuclein, but not oligomeric α-synuclein could promote IL-18 upregulation via a TLR1/2-dependent manner (Gustot et al., 2015). Microglial TLR4 also plays a significant role in α-synuclein-induced microglial activation, as α-synuclein could be uptake through TLR4, leading to pro-inflammatory cytokine release, and reactive oxygen species (ROS) production (Fellner et al., 2013). Furthermore, in the MPTP mouse PD model, TLR4 deficiency was neuroprotective (Noelker et al., 2013), indicating a detrimental role for TLR4 in PD pathogenesis.

Besides TLRs, NLRs mediate the process of the inactive IL-1β and IL-18 into active forms through inflammasomes activation, triggering pro-inflammatory responses (Latz et al., 2013). The NLR protein 3 (NLRP3) is the best-characterized inflammasome in PD, as in NLRP3-deficient mice, it was protective from MPTP-induced loss of DA neurons with decreased MPTP-induced caspase-1 activation and IL-1β release (Yan et al., 2015). There is extensive evidence that fibrillary α-synuclein, ATP, highmobility group box protein 1 (HMGB1), and lysophosphatidylcholine (LPC) could act as the activator of NLRs (Davalos et al., 2005; Freeman et al., 2017). Upon fibrillar α-synuclein insults, increased NLRP3 mRNA and protein expression were detected

(Gustot et al., 2015; Zhou et al., 2016). This study suggested the key role of NLRP3 in PD pathogenesis.

## CONCLUSION

In our present review, we mainly summarize six types of GPCRs and two types of innate immune receptors that are possibly related to disease progression of PD and their roles in mediating microglial action (**Table 1**). Some of these receptors, which consist of $A_1$, $A_3$, P2X4, P2Y13, Group III mGluRs, mGlu5, $\alpha_2$, $\beta_2$, CB1, CB2, MT1, and MT2, act as beneficial roles and inhibit microglial activation, while $A_{2A}$, $A_{2B}$, P2X7, P2Y1, P2Y12, P2Y6, TLR1/2/4, and NLRP3 play unfavorable roles and are capable of inducing neuroinflammation (**Figure 1**). Drugs that target multiple receptors to inhibit microglial activation show considerable prospects in clinical therapeutic potential in PD. Very strikingly, CB1 and MT1 receptors were both found on mitochondrial outer membrane in neuron (Hebert-Chatelain et al., 2016; Suofu et al., 2017). Besides, CB1 receptor was also located on astrocytic mitochondria (Jimenez-Blasco et al., 2020). The possibility of microglial mitochondria is worthy to explore.

In our opinion, as mitochondrial metabolic dysfunction is not only related to DA neuronal death directly but also closely involved in microglial activation, moreover, both crystal structures of MT1 and CB1 were analyzed recently, we believe that agonists that bind to both MT1 and CB1 receptors have great potential for the development of clinical treatment of PD drugs.

## AUTHOR CONTRIBUTIONS

CG and YJC wrote the manuscript. YC, ZZ, C-FL, and MW edited and revised the manuscript. All authors read and approved the final manuscript.

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REVIEW
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# Dynamic Diversity of Glial Response Among Species in Spinal Cord Injury

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The glial scar that forms after traumatic spinal cord injury (SCI) is mostly composed of microglia, NG2 glia, and astrocytes and plays dual roles in pathophysiological processes induced by the injury. On one hand, the glial scar acts as a chemical and physical obstacle to spontaneous axonal regeneration, thus preventing functional recovery, and, on the other hand, it partly limits lesion extension. The complex activation pattern of glial cells is associated with cellular and molecular crosstalk and interactions with immune cells. Interestingly, response to SCI is diverse among species: from amphibians and fishes that display rather limited (if any) glial scarring to mammals that exhibit a well-identifiable scar. Additionally, kinetics of glial activation varies among species. In rodents, microglia become activated before astrocytes, and both glial cell populations undergo activation processes reflected amongst others by proliferation and migration toward the injury site. In primates, glial cell activation is delayed as compared to rodents. Here, we compare the spatial and temporal diversity of the glial response, following SCI amongst species. A better understanding of mechanisms underlying glial activation and scar formation is a prerequisite to develop timely glial cell-specific therapeutic strategies that aim to increase functional recovery.

**Keywords: spinal cord injury (SCI), glial cells, immune cells, glial scar, glial bridge, rodents, primates, regenerative species**

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## INTRODUCTION

Traumatic injuries, including spinal cord injury in the adult mammalian central nervous system, induce a glial response that eventually forms a glial scar that is largely occupied by microglia, NG2 glia and astrocytes. The first glial cells to be activated, after injury, are microglia/macrophages that either proliferate and migrate toward the lesion site or, in the case of monocyte-derived macrophages, infiltrate from the periphery. The activated microglia/macrophages concomitantly express a full repertoire of molecules that modulate glial responses (including microglia/macrophages) but also immune-cell responses (for review, see David and Kroner, 2011; David et al., 2015, 2018). The response of astrocytes eventually leads to the formation of a dense astroglial border surrounding the lesion core, or fibrotic scar (for review, see Yang et al., 2020). In the past decade, the concept that the glial scar has both harmful and beneficial effects has emerged. Indeed, the scar acts as a chemical and physical obstacle to spontaneous axonal regeneration and thus prevents functional recovery. However, the glial scar also limits lesion extension. A better understanding of the complexity of individual cellular (glial and immune cells) and molecular mechanisms induced by SCI as well as their crosstalk remains a major challenge. The cellular dynamics induced by injury are closely reflected by tissue repair and functional

recovery. Remarkably, amphibians and fishes (for review, see Ghosh and Hui, 2018), but also embryonic/neonatal mammals, exhibit the capacity to both repair injured spinal cord tissues and to achieve functional recovery. Interestingly, these animals display rather limited (if any) glial scarring.

Here, we review the temporal diversity of the glial response, following SCI in rodents, primates, and species that display high regenerative capabilities. Due to the abundant literature on glial scarring, especially in rodents, we selected articles mainly focusing on descriptive characterisations of cellular and/or temporal events induced by SCI in order to highlight the consequences of glial-scar formation kinetics on functional recovery after injury. A better understanding of the mechanisms underlying the time line of glial activation and scar formation is a prerequisite to develop glial-cell-specific therapeutic strategies.

## MICE: A MAJOR GLIAL SCAR IS OBSERVED AFTER SPINAL CORD INJURY

Owing to the extensive availability of genetically modified animals, mice are the most widely used model to study the cellular and molecular responses of glia following SCI (**Figures 1**, **2** and **Table 1**).

In mice, immune-cell responses to SCI play a key role in the dynamics of the lesion. The recruitment of neutrophils, following contusion injury, displayed similar kinetics in four mouse strains. An early infiltration, starting as early as 6 h after injury, led to a peak of neutrophil number between 3 and 14 days post injury (dpi). This was followed by a decrease over the next 4 weeks. Neutrophil numbers, however, remained stable over the next 6 weeks of the study (Kigerl et al., 2006). Compression injury led to similar neutrophil kinetics with two waves of activation that peaked at 3 and 14 dpi (Mawhinney et al., 2012). Consistently, 3–12 h after contusion injury, expression of chemokines, such as KC (CXCL1) and MIP-2 (CXCL2) by astrocytes, was followed by the recruitment of neutrophils [and, to a lesser extent, monocytes] through MyD88/IL-1R1 signaling within damaged areas (Pineau et al., 2010). Analysis of the dynamics of cytokine expression after contusion injury has led to the suggestion that the early production (5–15 min) of IL-1β by astrocytes and microglia after injury orchestrates the recruitment of leukocytes (Pineau and Lacroix, 2007). Subsequently, the release of IL-1β and TNF-α (14–28 dpi) induces the recruitment of T lymphocytes (Pineau and Lacroix, 2007). This is in agreement with the biphasic T-cell influx reported after contusion injury, starting at 14 dpi, and then decreasing between 2 and 4 weeks and again increasing over the following 2 weeks to reach similar number as at 14 dpi (Kigerl et al., 2006).

Microglia and macrophages are the two predominant immune players in SCI. Resident microglia are within the spinal cord before injury, whereas the monocyte-derived macrophages (MDM) infiltrate the spinal cord from the periphery after the lesion. Crosstalk between both cell types modulates their respective responses to injury and, therefore, contributes to their functions. The dynamic orientation of microglial processes toward the lesion, within the white matter, has been observed by time-lapse two-photon imaging as early as 5 min after laser injury. This led, soon afterward, to the initiation of myelin debris phagocytosis (Stirling et al., 2014). Similarly, in several mouse strains, contusion and compression injuries induced an early macrophage activation at 6 h postlesion that further formed phagocytic clusters in the grey matter by 3 dpi (Kigerl et al., 2006; Mawhinney et al., 2012). At 1 day post contusion, microglia rapidly accumulated around the epicenter but decreased in number (cell death, partly by apoptosis) and retracted their processes at the lesion site (Bellver-Landete et al., 2019). From 4 dpi, microglia displayed a round shape and started to express phagocytic markers (Bellver-Landete et al., 2019). From 4 (Bellver-Landete et al., 2019) or 7 (Kigerl et al., 2006; Mawhinney et al., 2012) to 14 dpi, activated microglia and MDM peaked and then decreased but remained elevated for up to 6 weeks (Kigerl et al., 2006).

Microglia primarily and transiently proliferated after two severities of spinal cord section, as reflected by an upregulation of genes associated with proliferation at 3 days but not at 7 and 14 days after injury (Noristani et al., 2017). Consistently, after spinal cord contusion, Ki67 expression was observed in 50% of microglia at the lesion epicenter at 4 dpi; the peak of microglia proliferation occurred at 7 dpi and only few (2–6%) $Ki67^+$ microglia persisted at 14 and 35 days (Bellver-Landete et al., 2019). Additionally, microglia proliferated in greater numbers than infiltrating macrophages, and they initiated phagocytosis of damaged axons at 1 dpi (Bellver-Landete et al., 2019). Conversely, infiltrating macrophages started to phagocytose debris at 3–5 dpi and then progressively became the main phagocytic cells in the lesion and persisted chronically (up to 42 dpi) (Greenhalgh and David, 2014). In addition, the infiltrating macrophages repressed microglia-mediated inflammation and phagocytosis (Greenhalgh et al., 2018). Subsequent to proliferation, microglia were rapidly recruited around the lesion site and accumulated in the core of the lesion 3 days after hemisection (Tang et al., 2015). Similarly, 3 days after spinal cord contusion, $CD11b^+$ cells first occupied the periphery of the injury site before being preferentially located in the lesion site 5–56 dpi (Zhu et al., 2015a). Microglia, surrounding infiltrating cells, were located at the interface between infiltrating leukocytes and astrocytes, forming an immune interface through their interaction with both $GFAP^+$ astrocytes and blood-derived cells (Bellver-Landete et al., 2019). This "microglial scar" mostly visible from 14 to 35 dpi limited the spread of infiltrating cells outside of the lesion core and expressed IGF-1 that further promoted astrocytic proliferation and astrocytic scar formation (Bellver-Landete et al., 2019).

Analysis of activated microglia/macrophages revealed that, from 3 to 12 months post-injury, few cells were located in the core of the lesion as compared to the glial scar (Camand et al., 2004).

Finally, at a transcriptomic level, microarray experiments on spinal cord segments, centered on the contusion site, have revealed an induction of pro- and anti-inflammatory genes from 1 to 28 dpi. However, the upregulation of anti-inflammatory genes was more transient (up to 7dpi) than the pro-inflammatory genes (up to 1 month) (Kigerl et al., 2009). Three days after contusion injury, macrophage-specific transcriptomic analysis revealed an expression profile characteristic of cell

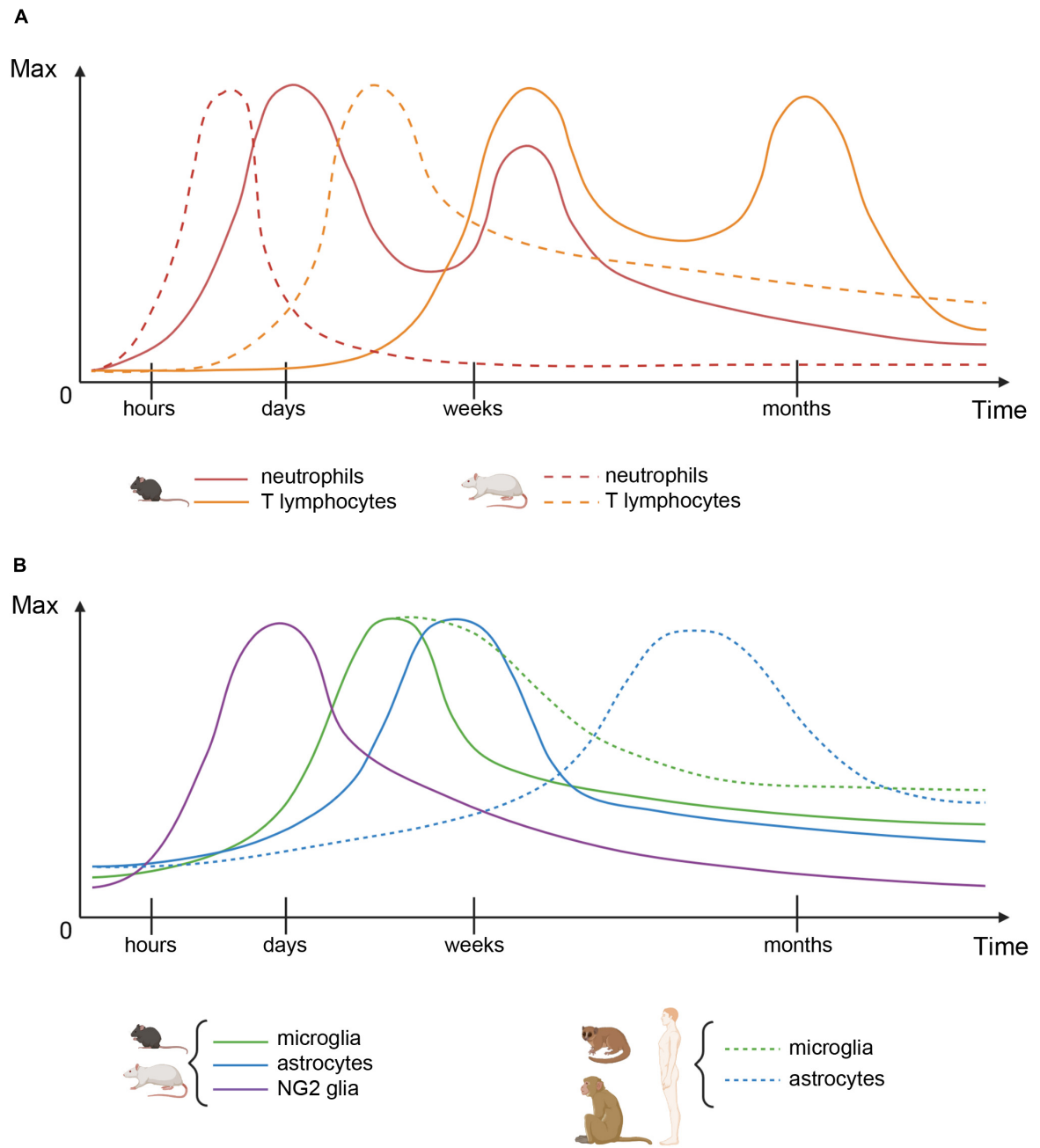


**FIGURE 1 |** Cellular dynamics after spinal cord injury. **(A)** Immune cell infiltration patterns in mice (plain lines) and rats (dashed lines). **(B)** Glial cell numbers in rodents (plain lines) and primates (dashed lines). For each cell type, both graphs represent the number of cells over time, relative to their maximum value.

migration that further evolved at 7 dpi to a typical profile of foam cells (Zhu et al., 2017). Lastly, using RNAseq of microglia/macrophages ($CX3CR1^{+}$ cells), following partial and complete spinal cord section, we have shown that microglial activation is dependent on the time post-injury but not on the lesion severity (Noristani et al., 2017). Indeed, the transcriptomic profile at 3 dpi reflected cell proliferation and was associated with neuroprotective genes, whereas, in the 7 and 14 dpi, the profile switched to neuroinflammation-associated gene expression. Interestingly, from 3 to 42 dpi, over 6% of microglia expressed astrocytic markers [glial fibrillary acidic protein (GFAP) and vimentin] that may reflect an SCI-induced glial differentiation (Noristani et al., 2017).

Astrocytes play a central role in the formation of the glial scar, following CNS injury. Five days after moderate spinal cord contusion, astrocytes, identified by their expression of GFAP, were seen in the vicinity of the lesion. From 7 dpi, astrocytes formed an astroglial scar surrounding the injury site

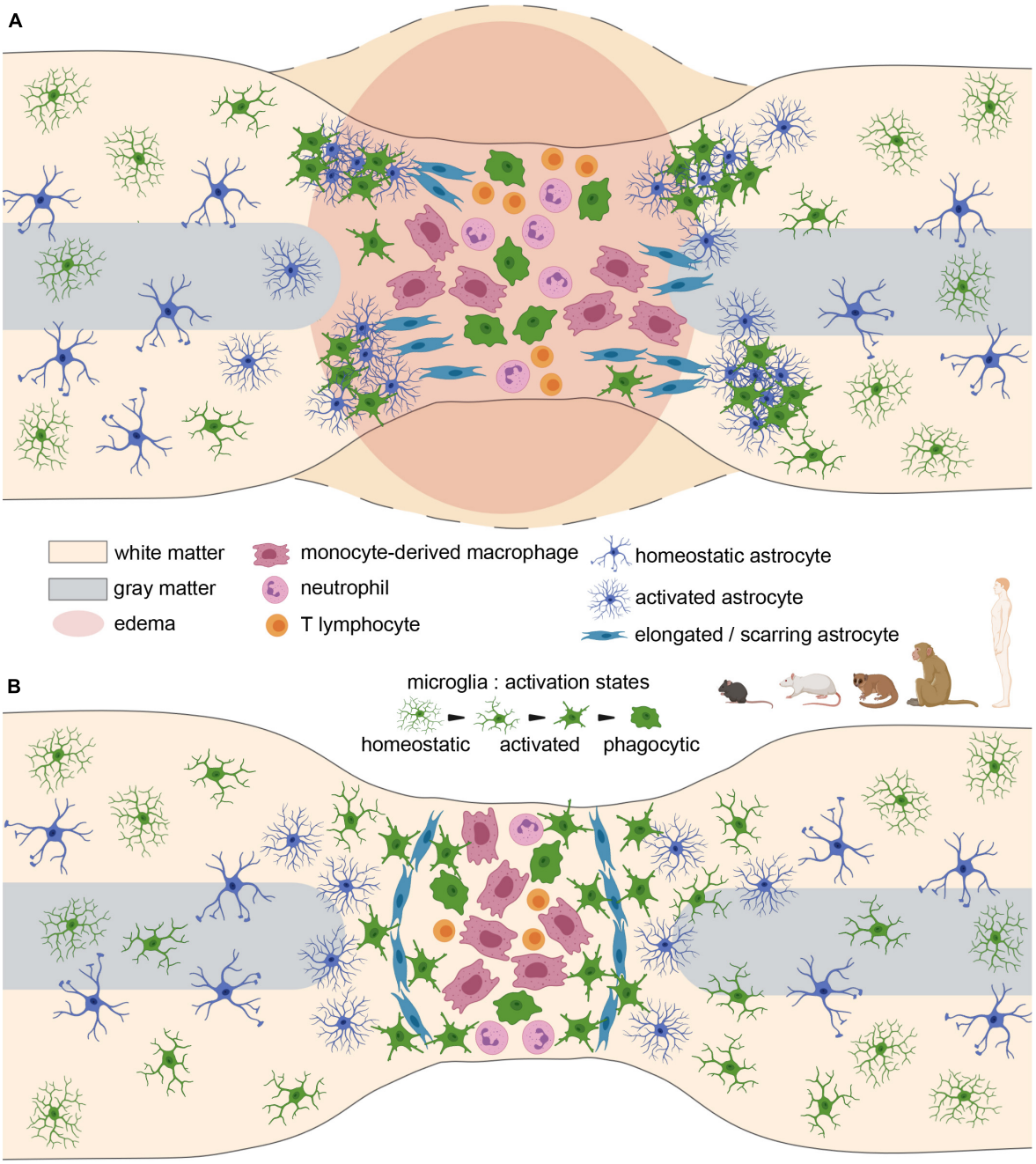


**FIGURE 2 |** Glial scar formation after spinal cord injury in rodents and primates. **(A)** Acute stage. Cellular infiltration, reactivity, proliferation, and edema at the lesion site. **(B)** Glial scar stabilisation at the subacute/chronic stage. Note the substantial role of scarring astrocytes in separating the lesion core from spared tissues.

that stabilised at 14 dpi (Zhu et al., 2015a). In the longer term (56 days after lesion), GFAP$^+$ cells were no longer observed in the lesion core (Zhu et al., 2015a). Likewise, 5–14 days following crush injury, astrocyte proliferation, together with the overlapping of astrocytic processes, started to form a dense scar. By 2 weeks postinjury, scar borders surrounded the lesion and restricted fibrotic and inflammatory cells to the core of the injury site, mainly included newly proliferative astrocytes. This "corral" organisation is STAT3 dependent (Wanner et al., 2013). In mice with spinal crush injury, selective ablation of scar-forming, reactive, and proliferating astrocytes hindered glial scar formation and led to an extensive influx of IBA1-positive microglia/macrophages. These findings highlight the constant cross-talk between glial cells and strongly suggest that reactive astrocytes modulate microglia/macrophage number and infiltration (Gu et al., 2019). This is consistent with the increased

number of proliferating microglia observed at 109 days after hemisection in adult MRL/MpJ mice that possess exceptional regeneration capabilities, which do not form a scar after injury and display a reduced astrocytic response (Thuret et al., 2012).

Eight days after dorsal hemisection of the spinal cord, an overall orientation of astrocytic processes within the rostro-caudal axis was observed immediately adjacent to the lesion. The core of the lesion, with only few astrocytes, remained rather wide from 8 days to 1 month after lesion and diminished by 50% from 3 to 12 months, following injury (Camand et al., 2004). In the vicinity of the lesion, hypertrophic astrocytes, displaying the classical "stellate shape," were present up to 6 months after injury. Thereafter, GFAP expression returned to a baseline value 6–12 months after injury (Camand et al., 2004). At 3–7 dpi after lateral crush injury, cavity-surrounding, reactive astrocytes have been shown to die by necroptosis. Moreover, induction of necroptotic, astrocytic markers partly resulted from the polarisation of M1 microglia/macrophages (Fan et al., 2016). Strikingly, 8–30 dpi, intense chondroitin sulfate proteoglycans (CSPG) expression was observed in astrocytes. This later almost disappeared. In parallel, PSA-NCAM, which is expressed by astrocytic end feet in the intact spinal cord, was increased in a subpopulation of reactive astrocytes from 8 to 30 dpi. This expression remained elevated at later time points (Camand et al., 2004). After severe crush injury, Cspg5 (neuroglycan C) and Cspg4 (NG2) were upregulated in scar-forming astrocytes. Furthermore, both NG2 and CSPG5 proteins were observed in the glial scar (Anderson et al., 2016), suggesting that astrocytes also participated in extracellular matrix dynamics.

The origin of scar-forming astrocytes remains to be elucidated. Newly formed astrocytes accumulated at the edge of the lesion by 7 days after moderate contusion injury and then remained at a constant level up to 49 days. Similarly, amongst the proliferative cells, an increased proportion of astrocytes was observed in the spared white matter (White et al., 2010). In parallel, radial glial cells ($BLBP^+$) presented an early and sustained increase in incidence at the edge of the lesion and in the preserved white matter conversely to their transient presence in the spared grey matter and central canal (White et al., 2010). There is an ongoing debate as to the origin of the newly proliferative scar-forming astrocytes. Indeed, scar-forming astrocytes were either reported to mainly (Sabelstrom et al., 2013) or minimally (Ren et al., 2017) originate from ependyma-derived progeny. This discrepancy on the ependymal contribution to newly scar-forming astrocyte may depend on whether or not the ependyma was directly damaged by the primary injury (Ren et al., 2017). Finally, we investigated astrocytic plasticity overtime using RNAseq analysis of a pure population of astrocytes, following hemi- or complete spinal cord section and demonstrated a time and severity-dependent deregulation of gene expression. However, in both injury severities, over 10% of mature (as opposed to newly formed) astrocytes underwent an injury-induced trans-differentiation toward neuronal progenitors (Noristani et al., 2016; Noristani and Perrin, 2016).

Finally, two NG2-expressing cell populations (glial cells and pericytes) also participate in scar formation. From 1 to 11 days after contusion injury, dividing oligodendrocyte progenitors, the $NG2^+$ glial cells, strongly outnumber dividing $NG2^+$ pericytes and were restricted at the lesion border and in the spared tissue (Hesp et al., 2018). From 8 days to 6 months, an increased expression of NG2 was also reported in the glial scar, following hemisection; it returned to control value 1 year after injury (Camand et al., 2004). Interestingly, ablation of $NG2^+$ cells induced a less-dense astrocytic border associated with macrophages infiltration (Hesp et al., 2018).

Overall, in mice, recruitment and infiltration of immune cells precede microglial and astrocytic responses (**Figure 1**). However, a complex molecular crosstalk between all cell populations orchestrates the formation of a well-defined and dense glial scar (**Figure 2** and **Table 1**).

## RATS: A MAJOR GLIAL SCAR IS ALSO OBSERVED AFTER SPINAL CORD INJURY BUT IMMUNE INFILTRATION APPEARS EARLIER THAN IN MICE

Rats display an overall pathophysiological response to SCI that mimics some features of the human response, such as the formation of cavities. This is not observed in mice. Rats are thus the most widely used model in SCI even if they are not predominant amongst rodents in studies focusing on glia (**Figure 2** and **Table 2**).

In rats, following spinal cord injury, the cellular response in the lesion is initiated by immune cells. The majority of studies have been carried out using immunohistochemistry, and only a few have resorted to flow cytometry. As early as 1–3 h, following partial spinal cord section, a few neutrophils adhere to the inner surface of blood vessels. Then, from 6 to 24 h, a large number of neutrophils are found at the site of the primary lesion. Thereafter, they disappear (Dusart and Schwab, 1994). Similarly, 1 day following contusion injury, the initial phase of inflammation consisted of an early neutrophil number peak that declines afterward. However, neutrophils persist for many months, and a positive correlation between contusion severity and the number of neutrophils has been reported (Beck et al., 2010). Finally, neutrophil and lymphocyte peaks were observed 3 days after dorsal hemisection of the spinal cord; neutrophils completely disappeared 7 days after lesion, whereas T cells displayed a strong decrease but remained present (Pruss et al., 2011). In agreement with this, following contusion injury, early T cell infiltration peaked between 3 and 7 dpi (Popovich et al., 1997; Sroga et al., 2003) and declined by 50% over the next 3 weeks (Sroga et al., 2003). Lymphocyte infiltration was paralleled by microglial activation (Popovich et al., 1997) and dendritic-cell influx (Sroga et al., 2003). Using flow cytometry, after contusion injury, Beck et al. show similar T cells dynamics, but with a slightly delayed infiltration (from 7 to 9 dpi peaking at Day 9), followed by a decrease at 10 dpi and persistence throughout the 6 months study follow-up (Beck et al., 2010).

Glial cell dynamics, including microglia/macrophages, oligodendrocytes, astrocytes, and NG2-expressing cells, have

**TABLE 1 |** Studies demonstrating roles of the glial and immune cells after SCI in mice.

| Injury, interval SCI-death, methods | Astrocyte | Microglia/ macrophage | Other glial cells | Immune cells | References |
|---|---|---|---|---|---|
| **Mice** | | | | | |
| Contusion T9. 3, 7, 21, 28&42dys. IHC | | CD11b MHCII | | CD3,CD4, CD8 | Sroga et al., 2003 |
| HS T8. 8, 30, 90, 180&365dys: IHC | GFAP PSA NCAM | Isolectin B4 | NG2 | | Camand et al., 2004 |
| Contusion T9. 3, 7, 14&42dys, IHC | | Mac1, MHCII | | LY6G,CD3, CD4, CD8 | Kigerl et al., 2006 |
| Contusion T10-11. 15&45mns, 3&24hrs, 2&14dys | GFAP | Iba1, CD11b | CA2 | CD45 | Pineau and Lacroix, 2007 |
| Contusion T9-10. 3, 7&28dys. Microarrays, IHC | | CD86, CD206, CD16, CD32, Arginase1 | | | Kigerl et al., 2009 |
| Contusion T10-11. 3&12hrs, 4&28dys IHC, FACS | GFAP | Iba1. FACS: CD11b, CD45, CD16, CD32 | CA2 | 7/4, LY6B FACS: F480, LY6C, LY6G, | Pineau et al., 2010 |
| Contusion T9. 3, 7&49dys BrdU, IHC | GFAP BLBP | CD11b | | | White et al., 2010 |
| Compression T5. 1, 3, 7, 14&42dys. | | | | LY6G F480 Tg: LysM | Mawhinney et al., 2012 |
| Dorsal HS T9. 1, 4&54dys. Microarrays, BrdU | GFAP | CD11b | NG2 | | Thuret et al., 2012 |
| Dorsal section C4. 7dys&14wks TgFoxJ1, IHC | GFAP | | | | Sabelstrom et al., 2013 |
| Crush L1-2. 5, 14&28dys TgSTAT3KO, BrdU, IHC | Tg: GFAP GFAP Aquaporin4 BLBP, RC2 | CD45 | SOX2 | | Wanner et al., 2013 |
| Contusion T11. 24hrs, 3, 7, 14&42dys. IHC | | Iba1, CD11b | | | Greenhalgh and David, 2014 |
| Laser injury. 5, 30&120mins, FACS | | Tg: CX3CR1 $CD11^{+}/Ly6C^{+}$ CD45 | | | Stirling et al., 2014 |
| HS T12. 30mins, 2, 8, 24, 48&72hrs. IHC | | F480 | | | Tang et al., 2015 |
| Contusion T8. 3, 5, 7, 14, 28&56dys. IHC | GFAP | Tg: CX3CR1 CD11b | | Tg: LysM | Zhu et al., 2015a |
| Crush T10. 2, 8&10wks RNAseq. Transgenic STAT3 KO, BrdU | Tg: GFAP GFAP | | NG2 | | Anderson et al., 2016 |
| Lateral crush T8. 3, 5, 7&14dys. | Tg: GFAP GFAP | CD11b | CC1 | | Fan et al., 2016 |
| HS and FT T9. 1&2wks. FACS, RNA-seq, IHC | Tg: Aldh111 GFAP, FGFR4 | | | | Noristani et al., 2016 |
| HS and FT T9. 72hrs, 1&2wks. FACS, RNA-seq, IHC | GFAP, Vim | Tg: CX3CR1 Iba1 | | | Noristani et al., 2017 |
| Crush and lateral stab T10. 2&8wks. Tg FoxJ1, BrdU, IHC | GFAP, Aldh111 | | | | Ren et al., 2017 |
| Contusion T8. 3&7dys. RNAseq, IHC | GFAP | | | Tg: LysM Tg:CD45, Tg: CD36 | Zhu et al., 2017 |
| Contusion T11. 1, 3, 4, 7&28dys. IHC. | | CD11b, CD86, Iba1,P2RY12, TMEM119 | | Tg: LysM Tg: CCR2 | Greenhalgh et al., 2018 |

*(Continued)*

**TABLE 1 |** (Continued)

| Injury, interval SCI-death, methods | Astrocyte | Microglia/ macrophage | Other glial cells | Immune cells | References |
|---|---|---|---|---|---|
| Lateral contusion C5. 1, 3, 7, 11, 14&21dys, IHC | GFAP | | Tg: NG2 ablation Olig2 | | Hesp et al., 2018 |
| Contusion T9-10 1, 4, 7, 14&35dys, IHC | GFAP SOX9 | R26-TdT Tg: LysM Tg: CX3CR1$^{cre}$ CD68,P2RY12, | | | Bellver-Landete et al., 2019 |
| Crush T8. 2, 4&6wks Lentiviral-induced ablation, BrdU, IHC | Lv-GFAP to ablate astrocytes | Iba1 | | | Gu et al., 2019 |
| Crush T10 3&7dys, 10wks,IHC, RNA-seq. | GFAP | CD68, P2Y12 RNA: CD11bTg: CX3CR1$^{cre}$ Tg: CSF1R$^{fl/fl}$ | | | Li et al., 2020 |

*FACS, flow cytometry; hrs, hours; min, minutes; dys, days; wks, weeks; mths, months; yrs, years; IHC, immunohistochemistry; C, cervical; T; thoracic; L, lumbar; HS, hemisection; FT, full transection; Tg, transgenic.*

been widely analysed in rat models of SCI. The partial section of the spinal cord first induced microglia/macrophage proliferation at the lesion site that predominated at 48 h, leading to a highest density between 4 and 8 dpi. Then, 2 weeks after injury, microglia progressively disappeared from the lesion site concomitantly with the formation of a cavity that was further surrounded by a scar composed of microglia and astrocytes (Dusart and Schwab, 1994). Similarly, microglial activation peaked within the contusion epicenter between 3 and 7 days (Popovich et al., 1997; Sroga et al., 2003) and plateaued between 7 and 28 dpi distal to the lesion (Popovich et al., 1997). Alongside, monocyte influx and macrophage activation started at 7 dpi (Popovich et al., 1997).

The number of contusion-induced microglia/macrophages increased with the injury severity and displayed a biphasic response, with a first peak at 7 dpi, followed by a very low cell number at 14 dpi, increasing to a second peak at 60 days; microglia/macrophage number then remained elevated throughout 180 dpi (Beck et al., 2010). In agreement with this, a peak of microglia/macrophages displaying thick and branched processes was observed 1 week after dorsal hemisection, followed by a slow decline in number; however, microglia/macrophages also remained elevated 70 days after the lesion (Pruss et al., 2011). Following contusion injury, microglia/macrophages located in the spared white matter proliferated from 1 to 7 days, reaching a maximum on Day 3. By 6 weeks postlesion, few remaining proliferative microglia/macrophages were present (Zai and Wrathall, 2005). Finally, after dorsal funiculotomy, in ascending and descending pathways undergoing Wallerian degeneration at both subacute (10 dpi) and chronic (30 dpi) stages, the numbers of microglia (OX42+) and macrophages (ED1$^{+}$) were higher than in sham animals. However, a decrease in cell number between subacute and chronic stages was seen only in the ascending tract (Wang et al., 2009). In the same animals, the number of astrocytes was also increased, *cf.* sham animals, at both stages but, conversely to microglia, remained stable between stages (Wang et al., 2009).

One week after dorsal hemisection, few astrocytes were located in the lesion site; however, several also began to surround the injury site. At 2 weeks, astrocytes and microglia then formed a scar (Dusart and Schwab, 1994). This is consistent with contusion injury (Popovich et al., 1997; Zhu et al., 2015b) where an astroglial scar surrounded the lesion, whereas cavitation sites were occupied by microglia and macrophages (Popovich et al., 1997). Interestingly, 1 and/or 4 months after injury, astrocytes expressed several proteins, such as gamma1- and alpha1-laminin, type IV collagen, and FGF2, which participated in the chronic persistence of the glial scar (Liesi and Kauppila, 2002). Likewise, 2 months after the complete section of the thoracic spinal cord, astrocytes produced CSPG in the scar (Li et al., 2018), thus suggesting that, as seen in mice, astrocytes contribute to extracellular matrix dynamics.

From 1 to 7 days following contusion injury, astrocytes, oligodendrocytes, and NG2 glial precursors proliferated in the spared white matter, with a peak on Day 3. About 50% of the astrocytes and oligodendrocytes located in the residual white matter, next to the injury site, however, were lost by 24 h (Zai and Wrathall, 2005). During the chronic phase (6 weeks after lesion), the remaining proliferative cells consist of mature astrocytes or oligodendrocytes (50%) and few expressing NG2 (Zai and Wrathall, 2005). After moderate contusion, the expression level of NG2 increased between 3 and 7 days post injury and remained chronically elevated. In contrast to the spared surrounding tissue, within the lesion site, few, if any, NG2$^{+}$ cells were oligodendrocytes (McTigue et al., 2006). Within areas undergoing Wallerian degeneration, following dorsal funiculotomy, oligodendrocyte density (Olig2) decreased at subacute (10 days) and chronic (30 days) stages, although Olig2$^{+}$ cells were still present (Wang et al., 2009).

Taken together, these results demonstrate that the glial response to SCI exhibits similar dynamics in rats and mice; however, the immune cell response occurs earlier in rats than in mice (**Figures 1A,B**).

**TABLE 2 |** Studies demonstrating roles of the glial and immune cells after SCI in rats.

| Injury, interval SCI-death, methods | Astrocyte | Microglia/ macrophage | Other glial cells | Immune cells | References |
|---|---|---|---|---|---|
| **Rats** | | | | | |
| Partial section, 1, 3, 6, 12, 24hrs and 2, 4, 8, 14&12wks. IHC, HC | GFAP | CD11b, ED1 | | Cresyl violet | Dusart and Schwab, 1994 |
| Contusion T8, 12, 72hrs, 7, 28dys IHC | GFAP | CD11b, ED1, MHCII | | CD5 | Popovich et al., 1997 |
| Stab dorsal 1&4mths | GFAP | | | | Liesi and Kauppila, 2002 |
| Contusion T9, 3, 7, 21, 28&42dys IHC | | CD11b, MHCII | | CD4, CD8, CD11c | Sroga et al., 2003 |
| Contusion T8 1, 3&7dys, 6wks BrdU, IHC | GFAP | CD11b | NG2 CC1 | | Zai and Wrathall, 2005 |
| Moderate contusion T8 3, 7, 28 &70dys | | | NG2 P75 P0 | | McTigue et al., 2006 |
| Dorsal funiculotomy T8. 1hr, 10&30dys IHC | GFAP | CD11b ED1 CD68 | Olig2 | | Wang et al., 2009 |
| Contusion T8 (3 severities) FACS: 0–10dys, 14, 90&180dys, IHC; 1, 7, 14&90dys | | FACS:ED1, CD11b IHC: ED1 | | FACS&IHCCD3, PME | Beck et al., 2010 |
| Dorsal HS 3, 7, 14&28dys, IHC | | ED1, CD8, CD86, CD206 | | MPO, CD43 | Pruss et al., 2011 |
| ContusionT8 56dys, IHC | GFAP | | | | Zhu et al., 2015b |
| FT T8 2, 8wks IHC | Morphology | | | | Li et al., 2018 |
| FT T9 48hrs IHC | | | Nr3c1, ependymal glia is a Glcc target | | Nelson et al., 2019 |

*FACS, flow cytometry; hrs, hours; min, minutes; dys, days; wks, weeks; mths, months; yrs, years; IHC, immunohistochemistry; HC, histochemistry, H&E, hematoxylin eosin; C, cervical; T, thoracic; L, lumbar; HS, hemisection; FT, full transection.*

## NONHUMAN PRIMATES: A MAJOR ASTROCYTIC SCAR IS NOT OBSERVED AFTER SPINAL CORD INJURY

The neuroanatomical organisation of the central nervous system and responses to injury differ between rodents and primates (Courtine et al., 2007); thus, several SCI models in various strains of nonhuman primate have been developed. However, investigation of the glial response following injury is sparse, particularly early after injury (**Figure 2** and **Table 3**).

One hour after spinal cord compression in *Macaca cynomolgus*, an increased IBA1 immunoreactivity was observed adjacent to the injury site; no modification in astrocytes was seen (Miller et al., 2012). Spatiotemporal investigation of cellular responses following lateral spinal cord hemisection in *Macaca fascicularis* highlighted that, 1 and 4 weeks post-injury, microglia displayed morphological changes and became amoeboid in the epicenter and the spared contralateral white matter (Wu et al., 2013). The number of IBA1 positive cells remained stable at 1 week and decreased 4 weeks after lesion in both locations. However, activated microglia/macrophages ($CD68^+$) increased in number at the two time points in the same locations. Concomitantly, at the lesion epicenter, a decreased astrocyte number was reported and astrocytes became hypertrophic contralateral to the lesion. Importantly, a major astrocytic glial scar surrounding the lesion site was never observed (Wu et al., 2013). In the same species and lesion model, 1 and 4 weeks after SCI, an increased number of microglia ($OX42^+$) was detected within areas undergoing Wallerian degeneration (Shi et al., 2009). Morphologically, microglia were branched but displayed a large cell body and short processes. None, although, were amoeboid (Shi et al., 2009). No modifications in astrocytic morphology or number were observed.

Longitudinal gene expression analysis following contusion of the cervical spinal cord in *Callithrix jacchus* (marmoset) revealed that the inflammatory response peaked at 1 week post SCI and

**TABLE 3 |** Studies demonstrating roles of the glial and immune cells after SCI in primates.

| Species, strain, sex, age | Interval SCIdeath, methods | Injury type, level | Astrocyte | Microglia/ macrophage | References |
|---|---|---|---|---|---|
| **Nonhuman primates** | | | | | |
| *Callitrhrix jacchus* (Marmoset), 20F, adults | 10wks, IHC | 3 contusion severities, C5 | GFAP | | Iwanami et al., 2005 |
| *Macaca fascicularis*9 M, 5–6yrs | 1&4wks, IHC | Lateral HS, T8-9 | GFAP | OX42 | Shi et al., 2009 |
| *Macacacynomolgus*1M | 1hr, IHC | Balloon compression | GFAP | Iba1 | Miller et al., 2012 |
| *Macaca fascicularis*4M, 4–6 yrs | 7&30dys, IHC | Lateral HS, T8-9 | GFAP | Iba1 CD68 | Wu et al., 2013 |
| *Callitrhrix jacchus* (Marmoset), 16F, 2yrs | 1, 2, 4&6wks, microarrays& RNA-seq. 1, 2&6wks, IHC | Contusion, C5 | GFAP | Iba1 | Nishimura et al., 2014 |
| *Macaca mulatta* 6M, 3.5–4.2 yrs | 6mths, IHC | 2 contusion severities, T9 | GFAP | | Ma et al., 2016 |
| *Chlorocebussabaeus* (african green monkey) 12M, 5–10yrs | 12wks, IHC | lateral HS, T9-10 | GFAP | Iba1 | Slotkin et al., 2017 |
| *Microcebus murinus* 8M, 2 yrs | 3mths, IHC | Lateral HS, T12-L1 | GFAP | Iba1 | Le Corre et al., 2018 |
| *Microcebus murinus* 10M, 2yrs | 3mths, IHC | Lateral HS, T12-L1 | GFAP | Iba1 | Poulen et al., 2021 |
| **Human** | | | | | |
| 27 cases, 5F&22M, 8–86 yrs | 8 dys-23yrs, IHC | Para- or tetraplegia C, T&L | GFAP | | Puckett et al., 1997 |
| 13 cases 21–85yrs | 2 dys- 30 yrs, IHC | Complete para- or tetraplegia C, T & L | GFAP | | Buss et al., 2004 |
| 180 cases Ratio 5:1 M:F 8 mths to 92yrs | Instantaneous-51yrs, IHC&HC | Predominantly C | GFAP | H&E | Norenberg et al., 2004 |
| 11 cases, 2F & 9M 18–83yr | 30min - 19dys, IHC | Para or tetraplegia. | GFAP | MHCII | Yang et al., 2004 |
| 1 case, 56yrs | 2yrs IHC | Complete C6 injury | GFAP | | Guest et al., 2005 |
| 28 cases, 8F&20M, 6–88yrs | Instantaneous - 1yr, IHC | Contusion, compression&lacerationC1-T12. | | CD68 | Fleming et al., 2006 |
| 3 cases, 1F&2M, 49, 59 and 80yrs | 15, 20, 60 dys, IHC | Contusion, C | | CD68 | Chang, 2007 |
| 1 case | 5dys, IHC | | GFAP | | Fan et al., 2016 |
| 22 cases, 6F&16M 15–80yrs | <1–413 dys, IHC | T & C | IBA1 | TMEM119 P2RY12 | Zrzavy et al., 2021 |

*hrs, hours; min, minutes; dys, days; wks, weeks; mths, months; yrs, years; IHC, immunohistochemistry; HC, histochemistry; H&E, hematoxylin eosin; M, male; F, female; C, cervical; T, thoracic; L, lumbar; HS, hemisection.*

remained elevated up to 6 weeks following injury (Nishimura et al., 2014). The inflammatory response thus required a longer time to occur than in rodents. Concomitantly, IBA1 positive cells and proliferative microglia were present at the lesion epicenter at 1 week, decreased at 2 weeks, and were absent 6 weeks after injury. The rim of the lesion was delineated by astrocytes only at 6 weeks. In the same species and lesion model but with graded severities, 10 weeks after trauma, GFAP was expressed in a severity-dependent manner at the border of the lesion (Iwanami et al., 2005).

Three months following hemisection of the thoracic spinal cord in *Chlorocebus sabaeus* (African green monkey), astrocytes and microglia/macrophages were present at the rim of the lesion (Slotkin et al., 2017). In *Microcebus murinus*, a small lemur, we have shown that, at 3 months following lateral hemisection of the thoracic spinal cord, the glial reactivity was increased adjacent to the lesion. Additionally, an increase in microglia/macrophage and astrocyte reactivity was present within the grey matter, only rostral to the lesion. Moreover, rostral to the lesion a marked increase in microglia/macrophage reactivity was also observed on the lesion side of the *dorsal funiculus* (Le Corre et al., 2018; Poulen and Perrin, 2018; Poulen et al., 2021).

Finally, in *Macaca mulatta*, 6 months following contusive injury of the thoracic spinal cord, the density of astrocytes was decreased in the lesion penumbra but increased in the spared white matter (Ma et al., 2016).

Overall, the microglial response appears similar as in rodents conversely to the astrocytic response that occurs slower and does not lead to the formation of a major astrocytic scar. In some species, the inflammatory response is also slower than in rats and mice (**Figure 1B**).

## HUMAN: A MAJOR ASTROCYTIC SCAR IS NOT OBSERVED AFTER SPINAL CORD INJURY AND ASTROCYTIC RESPONSE IS SLOWER THAN IN OTHER SPECIES

Similarly, to animal models of spinal cord injury, microglial/macrophage cells display the earliest cellular response to injury (**Figure 2** and **Table 3**). From 0 to 4 h after injury, a modest number of phagocytic microglia/infiltrating monocyte-derived macrophages were observed at the injury site (Fleming et al., 2006). Activated microglia have also been detected as early as 30 min (Yang et al., 2004) and 1 day (Norenberg et al., 2004; Fleming et al., 2006) after spinal cord injury. Consequently, activated microglia were observed in the surviving area 5 days after SCI (Yang et al., 2004), and numerous amoeboid microglia were present adjacent to areas of necrosis from 5 to 10 days post injury. These persisted for weeks (and up to 1 year) in the proximity of the injury site (Norenberg et al., 2004; Fleming et al., 2006; Chang, 2007). A recent analysis of 22 human SCI cases has highlighted a time-dependent activation of microglia and macrophages associated with a spatial-dependent inflammatory pattern composed of a predominantly pro-inflammatory lesion rim and a lesion core displaying a dual pro- and anti-inflammatory phenotype (Zrzavy et al., 2021). The initial loss of microglia within the core of the lesion at the acute stage (1–3 days post-SCI) was followed in the early subacute stage (4–21 days post-SCI) by a massive increase of IBA1-expressing cells in the core and the rim of the lesion. Within the lesion rim, the majority of these cells were microglia (80% $TMEM119^+$) conversely to the core where their proportion dropped to 10% and was associated with an amoeboid shape and a large number of $CD68^+$ macrophages. Importantly, $IBA1^+/TMEM119^+$ cells within the lesion rim mostly resulted from local microglial proliferation (Zrzavy et al., 2021). Later (21–90 days post-injury), the number of macrophages in the lesion core decreased but remained elevated and displayed a dispersed pattern in the lesion rim. At chronic stages (90 days to 1.5 years post-lesion), cystic cavitations appeared and were surrounded by a rim of activated astrocytes and macrophages. Overall, microglia are, thus, the predominant cells in the proximity of the injury during lesion maturation, and recruited monocytes/macrophages are dominant within the lesion core.

Only a few studies have investigated the temporal astrocytic response following spinal cord injury in man. The presence of activated astrocytes has been described to appear either early after the injury (from 4 days) (Buss et al., 2004; Norenberg et al., 2004) at 21–90 days post-injury in the lesion rim (Zrzavy et al., 2021) or as long as 4 months after lesion (Puckett et al., 1997). In one spinal cord sample, 5 days after SCI, necroptotic markers were found in $GFAP^+$ cells located in the lesion site, suggesting that reactive astrocytes may undergo necroptosis (Fan et al., 2016). Clusters of activated astrocytes were also observed one or two segments away from the lesion site in both white and grey matters from 4 to 12 days after SCI. Thereafter, activated astrocytes were evenly distributed over the whole section close to the lesion site from 24 days to 4 months after injury (Buss et al., 2004). Several days after injury, hypertrophic astrocytes appeared at the edge of the lesion and peaked at 2–3 weeks (Norenberg et al., 2004). Moreover, activated astrocytes surrounded cystic cavities from 90 days to 1.5 years post injury (Zrzavy et al., 2021). In another study, astrocytes displayed a slight increase in GFAP reactivity, in processes in contact with the phagocytes, 4 to 12 months after injury, followed by a hypointense GFAP signal, persisting up to 23 years after injury (Puckett et al., 1997). At longer post-injury time (1–30 years), dense GFAP-positive staining was present in the white matter that had undergone Wallerian degeneration (Buss et al., 2004). Two years following complete spinal cord injury, a dense GFAP reaction was observed in the peri-injury region (Guest et al., 2005). These differences may result from the heterogeneity of the lesions observed in man.

Overall, the astrocytic response in man seems to occur slower than in animal models, including nonhuman primates, and the astroglial processes that create an impenetrable barrier were almost never seen (Norenberg et al., 2004) (**Figures 1B**, **2**).

## SPECIES WITH HIGH REGENERATIVE CAPACITIES: A GLIAL BRIDGE MORE THAN A GLIAL SCAR

Interestingly, vertebrates, such as fishes, urodele amphibians, and some reptiles, that possess a remarkable capacity to regenerate injured spinal cord tissue and to recover associated functions also display rather limited (if any) glial scarring (**Figure 3** and **Table 4**). Radial glial cells are the main (and often the only) representant of astrocytes in lower vertebrates (for review, see Verkhratsky et al., 2019). Here, we kept the names "radial glia," "GFAP-expressing cells" or even "astrocytes" as they appeared in the original publications.

No reactive, fibrous astrocytes have been described at the injury site, after spinal cord lesion, in either juvenile or adult *Amybstoma mexicanum* (axolotl). Astrocytes, initially present in the white matter, first disappeared from the lesion site and reappeared 1 month after injury concomitantly with regenerating axons (O'Hara et al., 1992). In the same study, *in vitro* experiments suggested that the formation of a scaffold resulting from mesenchymal epithelial transition permits axon regeneration (O'Hara et al., 1992). Similarly, in the adult salamander, following the complete spinal cord section, axons regrew and crossed the lesion site (Zukor et al., 2011). No scar formation was observed; however, astrocytes were present but not hypertrophic. Astrocytic cells did not migrate into the injury site, but $GFAP^+$ processes crossed the lesion site, and axons appeared to regrow on this glial support. Additionally, a non-detrimental inflammatory response was

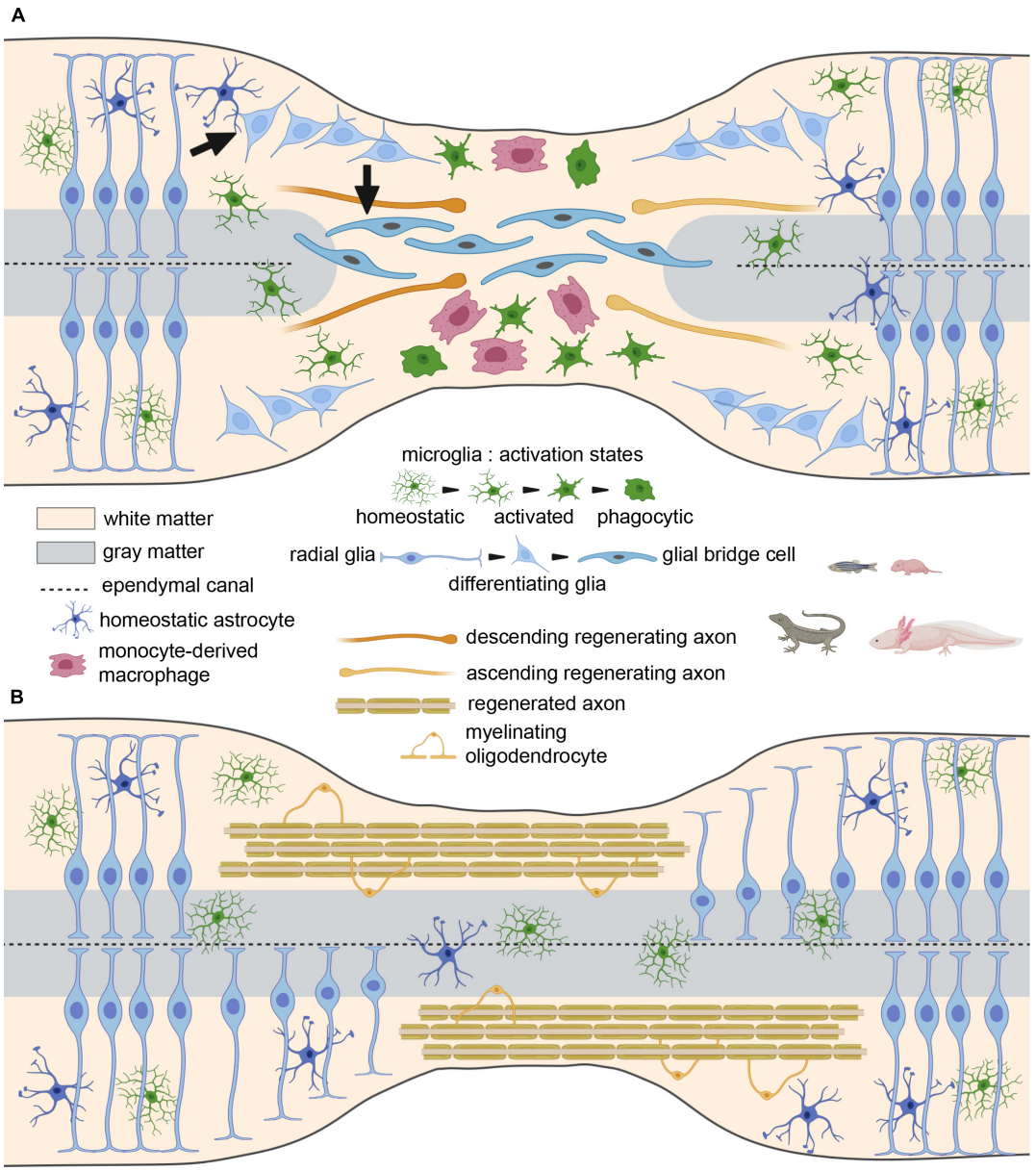


**FIGURE 3 |** The glial bridge after spinal cord injury in species with high regenerative capacities and perinatal mammals. **(A)** Tissue clearance, glial bridge, and axon sprouting at the acute/subacute stage. Arrows represent the involvement of radial glia in the glial bridge formation. **(B)** Remyelination and return to homeostasis at the chronic stage.

reported (Zukor et al., 2011). Likewise, following SCI in adult zebrafishes (Goldshmit et al., 2012) and larvae (Briona and Dorsky, 2014), GFAP-expressing cells became elongated and formed a "glial bridge" that joins the sides of the damaged spinal cord in the absence of glial scar formation. No reactive astrocytes were observed. In adults, within 3–5 days post injury, GFAP$^+$ glial cells proliferated in and around the central canal. Concomitantly, a few proliferative macrophages were also reported outside of the central canal (Goldshmit et al., 2012). Five days after injury, proliferative cells at the edge of the lesion expressed a low level of GFAP, and, from 7 to 10 days after SCI, GFAP$^+$ cells migrated into the site of the lesion and acquired a bipolar morphology. Then, from 2 to 3 weeks post SCI, a "glial bridge" formed of GFAP-expressing bipolar cells appeared in the lesion site. From 4 weeks post lesion, this permissive bridge supported axogenesis. Interestingly, by 3 (and up to 5) days post injury, oligodendrocyte precursors and motor neuron progenitors (olig2$^+$) bridged the injury site in zebrafish larvae (Anguita-Salinas et al., 2019). The mechanisms of bridge formation appeared to be Fgf- (Goldshmit et al., 2012) but also ctfg (connective tissue growth factor) dependent (reviewed in Cigliola et al., 2020). In zebrafish, bridge formation depends on

**TABLE 4 |** Studies demonstrating roles of the glial and immune cells after SCI in species with high regenerative capacities.

| Species, injury, interval SCI-death, methods | Astrocyte | Radial cells | Microglia/ macrophage | Infiltrating cells & other glia | References |
|---|---|---|---|---|---|
| **Regenerate embryonic/larvae** | | | | | |
| Rats: Adults *vs* E19, FT.T8-10. 3, 7, 21&35dys.IHC, ISH | GFAP | OX42 | | | Fujimoto et al., 2006 |
| Zebra larvae 5dpf. FT. ISH, IH | GFAP | Tg Dbx1a, | | Tg olig2 | Briona and Dorsky, 2014 |
| Rats: Adult *vs* E18. FT. T9-10. *Gekkos japonicus*, FT. L10-11. 1& 4wks. IHC | GFAP | | | | Gu et al., 2015 |
| Zebra larvae. 2dpf Mechanical lesion. IHC | | | L-Plastin 4C4 | Tg olig2 | Ohnmacht et al., 2016 |
| Zebra larvae. FT. ISH, IHC. | | | Tg: *mpeg1*/4C4$^{+}$ *mpeg1*/4C4$^{-}$ | TgMpx | Tsarouchas et al., 2018 |
| Zebra larvae. FT. BrdU. | | | Tg mpeg | Tg: Mpx olig2 | Anguita-Salinas et al., 2019 |
| Neonate rats Zebra larvae. 3dpf. Dexamethasone FT.6, 24, 48, 72&120 hrs | | | | Tg *cloche* Nr3c1 GFAP | Nelson et al., 2019 |
| Neonate mice P2. Crush. ISH, IHC, RNAseq | GFAP | | CX3CR1 Csf1r$^{flox,}$ CD68, P2RY12 | | Li et al., 2020 |
| *Xenopuslaevis* Regenerative and non-regenerative stages. FT. EM, IHC | Vimentin BLBP GS | | | | Edwards-Faret et al., 2021 |
| Zebra larvae. 3dpf. Stab injury. 12hrs. FACS, RNAseq. IHC. | Tg GFAP | | | SOX2 NG2 | Zeng et al., 2021 |
| **Regenerate adults** | | | | | |
| *Amybstoma mexicanum* (axolotl) Juvenile and adult. FT. 1, 2, 3, 4, 5&6wks. EM. IHC. | GFAP | | | | O'Hara et al., 1992 |
| Zebrafish, FT. IHC, EM | | | 4C4 | | Becker and Becker, 2001 |
| Newts (Salamander), FT. 1&3 dys; 1, 2, 3, 6&9 wks. IHC, HC, EM. | GFAP | | | | Zukor et al., 2011 |
| Zebrafish, FT. BrdU, IHC | GFAP, vimentin | | | | Goldshmit et al., 2012 |
| Zebrafish, FT.IHC, ISH, tissue clearing, EdU | GFAP | | | Tg(olig2:eGFP | Tsata et al., 2020 |

*FACS, flow cytometry; hrs, hours; dys, days; wks, weeks; IHC, immunohistochemistry; T, thoracic; L, lumbar; FT, full transection; EM, electronic microscopy; ISH, in situ hybridisation; dpf, day post fertilisation; Tg, transgenic; E, embryonic.*

the proliferation of ependymal glia. Remarkably, glucocorticoids directly inhibited the formation of *trans*-lesion glial bridges and prevented axon regrowth and functional recovery through activation of Nr3c1 signalling (Nelson et al., 2019). There is still debate as to whether the glial bridge is prerequisite to axonal regrowth or whether it forms concomitantly with regenerating axons (reviewed in Cigliola et al., 2020). Additionally, in the larval zebrafish, Dbx1a-expressing cells that persist as radial glia and represent a pool of neurogenic progenitors can be activated in response to injury and differentiate into neurons (Briona and Dorsky, 2014). In early developmental stages, radial glial cells displaying a bipolar shape are abundantly present in both mammals and salamanders. Following SCI in salamander, radial glia cells ligate both rostral- and caudal-sectioned ends of the spinal cord before proliferating and differentiating into other glial cells (including astrocytes and oligodendrocytes) and into neurons (reviewed in Tazaki et al., 2017). Along this line, in zebrafish embryos, stress-responsive regenerating cells that are induced by SCI and that play an essential role in axonal regeneration have been identified and further characterised as

mostly composed of radial glia (Zeng et al., 2021). In contrast, upon SCI, radial glial cells of adult mammalians generate astrocytes. Instead of stretching to build an ependymal bridge, these astrocytes participate in the formation of a glial scar and prevent axonal regeneration. Further experiments to investigate the role of radial glia in neonatal mammals after SCI would certainly provide interesting findings to develop therapeutic strategies to favour axonal regeneration.

In both adult and larval zebrafish, the recruitment of immune cells has been observed after SCI. In adults, reactive microglia were observed at 2–3 days and at 14 days after spinal cord injury (Becker and Becker, 2001). In zebrafish larvae, recruitment of immune cells was observed as early as 2 h following the complete spinal cord section with a peak of neutrophils accumulation at the injury site (Tsarouchas et al., 2018). A slightly different time window of activation has also been reported after the complete spinal cord section, with a strong neutrophil recruitment until 12 h post injury at the lesion site, followed by its disappearance 24 h post injury (Anguita-Salinas et al., 2019). Macrophages and microglia were reported to be increased at 48 h post injury (Ohnmacht et al., 2016; Tsarouchas et al., 2018; Anguita-Salinas et al., 2019) and were detected next to the transection site at 7 and 42 days post lesion (Tsata et al., 2020). A brief, pro-inflammatory macrophage response, followed by an anti-inflammatory state, was observed that may underlie rapid myelin debris clearance (reviewed in Ghosh and Hui, 2018) and has led to the hypothesis of a similarity between peripheral nervous system injury in mammals and CNS injury in zebrafish (Ghosh and Hui, 2018). Moreover, following the complete section of the adult zebrafish spinal cord, oligodendrocyte precursor cells survived, proliferated, and replaced lost oligodendrocytes that reestablished myelination (Tsata et al., 2020).

Comparison between regenerative (pre-metamorphosis stages) and non-regenerative (during metamorphosis) responses in *Xenopus laevis* highlighted that, in the same species, no glial scar was observed in regenerative stages conversely to the non-regenerative stage where a transient glial scar-like structure was formed (Edwards-Faret et al., 2021). Similarly, spinal crush injury in neonatal mice up to postnatal Day 2 led to scar-free healing, allowing axonal regrowth through the lesion (Li et al., 2020). When SCI occurred at 2 days post-natal (regenerative response), amoeboid activated microglia first accumulated in the stumps 2–3 dpi and quickly returned to a ramified "resting" morphology by 2 weeks post-injury, when spinal cord regeneration was complete. When SCI occurred after 7 days post-natal (the non-regenerative stage), microglia remained highly activated for at least 2 weeks. Moreover, RNA sequencing at 2 days post-natal highlighted that this transient microglial activation permitted the formation of a temporary fibronectin bridge that ligated the two ends of the spinal cord and allowed axon regeneration (Li et al., 2020). Likewise, an intra-uterine complete section of the spinal cord at embryonic Day 19 in rats led to an absence of glial scar formation conversely to the same injury in adults (Fujimoto et al., 2006). Time course analysis showed an increase in the number of astrocytes and microglia/macrophages ($OX42^+$) in adults from 3 to 35 days after injury. Conversely, fetal injury led to a transient and rather limited increase in the number of astrocytes and microglia/macrophages at 3 and 3–7 days after injury, respectively. Additionally, leucocyte and macrophage infiltration were reported 3 and 7 days after SCI only in adults. In rodents, fetal and postnatal Day 2 injury thus led to a transient and limited activation of glial cells in the surrounding of the lesion contrariwise to SCI at the adult stage.

Comparative studies have been carried out in species, displaying high and low regenerative capabilities. One study characterised GFAP expression, following the complete spinal cord section in the adult gecko (*Gekko japonicum*), a reptile that displays a remarkable capacity for tail restoration, and adult rats. Concomitantly, astrocytic response was compared, following an *in vitro* scratch assay in adult geckos and rats and embryonic rats (Gu et al., 2015). In adult rats, GFAP expression was continuously increased from 1 to 4 weeks after SCI, while geckos displayed a transient expression peak at 1 week, followed by a decrease at 4 weeks. Moreover, astrocytes subjected to *in vitro* scratch wound displayed a higher GFAP expression and higher proliferative ability in adult rats than in embryonic rats and adult geckos. Lastly, it has been demonstrated, in zebrafish and rat, that the opposing regulation of the ependymal glial glucocorticoid receptor (Nr3c1), after complete spinal cord injury, participated in the differential responses between species (Nelson et al., 2019).

Taken together, these studies demonstrate that glial cells are present after spinal cord injury in non-mammal species and mammalian developmental stages that display spinal cord regeneration but respond differently as compared to the adult mammalian nervous system and seem to favour axon regeneration instead of hindering regrowth (**Figure 3**).

## CONCLUDING COMMENTS AND FUTURE DIRECTIONS

Responses of glial and immune cells following spinal cord injury display similarities and differences across species that are strongly correlated with functional recovery. The overall dynamics of the glial response to SCI in adult rodents and primates, which present extremely limited tissue repair and functional recovery, is comparable across species. Indeed, at acute and subacute stages, an early activation of microglia/macrophages precedes immune-cell infiltration and astrocyte activation (**Figures 1A,B**, **2A**). Both microglia/macrophages and astrocytes proliferate and migrate toward the lesion site. At later stages, astrocytes form an astroglial barrier that surrounds the lesion core. Microglia and NG2 cells also constitute the stabilised scar with tight interlacing between all cell populations (soma and processes) (**Figure 2B**). The core of the lesion is composed of a fibrotic scar with monocyte-derived macrophages, infiltrating immune cells and a few activated microglia (**Figure 2B**).

Strikingly, temporal dynamics and levels of activation differ across species. In rodents, rats exhibit an earlier and monophasic infiltration of immune cells conversely to mice that display a delayed biphasic neutrophil and T cell infiltration (**Figure 1A**). Interestingly, in man, the peak number of neutrophils bears more similarity to mice than rats (Mawhinney et al., 2012). Another major difference is that cystic cavities are observed only (or at

least predominantly) in rats and primates. Moreover, in primates, the astrocytic response is delayed and displays a lower level of activation as compared to rodents (**Figure 1B**).

The dynamics of the glial response to SCI is different in non-mammal species/mammalian developmental stages that exhibit high regenerative capacities and functional recovery. In particular, astroglial activation differs drastically, since astrocytes migrate toward the lesion site but form a bridge (**Figure 3**), and not a scar (**Figure 2**), which permits axonal regrowth through the lesion site. Interestingly, similar mechanisms are observed in embryonic and fetal mammals. The inflammatory response to SCI seems slightly different and leads to a faster myelin clearance that may resemble peripheral nervous system injury in adult mammals.

Recent findings have highlighted a sexual dimorphism in glial and immune cell responses present in pain signalling (for review, see Midavaine et al., 2021); thus, future investigations of the sex-dependent glial response and its crosstalk with immune cells, following SCI, are of great interest. The analysis of cellular dynamics following SCI in different contexts (species, age, sex, etc.) will help in the design of efficient therapeutic strategies used concomitantly, or sequentially, to improve recovery after CNS lesion.

## AUTHOR CONTRIBUTIONS

J-CP participated in the design of the review, analysed the data, and prepared the figures. YG contributed to the design of the review and the analysis of the data. FP conceptualised the design of the review, participated in the analysis and data interpretation, wrote the manuscript, and approved the final review. All authors contributed to the article and approved the submitted version.

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# Does Soluble TREM2 Protect Against Alzheimer's Disease?

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Triggering Receptor Expressed in Myeloid Cells 2 (TREM2) is a pattern recognition receptor on myeloid cells, and is upregulated on microglia surrounding amyloid plaques in Alzheimer's disease (AD). Rare, heterozygous mutations in TREM2 (e.g., R47H) increase AD risk several fold. TREM2 can be cleaved at the plasma membrane by metalloproteases to release the ectodomain as soluble TREM2 (sTREM2). Wild-type sTREM2 binds oligomeric amyloid beta (Aβ) and acts as an extracellular chaperone, blocking and reversing Aβ oligomerization and fibrillization, and preventing Aβ-induced neuronal loss *in vitro*. Whereas, R47H sTREM2 increases Aβ fibrillization and neurotoxicity. AD brains expressing R47H TREM2 have more fibrous plaques with more neuritic pathology around these plaques, consistent with R47H sTREM2 promoting Aβ fibrillization relative to WT sTREM2. Brain expression or injection of wild-type sTREM2 reduces pathology in amyloid models of AD in mice, indicating that wild-type sTREM2 is protective against amyloid pathology. Levels of sTREM2 in cerebrospinal fluid (CSF) fall prior to AD, rise in early AD, and fall again in late AD. People with higher sTREM2 levels in CSF progress more slowly into and through AD than do people with lower sTREM2 levels, suggesting that sTREM2 protects against AD. However, some of these experiments can be interpreted as full-length TREM2 protecting rather than sTREM2, and to distinguish between these two possibilities, we need more experiments testing whether sTREM2 itself protects in AD and AD models, and at what stage of disease. If sTREM2 is protective, then treatments could be designed to elevate sTREM2 in AD.

**Keywords: TREM2, sTREM2, microglia, Alzheimer's disease, amyloid beta, neuroinflammation, neurodegeneration, neuroprotection**

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## INTRODUCTION

### TREM2

Triggering Receptor Expressed in Myeloid Cells 2 (TREM2) is a pattern recognition receptor found on the plasma membrane of myeloid cells. When activated by ligands, such as phospholipids, lipoproteins, and amyloid beta peptide (Aβ), TREM2 induces an innate immune response, which includes phagocytosis, chemotaxis, and transcriptional changes (Keren-Shaul et al., 2017; Deczkowska et al., 2020; Kulkarni et al., 2021). TREM2 signaling is mainly *via* binding DAP12 (DNAX-activating protein of 12 kDa), which activates Syk tyrosine kinase (Deczkowska et al., 2020). Within the brain, TREM2 is almost uniquely expressed by microglia, and is upregulated on microglia around amyloid plaques in AD (Giraldo et al., 2013; Yuan et al., 2016; Brendel et al., 2017). Rare, heterozygous mutations of TREM2 are known to affect AD risk, including the R47H

mutation, which increases AD risk several fold (Guerreiro et al., 2012; Giraldo et al., 2013; Jonsson et al., 2013; Kulkarni et al., 2021). These mutations are thought to increase AD risk by reducing the protective roles of microglial TREM2, in particular by reducing microglial phagocytosis of amyloid plaques (Condello et al., 2015; Yuan et al., 2016).

## sTREM2

TREM2 is a single-pass type I transmembrane protein with a small C-terminal on the cytosolic side of the plasma membrane, and an N-terminal ectodomain that includes the ligand binding site (Zhong and Chen, 2019; Yang et al., 2020). However, the ectodomain of TREM2 is shed from cells expressing full-length TREM2 into the extracellular medium, and is then known as soluble TREM2 (sTREM2) (Piccio et al., 2008; Wunderlich et al., 2013). The turnover of full-length TREM2 on macrophages is very rapid with a half-life of $<1$ h, because of constitutive cleavage of full-length TREM2 and shedding of sTREM2 (Thornton et al., 2017). The proteases responsible for shedding sTREM2 include A Disintegrin And Metalloproteases 10 and 17 (ADAM10 and ADAM17), and this cleavage occurs at the H157-S158 peptide bond (Schlepckow et al., 2017; Thornton et al., 2017). ADAM10 and 17 appear to be responsible for sTREM2 release induced by lipopolysaccharide (LPS), whereas the protease meprin β constitutively cleaves TREM2 (predominately at the R136-D137 peptide bond) to release sTREM2 from macrophages (Berner et al., 2020). However, it is unclear whether meprin β can generate sTREM2 in microglia. After shedding of sTREM2, the remaining part of TREM2 may be cleaved within the membrane by γ secretase (Wunderlich et al., 2013). The very rapid and inducible turnover of TREM2 to generate sTREM2 suggests either that (i) TREM2 levels need to be regulated very rapidly, or (ii) that sTREM2 has a function, and full-length TREM2 is a precursor of this functional sTREM2.

## Regulation of sTREM2 Shedding

Conditions that increase or decrease sTREM2 shedding from full-length TREM2 are not clear, but LPS or IL-1β can induce sTREM2 release from primary mouse microglia (Zhong et al., 2019). Also, oligomeric Aβ, which can bind both full-length TREM2 and sTREM2, induced shedding of sTREM2 for TREM2-overexpressing cells (Vilalta et al., 2021), suggesting that sTREM2 shedding may be induced prior to and during AD as a result of Aβ oligomerization. CSF sTREM2 levels increase in amyloid mouse models and correlate with microglial activation (Brendel et al., 2017). Viral infection of the lungs can increase sTREM2 levels post-infection, due to IL-13 or IL-4 induced sTREM2 shedding (Wu et al., 2015). And HIV viral infection of the brain increases CSF levels of sTREM2 (Gisslén et al., 2018). sTREM2 levels in CSF are thought to be a biomarker of microglial activation, although there is limited evidence for this *in vivo* (Bekris et al., 2018; Rauchmann et al., 2020; Pascoal et al., 2021), and sTREM2 may itself cause microglial activation (see below). CSF sTREM2 levels rise with age in humans from about 2 ng/ml at 43 years to 6 ng/ml at 80 years of age (Henjum et al., 2016).

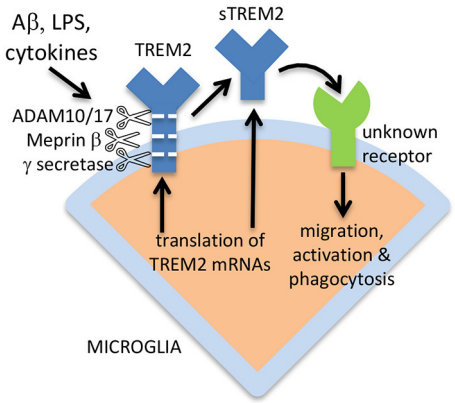


**FIGURE 1 |** Release of sTREM2 from microglia, and activation of microglia by sTREM2. sTREM2 may be generated by ADAM10/17 or meprin β proteolysis of full-length TREM2, or from expression of an isoform lacking the transmembrane domain. γ secretase can cleave the remains of TREM2 within the membrane to degrade it. Released sTREM2 can chemoattract and activate microglia *via* unknown receptors.

## Alternative Forms of sTREM2

*TREM2* can be expressed *via* alternative splicing as a soluble isoform, lacking the transmembrane form, and this alternative sTREM2 may constitute 25% of total sTREM2 in the brain (Ma et al., 2016; Del-Aguila et al., 2019). This again suggests that sTREM2 has a function, rather than being simply a degradation product of full-length TREM2. The sTREM2 generated by alternative splicing would be 219 amino acids residues long, the sTREM2 generated by ADAM10 or 17 would be 157 amino acids residues long, and the sTREM2 generated by meprin β would be 136 amino acids residues long (plus shorter forms) (Berner et al., 2020), although removal of the signal peptide would shorten all these sTREM2 forms by 18 amino acid residues. The ectodomain of TREM2 and sTREM2 is highly glycosylated at Asn20 and Asn79, so the apparent molecular weight of full-length TREM2 on electrophoresis gels is about 50 kDa when fully glycosylated, and about 25 kDa when deglycosylated (Ma et al., 2016). The apparent molecular weight of sTREM2 in CSF is 30–35 kDa (Ma et al., 2016), implying that almost half the apparent weight of sTREM2 is sugars, and that different glycosylation states coexist. The alternative mechanisms of sTREM2 generation are illustrated in **Figure 1**.

## sTREM2 Degradation

Processes responsible for degradation and clearance of extracellular sTREM2 are unclear, although it has been found that macrophages readily take up sTREM2 (Wu et al., 2015), and sTREM2 injected into mouse brain is cleared from the brain within 3 days (Zhong et al., 2019). Membrane-attached meprin β generates sTREM2 constitutively, but inflammation-induced ADAM10/17 releases soluble meprin β, which can rapidly degrade sTREM2 (Berner et al., 2020). However, it is unclear whether meprin β contributes to sTREM2 production or degradation in the brain.

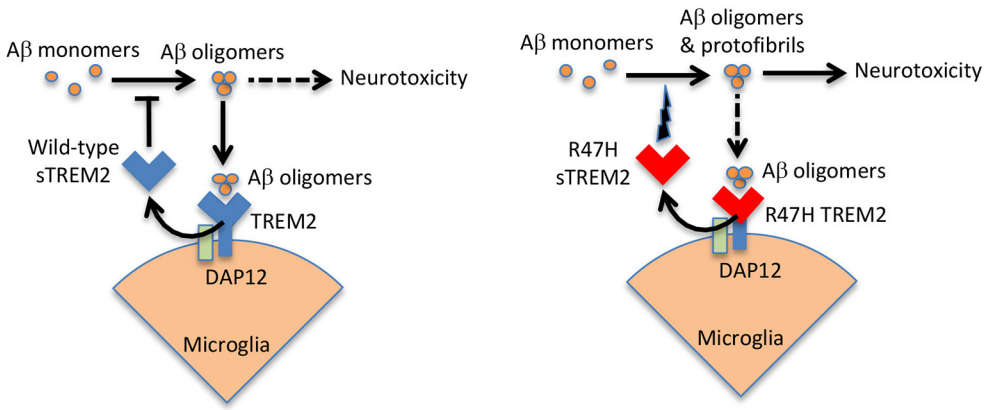


**FIGURE 2 |** Wild-type sTREM2 blocks Aβ pathology, but R47H TREM2 does the opposite. Aβ oligomers bind to TREM2 and induce shedding of sTREM2. Wild-type sTREM2 blocks Aβ oligomerization, fibrillization and neurotoxicity. R47H sTREM2 increases Aβ oligomerization, fibrillization and neurotoxicity. Thus, wild-type sTREM2 may protect against amyloid pathology, while R47H TREM2 exacerbates amyloid pathology. This might help explain why a single copy of the R47H TREM2 gene increases AD risk several fold.

# ACTIONS OF sTREM2

## sTREM2 Activates Microglia

sTREM2 treatment of macrophages induced phosphorylation of ERK1/2 (extracellular signal-regulated kinases 1 and 2) and inhibited apoptosis (Wu et al., 2015). Similarly, sTREM2 treatment of microglia in culture promoted survival by inhibiting apoptosis, apparently *via* activation of Akt (Zhong et al., 2017). In addition, sTREM2 induced inflammatory activation of cultured microglia *via* nuclear factor-κB, resulting in morphological activation and release of pro-inflammatory cytokines (Zhong et al., 2017). sTREM2 also stimulated migration and phagocytosis by primary microglia in culture (Zhong et al., 2019). Injection of sTREM2 into the brains of mice expressing the amyloid precursor protein (APP) induced activation and proliferation of microglia, plus increased expression of pro-inflammatory cytokines, and increased microglial phagocytosis of Aβ (Zhong et al., 2019). Injection of sTREM2 into the brains of healthy mice also induced expression of pro-inflammatory cytokines (Fassler et al., 2021). A fragment of sTREM2 (amino acids 51–81) was sufficient to activate microglia (Sheng et al., 2021). Thus, sTREM2 activates microglia, although the mechanism of this activation is unclear.

## sTREM2 Blocks Aβ Aggregation and Neurotoxicity

sTREM2 is known to bind oligomeric Aβ, with minimal binding to monomeric or fibrillar Aβ (Lessard et al., 2018; Zhao et al., 2018; Zhong et al., 2018; Vilalta et al., 2021). Subsequently, it was found that sTREM2 blocked Aβ oligomerisation and fibrillization at a molar ratio of 1 sTREM2 to 100 Aβ (Kober et al., 2021; Vilalta et al., 2021), and at higher molar ratios sTREM2 disaggregated Aβ oligomers and fibrils (Vilalta et al., 2021). Wild-type sTREM2 also inhibited Aβ-induced permeabilization of artificial membranes, and inhibited Aβ-induced neuronal loss in glial-neuronal cultures (Vilalta et al., 2021). These results suggest that wild-type sTREM2 may act as extracellular chaperone for Aβ, blocking its folding into aggregatable forms and refolding aggregates into soluble forms, thereby inhibiting the neurotoxicity of Aβ. In contrast, R47H sTREM2 bound less to Aβ oligomers, but increased Aβ aggregation into protofibrils, and increased Aβ-induced neuronal loss in glial-neuronal cultures (Vilalta et al., 2021). Thus, R47H sTREM2 may not only loose a neuroprotective function, but also gain a neurotoxic function in the presence of Aβ, probably by folding Aβ into more toxic forms (see **Figure 2**).

## sTREM2 Protects Against Amyloid Pathology in Mice

sTREM2 injection into the brains of mice expressing APP reduced amyloid plaque load (Zhong et al., 2019). Furthermore, viral expression of sTREM2 in the APP-expressing mice, reduced plaque load and reversed deficits of spatial memory and long-term potentiation (Zhong et al., 2019). Thus, sTREM2 is protective against amyloid pathology in mice, and this might be by sTREM2 affecting Aβ aggregation and/or sTREM2 activating microglia to phagocytose plaques. A fragment of sTREM2 (amino acids 51–81) was sufficient to activate microglia, but not to bind Aβ and reduce amyloid pathology *in vivo*; whereas a 41–81 fragment of sTREM2 bound Aβ and reduced amyloid pathology *in vivo* better than full-length sTREM2 (Sheng et al., 2021). This suggests that sTREM2 protects against amyloid pathology mainly by binding Aβ.

TREM2 knockout mice, crossed with APP-expressing mice, have more fibrous and less compact plaques (Condello et al., 2015; Wang et al., 2016; Yuan et al., 2016; Song et al., 2018), and while this has been attributed to less microglial phagocytosis of the plaques because of less full-length TREM2, the result might alternatively be due to sTREM2 blocking Aβ aggregation and/or sTREM2 activating microglia to phagocytose plaques. TREM2 knockout mice have increased Aβ seeding (Parhizkar et al., 2019), which again could be explained by reduced microglial phagocytosis of Aβ seeds mediated by full-length TREM2, or

reduced blocking of Aβ aggregation by sTREM2. In 5xFAD mice expressing wild-type human TREM2, sTREM2 was found bound to the amyloid plaques (Song et al., 2018), consistent with sTREM2 having a role in regulating plaques. Note that the ability of sTREM2 to block Aβ aggregation and to disaggregate Aβ, might be shared with full-length TREM2, as they both bind Aβ oligomers (Vilalta et al., 2021), but this has not been tested. Humans (and mice) with heterozygous R47H TREM2 have more fibrous plaques with more neuritic pathology (Yuan et al., 2016), which again might be explained by either R47H sTREM2 promoting Aβ fibrillation, or by reduced microglial phagocytosis of plaques.

## EVIDENCE THAT sTREM2 IS PROTECTIVE AGAINST AD IN HUMANS

CSF levels of sTREM2 fall significantly in early pre-symptomatic stages prior to AD diagnosis (when amyloid is aggregating), but rise during mild cognitive impairment (MCI) and AD (when tau is aggregating), and fall again during the dementia stages of AD (Heslegrave et al., 2016; Piccio et al., 2016; Suárez-Calvet et al., 2016, 2019; Bekris et al., 2018; Liu et al., 2018; Nordengen et al., 2019; Rauchmann et al., 2019; Ma et al., 2020). People with higher CSF levels of sTREM2 progress more slowly through MCI and AD, in terms of memory loss, clinical score and brain atrophy (Ewers et al., 2019, 2020; Edwin et al., 2020; Franzmeier et al., 2020). And this apparent protective effect of sTREM2 correlated with reduced amyloid and Tau aggregation measured by PET (Ewers et al., 2020), consistent with sTREM2 reducing amyloid aggregation and pathology.

However, these apparent protective effect of high sTREM2 has been attributed to full-length TREM2, rather than sTREM2, on the untested assumption that high sTREM2 levels indicates high TREM2 levels, as a result of constant shedding. However, if elevated sTREM2 results from elevated shedding, which is for example induced by oligomeric Aβ (Vilalta et al., 2021), then this will reduce full-length TREM2. Thus, elevated levels of sTREM2 do not necessarily indicate that levels of full-length TREM2 are elevated, and the apparent protective effect of sTREM2 against AD may be more simply explained by sTREM2 itself being protective.

GWAS studies of gene variants that affect the CSF levels of sTREM2 identified the membrane-spanning 4-domains superfamily A (*MS4A*) gene cluster as key determinants of sTREM2 levels in CSF (Piccio et al., 2016; Deming et al., 2019; Hou et al., 2019). This gene region had previously been linked to AD risk (Naj et al., 2011). For example, rs1582763 increased brain expression of *MS4A4A* and *MS4A6A* genes, increased sTREM2 levels in CSF, reduced AD risk and increased age of AD diagnosis. While rs6591561 resulted in a loss-of-function *MS4A4A*, reduced CSF sTREM2 levels, increased AD risk and reduced age at AD onset (Deming et al., 2019). MS4A4A and TREM2 were found to colocalize at the plasma membrane, and overexpression of *MS4A4A* increased sTREM2 levels, whilst silencing of *MS4A4A* reduced sTREM2 levels (Deming et al., 2019). This suggests that MS4A4A may affect AD risk by promoting sTREM2 shedding, and if so, indicating that sTREM2, rather than full-length TREM2 is protective against AD. However, further work is required to establish whether MS4A4A directly affects sTREM2 shedding.

## EVIDENCE AGAINST THE HYPOTHESIS THAT sTREM2 PROTECTS

One piece of evidence potentially contradicting a protective role of sTREM2 in AD, is that the H157Y mutation of TREM2 expressed in cells significantly increased sTREM2 shedding relative to wild-type TREM2, resulting in increased sTREM2 and decreased full-length TREM2, but is associated with increased AD risk (Schlepckow et al., 2017; Thornton et al., 2017). This suggests that the increased AD risk associated with the H157Y mutation is due to decreased full-length TREM2 or increased sTREM2, contradicting the hypothesis that sTREM2 is protective against AD. However, the H157Y mutation only increased shedding by about 50%, and this was from HEK293 cells (Schlepckow et al., 2017; Thornton et al., 2017), so it may be difficult to extrapolate to sTREM2 levels in human brains. Additionally, the H157Y mutation would constitute the C-terminal of sTREM2, and might affect its properties, such as its interactions with Aβ. Thus, it would be important to determine whether this mutation does indeed increase CSF levels of sTREM2 in humans, and whether H157Y sTREM2 has the same protective properties as wild-type sTREM2.

Other evidence potentially contradicting the hypothesis that sTREM2 protects against AD is the finding of Schlepckow et al. (2020) that an antibody binding to the ADAM cleavage site of TREM2 prevented sTREM2 release, but reduced plaques load in an amyloid mouse model. However, the antibody used directly activated TREM2 signaling, so the reduced plaque load may result from this signaling (Schlepckow et al., 2020). Additionally, the compaction of these plaques, neuritic pathology and memory loss were not tested in this model.

## DISCUSSION

### Is TREM2 or sTREM2 Protective in Alzheimer's Disease?

It appears that either TREM2 or sTREM2 are protective in Alzheimer's disease, but which? TREM2 is thought to be protective by (i) recruiting and activating microglia into a protective state around amyloid plaques, and (ii) compacting amyloid plaques by phagocytosis of Aβ, preventing the plaques inducing neuritic pathology (Condello et al., 2015; Yuan et al., 2016; Keren-Shaul et al., 2017). Whereas, sTREM2 is thought to be protective by: (i) stimulating microglial recruitment, activation and phagocytosis of Aβ, and/or (ii) blocking and reversing Aβ aggregation, preventing neurotoxicity (Zhong et al., 2019; Vilalta et al., 2021). Thus, the putative protective effects of TREM2 and sTREM2 are complimentary rather than antagonistic, and potentially both may be protective against Alzheimer's disease. However, it is still important to verify that TREM2 and/or sTREM2 are in fact protective.

## Key Experiments to Determine Whether sTREM2 Is Protective Against AD

Some of evidence indicating that sTREM2 is protective against AD, may alternatively be interpreted as full-length TREM2 is protective. Thus, there is a need for experiments that distinguish between these possibilities, or directly show that sTREM2 is protective. The most direct way to show that is to add or express sTREM2 independent of full-length TREM2 and test whether this is protective in AD models. This has been done for a mouse amyloid model and found to be protective (Zhong et al., 2019), but this was relatively acute model, and it would be important to test this in other models, particularly more chronic and AD-relevant models. Within such models, it would be important to test whether sTREM2 can block Aβ aggregation, or disaggregate preformed plaques or oligomers. It would also be useful to know whether Aβ oligomers in AD CSF are significantly bound to sTREM2, and whether physiological levels of sTREM2 can disaggregate Aβ aggregation in CSF. Further, it would be worth knowing whether the different types of sTREM2 behave differently, including sTREM2 generated by ADAM and meprin β, or by alternative splicing, or H157Y and R62H sTREM2.

## Potential Treatment Strategies

Current strategies targeting TREM2 in AD have focused on agonistic antibodies to activate TREM2 with the aim of increasing microglial phagocytosis of amyloid plaques (Wang et al., 2020; Fassler et al., 2021). These antibodies will also bind sTREM2 and potentially block the protective effects of sTREM2 (Fassler et al., 2021). If sTREM2 is indeed more protective against AD than full-length TREM2, then antibodies that increased sTREM2 shedding might be beneficial, or other treatments designed to activate sTREM2 shedding e.g., by activating ADAM10 and ADAM17. Blocking sTREM2 degradation (e.g., by inhibiting meprin β) might increase sTREM2 levels without decreasing full-length TREM2. sTREM2 and sTREM2 fragments injected into the brain were protective in mouse models of AD (Zhong et al., 2019; Sheng et al., 2021), but may be difficult to deliver practically in humans. However, viral vectors expressing sTREM2 in the brain were protective in these mouse models of AD, and thus might be protective in humans with AD (Zhong et al., 2019).

## AUTHOR CONTRIBUTIONS

GB wrote the article. PG-H reviewed and adjusted the article. Both authors were responsible for its content.

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OPINION
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# Contribution of "Genuine Microglia" to Alzheimer's Disease Pathology

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## HETEROGENEITY OF MYELOID CELLS IN THE BRAIN

In the late 1980's, McGeer et al. observed that major histocompatibility complex (MHC) class II-immunopositive cells with an amoeboid shape were concentrated in the vicinity or center of amyloid plaques in postmortem brains of patients with Alzheimer's disease (AD) (McGeer et al., 1987; Itagaki et al., 1988). This historical neuropathological finding was interpreted to mean that microglia were activated in lesioned areas, and led to the neuroinflammation hypothesis suggesting that activated microglia significantly contribute to AD pathogenesis. Recently, genome-wide association studies have identified variants of the myeloid cell genes triggering receptor expressed on myeloid cell 2 (*TREM2*) (Jonsson et al., 2013), complement receptor 1 (Lambert et al., 2009), and *CD33* (Hollingworth et al., 2011) as novel AD risk genes, sparking renewed interest in microglia from the aspect of genetics (Hashioka et al., 2020).

However, microglia are not the only myeloid cells expressing MHC class II in the brain. Besides parenchymal microglia, the intact brain hosts non-parenchymal specialized myeloid cells such as perivascular, meningeal, and choroid-plexus macrophages, which are referred to as CNS-associated macrophages (CAMs) (Kierdorf et al., 2019). In addition, circulating monocytes are believed to infiltrate the brain and differentiate into macrophages under pathological conditions (Martin et al., 2017). Human microglia, CAMs, and infiltrating monocytes/macrophages express MHC class II as well as certain pan-macrophage markers, such as Iba1 (ionized calcium-binding adapter molecule 1), CD11b, and the fractalkine receptor CX3CR1 (Prinz et al., 2017; Bottcher et al., 2019; Kierdorf et al., 2019). Identification of these brain mononuclear phagocytes was based on their location, morphology, and a small set of surface markers. Such mononuclear cells, therefore, used to be mingled in conventional bulk analyses.

Accumulating evidence indicates that ontogeny and longevity are prominent properties shared by microglia and CAMs, but not by infiltrating monocytes/macrophages (Prinz et al., 2017; Kierdorf et al., 2019). Microglia and CAMs, excluding choroid-plexus macrophages, arise solely from erythromyeloid progenitor cells in the extraembryonic yolk sac and possess extreme longevity and self-renewal potential, without replacement by circulating monocytes (Ginhoux et al., 2010; Goldmann et al., 2016). As an exception for CAMs, choroid-plexus macrophages show mixed ontogeny and a substantial contribution from circulating monocytes (Goldmann et al., 2016). Immigrating monocytes/macrophages, which express a unique monocytic marker Ly6C in mice (Geissmann et al., 2003), originate from the myeloid progenitor lineage in the bone marrow and exhibit a short life with high turnover (Prinz et al., 2017).

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## SPECIFIC MARKERS SEGREGATING MYELOID CELLS IN THE BRAIN

Although microglia and most CAM populations share the same prenatal origin (i.e., yolk sac), recent studies with single-cell RNA sequencing have clearly segregated the transcriptome signature specific for microglia from that for CAMs. Specifically, *TMEM119* (transmembrane protein 119) and *P2RY12* (P2Y purinergic receptor 12) have been identified as core genes specific to microglia in humans (Masuda et al., 2019; Sankowski et al., 2019), while *Mrc1* (mannose receptor 1, also called CD206) and *Pf4* (platelet factor 4) have been considered as core genes specific to CAMs in mice (Zeisel et al., 2015; Jordao et al., 2019) (**Table 1**). Indeed, selective microglial expression of TMEM119 and P2RY12 has been confirmed at the protein level in humans. Immunohistochemical analysis of postmortem human brains showed that parenchymal Iba1-immunopositive cells expressed TMEM119 (Bennett et al., 2016; Satoh et al., 2016). On the other hand, the $Iba1^+$ or $CD68^+$ cells, which were presumed to be infiltrating monocytes, did not express TMEM119 in active demyelinating lesions of multiple sclerosis (MS) or necrotic lesions of cerebral infarction (Satoh et al., 2016). P2RY12 immunoreactivity was also observed in parenchymal $Iba1^+$ ramified cells that were supposed to be microglia, but not in $CD14^+$ and $CD16^+$ cells in blood vessels and in the meninges. These cells most likely correspond to peripherally derived monocytes and meningeal macrophages, respectively (Mildner et al., 2017). Postmortem brain study demonstrated that such microglial expression of P2RY12 was decreased in the brains of AD patients and those of MS patients (Mildner et al., 2017). More critically, another advanced single-cell technology, namely cytometry by time of flight (CyTOF), showed that TMEM119 and P2RY12 were expressed on microglia isolated from postmortem human brains and were absent from myeloid cells in human blood and cerebrospinal fluid (Bottcher et al., 2019). Accordingly, in humans, it is tempting to regard TMEM119 and P2RY12 as the most reliable markers that can identify "genuine microglia" in humans, while *Siglech* (sialic-acid-binding immunoglobulin-like lectin-h) (Bedard et al., 2007; Konishi et al., 2017) and *Hexb* (beta-hexosaminidase subunit beta) (Masuda et al., 2020; Jia et al., 2021) are also considered as microglia-enriched genes (**Table 1**).

## PATHOLOGICAL ROLES OF HETEROGENOUS BRAIN MYELOID CELLS IN ALZHEIMER'S DISEASE

The heterogeneous nature of brain myeloid cells raises the question as to whether or not there are differences in pathological roles between microglia, CAMs, and infiltering monocytes in AD. Do "genuine microglia" play specific roles in AD pathogenesis? The answer seems to be yes, since it was demonstrated that macrophages transplanted from the bone marrow in donors could adopt some features of endogenous microglia, but such macrophages were not able to fully recapitulate all microglial properties, such as increased expression of microglial identity genes, even after prolonged residence in the recipient brain (Bennett et al., 2018).

In addition, several studies have reported conflicting results concerning the contribution of microglia, CAMs, and recruited monocytes to AD pathology. For instance, infusion of wildtype monocytes derived from the bone marrow to the peripheral blood of AD transgenic mice led to spontaneous migration of monocytes to amyloid lesions in the absence of irradiation, genetic manipulation, or chemotherapy. Such treated mice showed a decrease in cerebral Aβ levels, which seemed to be associated with monocytic phagocytosis, and ameliorated cognitive deficits (Koronyo et al., 2015). On the other hand, a study using AD transgenic mice demonstrated that peripheral monocytes distinguished from microglia by parabiosis were not significantly recruited to Aβ plaques, whereas resident microglia gathered to surround Aβ plaques (Wang et al., 2016). This controversy may stem from limitations of conventional analytical methods, such as immunohistochemistry and flow cytometry, employed in the aforementioned studies to characterize myeloid cells. These methods can only probe a few preselected proteins as cell surface markers.

But now, can "genuine microglia" reliably be typified by the microglia-specific markers TMEM119 and P2RY12, which were established by the latest single-cell profiling technologies? There seems to be no clear answer, since expression levels of TMEM119 and P2RY12 depend on the microglial activation status. Microglia highly express microglia core genes *TMEM119* and *P2RY12* in the homeostatic state. After loss of their homeostatic phenotype,

**TABLE 1** | Markers of myeloid cells in the brain.

| Marker | Target Cell Type | Origin of Target Cell | References |
|---|---|---|---|
| TMEM119 | Homeostatic microglia | Yolk sac | Bennett et al., 2016; Bottcher et al., 2019; Masuda et al., 2019 |
| P2RY12 | Homeostatic microglia | Yolk sac | Mildner et al., 2017; Bottcher et al., 2019; Sankowski et al., 2019 |
| Siglech | Homeostatic microglia | Yolk sac | Bedard et al., 2007; Konishi et al., 2017 |
| Hexb | Microglia during homeostasis and disease | Yolk sac | Masuda et al., 2020; Jia et al., 2021 |
| Clec7a | DAM/MGnD | Yolk sac | Keren-Shaul et al., 2017; Krasemann et al., 2017 |
| Mrc1 (CD206) | CAMs (except for some choroid-plexus macrophages) | Yolk sac | Zeisel et al., 2015; Jordao et al., 2019 |
| Pf4 | CAMs (except for some choroid-plexus macrophages) | Yolk sac | Jordao et al., 2019 |
| Ly6C | Infiltrating monocytes/macrophages | Bone marrow | Geissmann et al., 2003 |

*DAM, disease-associated microglia; MGnD, microglial neurodegenerative phenotype; CAMs, CNS-associated macrophages.*

however, microglia suppress the expression of *TMEM119* and *P2RY12* in an activated state referred to as disease-associated microglia (DAM) (Keren-Shaul et al., 2017) or microglial neurodegenerative phenotype (MGnD) (Krasemann et al., 2017). Such DAM/MGnD microglia are closely associated with Aβ plaques and possess pro-inflammatory signatures (Keren-Shaul et al., 2017; Krasemann et al., 2017). Based on these findings, TMEM119 and P2RY12 should be regarded as homeostatic microglia molecules. Therefore, immunohistochemical analysis appears to be difficult to distinguish between the absence of microglia themselves and the presence of microglia in the DAM/MGnD activation state in lesions showing poverty of sole TMEM119 or P2RY12 immunoreactivity.

A recent study using immunocytochemical electron microscopy has uncovered a new microglial phenotype called dark microglia, which are associated with amyloid plaques in APP/PS1 mice (Bisht et al., 2016). Dark microglia display condensed, electron-dense cytoplasm and nucleoplasm, a characteristic giving them a "dark" appearance. They also show a downregulated expression of the homeostatic marker P2RY12 (Bisht et al., 2016). Therefore, gene expression profiles and biological characteristics of dark microglia may be similar to those of the DAM/MGnD microglia.

## NOVEL GENETIC APPROACHES TO DEFINE PATHOLOGICAL ROLES OF MICROGLIA

Advances in genetic manipulation have established certain transgenic or knock-in mouse lines that genetically target microglia. Such mouse lines have been shown to monitor precisely and manipulate microglia regardless of their activation state. In addition, CyTOF combined with fate mapping on APP/PS1 mice has detected a subset of microglia associated with AD-prone neurodegeneration (Mrdjen et al., 2018).

The mice with the recombinase *CreERT2* inserted into the locus *TMEM119* and the *TMEM119-tdTomato* knock-in mice have shown clear discrimination of microglia from CAMs, even though non-myeloid brain cells, such as endothelial cells and fibroblasts, and some choroid-plexus macrophages can also be targeted (Kaiser and Feng, 2019; Ruan et al., 2020). In the brains of *Tmem119-tdTomato* reporter mice that were treated by laser ablation, tdTomato-positive microglia, which were presumably activated, entered the site of injury and dramatically changed their process length without losing the TMEM119-tdTomato signal (Ruan et al., 2020). It is yet to be clarified why TMEM119 expression was preserved even in microglia activated in lesioned areas. Also, *P2RY12-CreERT2* knock-in mice were able to specifically label microglia, as shown by *P2ry12-CreERT2*; *Rosa26*$^{Rosa26Ai14}$ reporter mice that expressed TdTomato upon Cre-dependent recombination with minor effects on CAMs (McKinsey et al., 2020). When *P2RY12-CreERT2*; *Rosa26*$^{Rosa26Ai14}$ mice underwent middle cerebral artery occlusion, tdTomato$^{+}$ microglia exhibited reduced immunoreactivity for P2RY12 and TMEM119 in the ischemic core and penumbra (McKinsey et al., 2020).

To define the pathological roles of "genuine microglia" in AD, it is tempting to apply these mouse lines targeting microglia genetically to experimental AD models. For instance, intrahippocampal injection of Aβ into such mice seems to be technically feasible and to facilitate the transcriptional and functional analysis of microglia in response to Aβ. It should be noted that there is no AD animal model sufficient to reflect all aspects of AD pathology (Drummond and Wisniewski, 2017). In fact, intrahippocampal Aβ injection appears to exaggerate inflammatory responses and to represent an acute brain insult (McLarnon and Ryu, 2008), even though chronic inflammation is considered a critical aspect of AD pathology. Nevertheless, further studies along this line are warranted to elucidate the potential of microglia as key therapeutic targets in AD.

Tracing DAM/MGnD microglia in the brain of AD animal models could also help to reveal the pathological roles of microglia. DAM/MGnD microglia have been identified by high expression of the DAM/MGnD marker Clec7a combined with low expression of the homeostatic microglia marker P2RY12 (Keren-Shaul et al., 2017; Krasemann et al., 2017). While clarifying microglial ontogeny in humans could help to clarify the pathological roles of human microglia, there is no approach to address this issue directly for obvious reasons. However, using single-cell transcriptional profiling approaches, a recent study on macrophages from aborted fetuses implies that human microglia are derived largely from yolk sac progenitors (Bian et al., 2020).

## AUTHOR CONTRIBUTIONS

SH wrote the manuscript. All authors discussed, edited the manuscript, read, and approved the final manuscript.

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