CELL-CELL INTERACTIONS CONTROLLING NEURONAL FUNCTIONALITY IN HEALTH AND DISEASE

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EDITORIAL
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Editorial: Cell-Cell Interactions Controlling Neuronal Functionality in Health and Disease

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Editorial on the Research Topic

Cell-Cell Interactions Controlling Neuronal Functionality in Health and Disease

In the central nervous system (CNS), several cell types interact with each other to promote and protect the homeostatic functions of neuronal cells. Neurons are equipped with the unique capabilities of decoding the signals associated with sensory stimuli and controlling body movement and cognition. The activity of neurons is regulated at multiple levels, and depends on their interaction with CNS-resident microglia, astrocytes, and oligodendrocytes, as well as on the flux of nutrients through the blood-brain barrier (BBB). However, detrimental events may compromise the CNS milieu and cytoarchitecture, and affect essential neuronal activities, ultimately causing neuronal death, loss of motor functions, cognitive decline, and systemic body failure. In this Research Topic, we provide a collection of 11 manuscripts highlighting how peripheral and CNS-local events can cause neuronal dysfunctions.

Development of the CNS is a finely regulated process, and any alteration in it may lead to dramatic consequences in newborn children, who thus may experience neurodevelopmental disorders (ND). ND can have a genetic basis, but environmental factors have also been linked to their appearance (Homberg et al., 2016). In particular, alcohol consumption by pregnant women is a well-known risk factor for ND in children. Licheri and Brigman discuss the effect of prenatal alcohol exposure on the expression of cell adhesion molecules in the developing brain, which may contribute to defective CNS development, impact neuronal activity, and lead to the onset of ND.

The adult CNS is "isolated" from the surrounding tissue by the presence of a vascular barrier (in the brain, the BBB) that tightly controls nutrient supply and limits the trafficking of blood-borne cells into the CNS parenchyma. When this barrier is altered, the homeostasis of the CNS may be lost, and neuronal cells can suffer or die (Sweeney et al., 2019). Hudson and Campbell describe how endothelial tight junctions (TJs) regulate BBB and inner blood-retinal barrier (iBRB) functionality in the brain and in the retina, respectively, and how altered expression or functionality of such TJs have been implicated in neurological diseases such as multiple sclerosis, Alzheimer's disease, and stroke.

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The CNS contains local phagocytic immune cells, namely microglia, which control neuronal activity in homeostatic conditions and protect the CNS from potentially pathogenic events such as infections and accumulation of toxic products. However, dysregulated microglia activity may also cause neuroinflammation and neurodegeneration (Prinz et al., 2021). Carrier et al. discuss how the cross-talk between microglia and neurons is altered as a consequence of chronic psychological stress, which can accelerate cellular aging, cause neuronal dysfunction, and promote the development of depressive disorder and cognitive decline. Noteworthy, microglia interact not only with neurons, but also with other glial cells, including astrocytes and oligodendrocytes, regulating their functional properties and indirectly impacting neuronal activity. In their review, Zhao et al. describe how the microglia and macroglia (astrocytes and Müller cells) interact in the eye to support optic axon activity, and how this process is affected in glaucoma pathogenesis.

The CNS has long been considered an "immuno-privileged" site, but this view has been challenged in the last years. We now know that the CNS hosts several resident immune cell populations other than microglia, and that peripheral immune cells infiltrate the CNS upon inflammation and leakage of the vascular barrier. Once activated in the CNS, local or infiltrating immune cells may promote and sustain CNS inflammation, causing neuronal death by direct cell-cell contact or through the release of inflammatory mediators such as cytokines (Ramaglia et al., 2021). In their original work, Donninelli et al. analyzed the expression of 27 immune soluble factors in the cerebrospinal fluid of patients with primary progressive or secondary progressive multiple sclerosis. The authors didn't identify major differences between the two disease forms, but were able to stratify and correlate the levels of some cytokines and chemokines according to active or inactive magnetic resonance imaging. In another manuscript, Mai et al. summarize previous studies suggesting a significant effect of soluble mediators released by CNS-infiltrating mast cells on neuronal activity, and their ability to induce inflammation-associated pain. Finally, Kopeikina and Ponomarev highlight how platelets can infiltrate the CNS during inflammatory responses, and how they directly modulate neuronal activity and induce neurodegeneration. All these works support the notion that targeting inflammation induced by infiltrating blood cells or pro-inflammatory mediators may preserve neuronal fitness.

In recent years, studies in the field of immunometabolism highlighted how the immune system and systemic metabolism are heavily interconnected. Inflammation is indeed involved in the development of metabolic disorders, while metabolic hormones or circulating metabolites can affect immune cell function (Hotamisligil, 2017; Zasłona and O'Neill, 2020). This is particularly evident in obesity, and has implications also in neurological research, as the systemic metabolic state can alter brain functionality (Salas-Venegas et al., 2022). Importantly, previous studies strongly supported a beneficial effect for physical exercise in CNS health, via local and systemic changes that regulate body metabolism, limit inflammation, and promote the production of neuron-surviving factors. In their review, Consorti et al. summarize our knowledge on how different types of physical exercise can modulate neuronal functionality, through local and systemic effects. On the other side, immune cell functionality and pathogenicity are regulated by alterations in their intracellular metabolic profile (Makowski et al., 2020), and infiltrating or local CNS immune cells can induce neuroinflammation and neuronal damage upon activation and metabolic remodeling (Runtsch et al., 2021; Yang et al., 2021). Fessler and Angiari discuss how intracellular metabolic remodeling controls the appearance of a senescent phenotype in T cells, which is characterized by cellular stress and secretion of pro-inflammatory mediators. They also highlight the potential connection between cellular metabolism, T cell senescence, neuroinflammation, and neurodegeneration.

The CNS can undergo structural changes and cellular reprograming events during its entire lifespan, driven by environmental or genetic triggers. These can cause the appearance of CNS tumors such as gliomas, a group of brain cancers with limited therapeutic opportunities and high mortality rates (Kannan et al., 2022). Of particular relevance for gliomas is the effect of tumor cells on the surrounding brain tissue. Indeed, glioma cell growth not only causes structural changes in the brain, but also alters neuronal functionality, leading to tumor-associated neurological disorders such as epilepsy. Parmigiani et al. provide an overview of the communication between tumor cells, microglia, macroglia, infiltrating immune cells, and neurons in gliomas, highlighting potential targets for glioma therapy. A particularly severe type of gliomas is glioblastoma, which is known for being immunologically "cold", displaying high amounts of infiltrating suppressive immune cells in the tumor microenvironment (TME). Recent studies have shown that immunotherapy could overcome the inhibiting effect of the TME on immune cell infiltration and anti-tumor activity (Waldman et al., 2020). Supporting the idea that immunotherapy may also represent a valuable approach to tackle glioblastoma in humans, Bufalieri et al. discuss the potential of RIG-I-like receptor (RLR) agonists as immune-stimulators for the treatment of this life-threatening tumor.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Immune Soluble Factors in the Cerebrospinal Fluid of Progressive Multiple Sclerosis Patients Segregate Into Two Groups

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Primary-progressive (PP) and secondary-progressive (SP) multiple sclerosis (MS) are characterized by neurological deficits caused by a permanent neuronal damage, clinically quantified by the expanded disability status scale (EDSS). Neuronal tissue damage is also mediated by immune infiltrates producing soluble factors, such as cytokines and chemokines, which are released in the cerebrospinal fluid (CSF). The mechanisms regulating the production of a soluble factor are not completely defined. Using multiplex bead-based assays, we simultaneously measured 27 immune soluble factors in the CSF collected from 38 patients, 26 with PP-MS and 12 with SP-MS. Then, we performed a correlation matrix of all soluble factors expressed in the CSF. The CSF from patients with PP-MS and SP-MS had similar levels of cytokines and chemokines; however, the stratification of patients according to active or inactive magnetic resonance imaging (MRI) unveils some differences. Correlative studies between soluble factors in the CSF of patients with PP-MS and SP-MS revealed two clusters of immune mediators with pro-inflammatory functions, namely IFN-γ, MCP-1, MIP-1α, MIP-1β, IL-8, IP-10, and TNF-α (group 1), and anti-inflammatory functions, namely IL-9, IL-15, VEGF, and IL-1ra (group 2). However, most of the significant correlations between cytokines of group 1 and of group 2 were lost in patients with more severe disability (EDSS $\geq$ 4) compared to patients with mild to moderate disability (EDSS $<$ 4). These results suggest a common regulation of cytokines and chemokines belonging to the same group and indicate that, in patients with more severe disability, the production of those factors is less coordinated, possibly due to advanced neurodegenerative mechanisms that interfere with the immune response.

Keywords: progressive multiple sclerosis, cytokines, chemokines, expanded disability status scale, cerebrospinal fluid

INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS). Approximately 85% of patients with MS show a relapsing-remitting course of the disease (RR-MS), which in 50% of cases turn to a progressive course, termed secondary-progressive MS (SP-MS) (1). The remaining 10–15% of patients suffer from a progressive onset of the disease without relapses, termed primary progressive MS (PP-MS) (2, 3). Both progressive forms of MS are characterized by irreversible neurological decline caused by neuronal and axonal loss, failure of repair mechanisms, and a continuous accumulation of disability that can be measured by the expanded disability status scale (EDSS), a 0.5-point step-based scale with values ranging from 0 to 10 (4–6).

However, a clear comprehension of pathophysiology characterizing progressive MS is still missing. Known pathogenic mechanisms that possibly drive progression include specific immunological processes that are responsible for chronic inflammation at the leptomeningeal space and cerebral blood vessels (5). In the CNS, the main mediators of neuroinflammation are resident innate immune cells, such as microglia (7) and astrocytes (8), as well as monocyte-derived macrophages (9), B cells (10), and T lymphocytes (11), and each of them can promote the pathology by releasing cytokines and chemokines in the cerebrospinal fluid (CSF). Cytokines and chemokines are essential for activating an immune response and play a pivotal role in establishing and maintaining the inflammatory milieu by serving as chemotactic factors, restraining cell-to-cell communication and regulating proliferation and an activation state of immune, as well as neuronal and glial cells (12). Since CSF reflects the specific CNS immune microenvironment, the analysis of its soluble factors can reveal the altered balance between pro-inflammatory and anti-inflammatory cytokines or between neuroprotective and neurotoxic factors involved in the progression of MS. Several studies have investigated the potential role of CSF cytokines and chemokines in MS, with the final aim of better clarifying the pathogenesis of the disease, suggesting biomarkers for diagnosis, prognosis, and eventually predicting the response to therapies (13). However, most of them focused on RR-MS patients, and few studies investigated patients with progressive MS (14–20); moreover, most studies analyzed a limited cohort of patients (16, 21–23), and some studies included patients treated with immunomodulatory therapies that could affect the CSF environment (14, 21, 22, 24). Thus, an extensive analysis of the CSF composition in a large cohort of untreated PP-MS and SP-MS patients is useful. Our study performed on 38 patients with untreated progressive MS revealed that multiple cytokines and chemokines with distinct functions are released in the CSF. Through correlative studies, we highlighted a differential regulation of immune mediators expressed in the CSF of patients with moderate and severe disability. These results shed light on the potential interference between immunological and neurodegenerative mechanisms behind the progressive forms of MS.

Abbreviations: MS, multiple sclerosis; CNS, central nervous system; RR, relapsing remitting; PP, primary progressive; SP, secondary progressive; MRI, magnetic resonance imaging; EDSS, expanded disability status scale; CSF, cerebrospinal fluid; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; RANTES, regulated on the activation, normal T cell expressed and secreted; IP, interferon gamma-induced protein.

TABLE 1 | Demographic and clinical characteristics of progressive and relapsing-remitting MS subjects at the time of CSF collection.

Parameter	PP-MS	SP-MS	RR-MS
Number	26	12	11
Gender (male/female)	12/14	4/8	2/9
Age at sample collection (years)	51.3 ± 1.9	48.4 ± 2.7	30.8 ± 2.5
Disease duration (years)	3.8 ± 0.5	10.2 ± 2.4	1.8 ± 0.9
EDSS	3.7 ± 0.3	4.5 ± 0.5	1.8 ± 0.3
MRI (gadolinium+/gadolinium −)	5/21	3/9	–
N. of gadolinium+ lesions	3.5 ± 1.8	1.5 ± 0.5	–

Data for age at sample collection, disease duration and EDSS are shown as means ± SEM. PP-MS, primary progressive multiple sclerosis; SP-MS, secondary progressive multiple sclerosis; RR-MS, relapsing-remitting multiple sclerosis.

MATERIALS AND METHODS

MS Subjects for the CSF Collection

Patients with PP-MS ($n = 26$), SP-MS ($n = 12$), and RR-MS ($n = 11$) according to the established criteria (25) were recruited in the study from the Neurological Institute Carlo Besta (Milan, Italy) and the University of Genova/IRCCS Ospedale Policlinico San Martino Hospital (Genova, Italy). Demographic and clinical data of patients with progressive and RR-MS included in the study are described in **Table 1**. Approval by the Ethics Committee of the Neurological Institute Carlo Besta (n.40 of April 27, 2017) and Regione Liguria (n.185/2018 of May 28, 2018) and written informed consent forms in accordance with the Declaration of Helsinki from all participants were obtained before study initiation. At the time of the CSF collection, all patients underwent a full neurological assessment and a brain MRI scan; all subjects were positive to the detection of the oligoclonal band and were not treated with any disease modifying drugs or immunosuppressants.

Demographic and clinical data were derived from medical records. The onset of the MS disease was defined as the first episode of focal neurological dysfunction indicative of MS for patients with SP-MS, or the onset of the progressive symptoms for those affected with PP-MS. Disease duration was estimated as the number of years from the onset to the last assessment of disability. Disability at the time of the CSF collection was evaluated by means of the EDSS (4). Active disease was

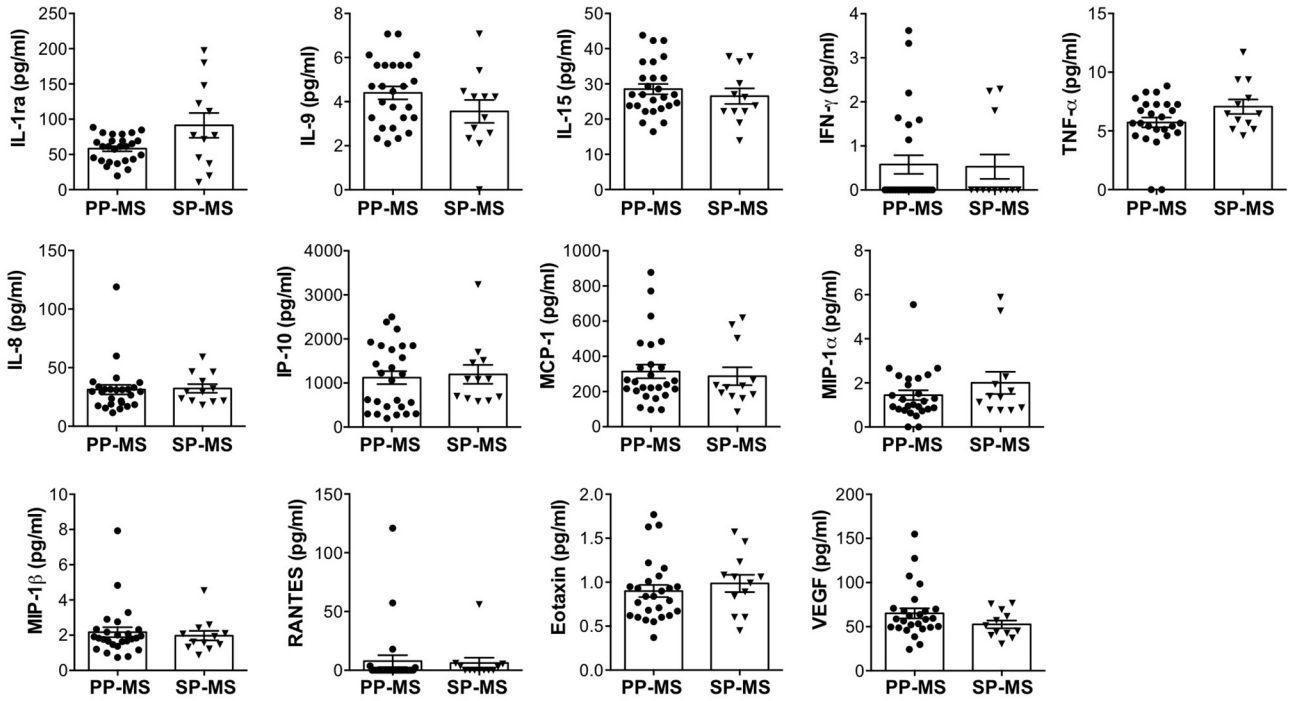

FIGURE 1 | Similar levels of immune soluble factors in the cerebrospinal fluid (CSF) of patients with primary progressive multiple sclerosis (PP-MS) and secondary progressive multiple sclerosis (SP-MS). The levels of interleukin (IL)-1ra, IL-9, IL-15, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-8, interferon-gamma induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on the activation, normal T cell expressed and secreted (RANTES), eotaxin, and vascular endothelial growth factor (VEGF) were simultaneously measured in the CSF of PP-MS and SP-MS by multiplex assay. Each CSF sample was analyzed in duplicate and means are represented. Data are reported as mean ± SEM.

defined according to the presence of lesions with gadolinium enhancement at baseline MRI.

MRI

MRI examination (1.5 Tesla) consisted of dual-echo proton density, fast fluid-attenuated inversion recovery, T2-weighted spin echo images, and pre-contrast and post-contrast T1-weighted spin-echo images. All images were acquired in the axial orientation with 3-mm-thick contiguous slices. The presence of gadolinium (0.2 ml/kg i.v.)-enhancing lesions was evaluated by a neuroradiologist who was unaware of the clinical details of patient.

CSF Cytokine and Chemokine Analysis

The profiles of CSF cytokines and chemokines were analyzed using Bio-Plex multiplex system (Bio-Rad, Hercules, CA, USA) of magnetic bead-based antibody detection kits, following the manufacturer's instructions. Specifically, Bio-Plex Pro Human Cytokine 27-plex (#M50-0KCAF0Y) was used for the detection of the following analytes: interleukin (IL)-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, eotaxin, basic fibroblast growth factor (FGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte CSF (G-CSF), interferon-gamma (IFN-γ), interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, regulated on activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor-alpha (TNF)-α and vascular endothelial growth factor (VEGF).

The aliquots of CSF (50 μl) were used for analysis, with a minimum of 50 beads per analyte acquired. Each CSF sample was analyzed in duplicate. Median fluorescence intensities were measured using the Luminex 200 System. Standard curves and values were calculated using xPONENT 4.2 software for MAGPIX®. Data were analyzed and reported as concentration readings (pg/ml).

Statistical Analysis

For pair-wise comparisons of different groups of patients, we used a non-parametric Mann–Whitney *U*-test. Data were presented as mean ± standard error (SEM). The Pearson's correlation coefficient ($\geq$ 0.5 or $\leq$ 0.5) was considered for statistical analyses of correlations between cytokines. The significance level was $p \leq 0.05$ without correction for multiple testing.

RESULTS

CSF From PP-MS and SP-MS Patients Contains Immune Mediators

In order to define the wide CSF profile of patients with progressive MS, we measured the levels of 27 immune soluble factors in CSF from 26 patients with PP-MS and 12 patients

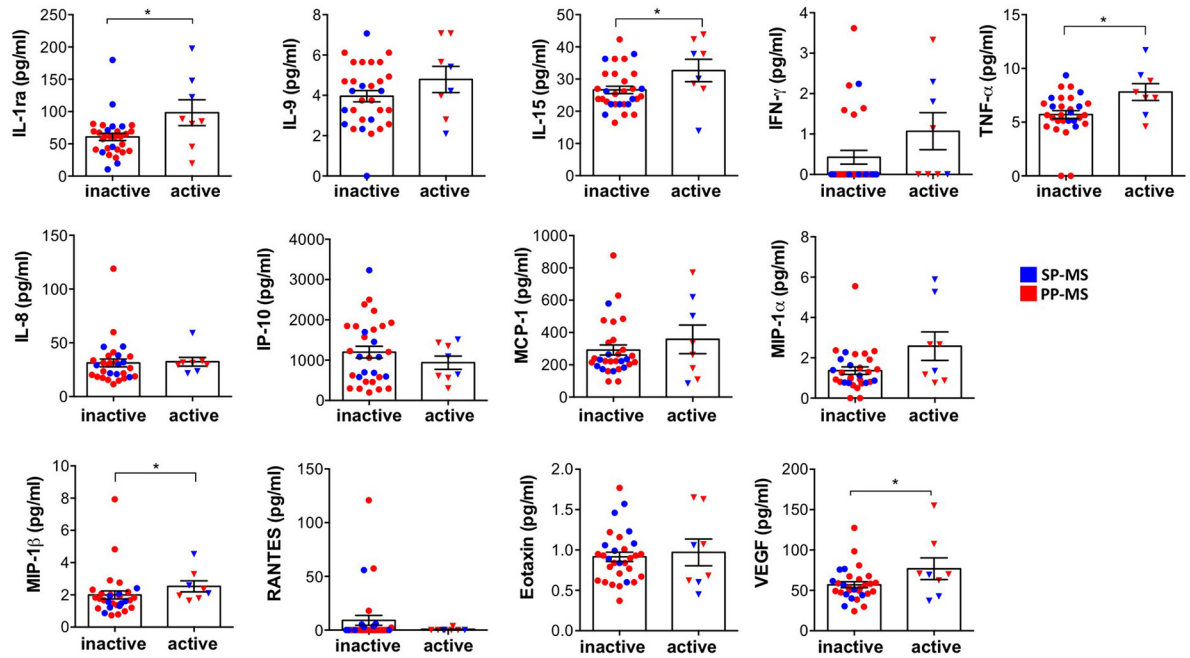

FIGURE 2 | The differential expression of immune soluble factors released in the CSF of patients with progressive MS in active and inactive diseases. The levels of IL-1ra, IL-9, IL-15, IFN-γ, TNF-α, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, eotaxin, and VEGF were measured, by multiplex assay, in the CSF of subjects with progressive MS distinct by the absence (inactive) or the presence (active) of gadolinium detection on MRI. Each CSF sample was analyzed in duplicate and means are represented. Mann–Whitney *U*-test was used to compare different conditions. Data are reported as mean ± SEM. $^*p < 0.05$.

with SP-MS (**Table 1**), by using a multiplex bead-based assay. We observed that the CSF of both progressive forms of MS contains detectable levels of five cytokines, namely IL-1ra, IL-9, IL-15, TNF-α, and IFN-γ, seven chemokines, namely IL-8, IP-10, MCP-1, MIP-1α, MIP-1 β, RANTES, eotaxin, and the growth factor, VEGF (**Figure 1**). Interestingly, these soluble factors were found at similar concentrations in the CSF of patients with PP-MS and SP-MS (**Figure 1**). Additionally, 14 soluble factors (IL-1 β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17A, FGF, GM-CSF, G-CSF, and PDGF-BB), were undetectable in the CSF of patients with both PP-MS and SP-MS (data not shown). These results indicate that a similar pattern of soluble factors characterizes the two progressive forms of MS, suggesting that similar mechanisms regulate the immune response in patients with PP-MS and SP-MS.

Next, we divided progressive patients into active and inactive MS based on the presence or absence of contrast-enhancing lesions at MRI. We found that the levels of IL-1ra, IL-15, TNF-α, MIP-1β, and VEGF are significantly higher in patients with active MS compared to patients with inactive progressive MS (**Figure 2**).

Two Clusters of Immune Soluble Factors Characterize the CSF of Patients With Progressive MS

In order to investigate whether the expression of immune soluble factors in the CSF of progressive patients is associated with disease severity, we categorized patients in two groups based on disability: mild to moderate (EDSS < 4) and severe (EDSS ≥ 4) disabilities. We obtained two numerically comparable categories of patients, composed of 17 and 21 subjects. The analysis revealed that the levels of CSF soluble factors were not affected by the degree of disability (**Figure 3**). In fact, IL-1ra, IL-9, IL-15, IFN-γ, TNF-α, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, eotaxin, and VEGF were similarly expressed in patients with mild to moderate or severe disability. Similarly, there was no association between the levels of CSF soluble factors and disease duration (**Supplementary Figure 1**).

Next, we performed correlative studies between soluble factors expressed in the CSF of patients with progressive MS. This analysis revealed two clusters of molecules significantly correlated in mild to moderate (EDSS < 4) and not in severe (EDSS ≥ 4) disabilities.

Specifically, group 1 contains IFN-γ, MCP-1, IL-8, MIP-1α, MIP-1β, TNF-α, and IP-10, which are positively correlated, and group 2 contains IL-9, IL-15, VEGF, and IL-1ra, which are positively correlated (**Figure 4A**). Interestingly, soluble factors of group 1 are involved in inflammatory functions, such as the recruitment and activation of T lymphocytes, monocytes, macrophages, and neutrophils, while group 2 contains proteins with anti-inflammatory properties. These results indicate that two classes of soluble factors with opposite functions are simultaneously produced and released in the CSF. Moreover, positive correlations between factors of group 1 suggest that common mechanisms regulate the production of IFN-γ,

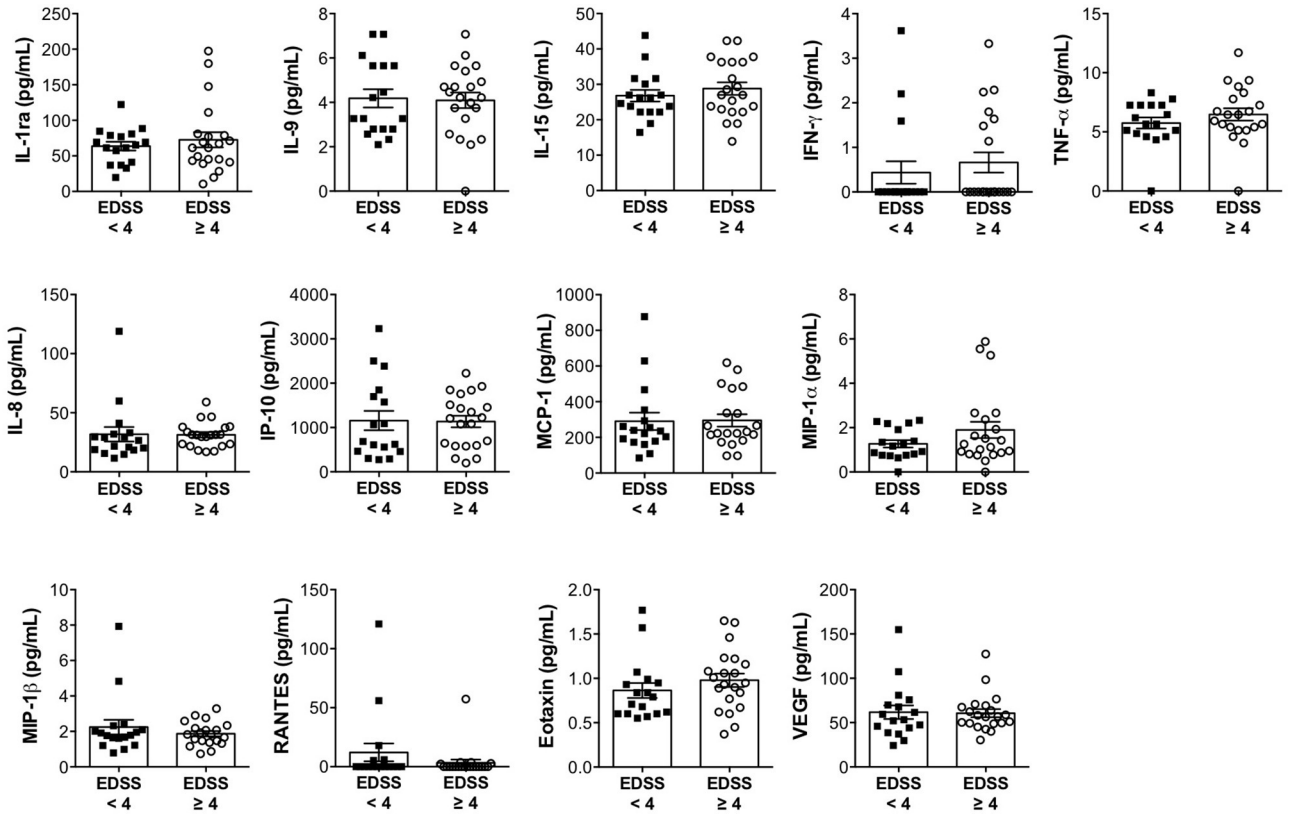

FIGURE 3 | The expression levels of immune soluble factors released in the CSF of progressive MS disease are not associated with expanded disability status scale (EDSS). The levels of IL-1ra, IL-9, IL-15, IFN-γ, TNF-α, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, eotaxin, and VEGF were measured in the CSF of patients with progressive MS with minimal-to-moderate disability (EDSS < 4) or with high disability (EDSS ≥ 4), by multiplex assay. Each CSF sample was analyzed in duplicate and means are represented. Data are reported as mean ± SEM.

MCP-1, IL-8, MIP-1α, MIP-1β, TNF-α, and IP-10. Similarly, correlations between factors belonging to group 2 (IL-9, IL-15, VEGF, and IL-1ra) suggest common mechanisms regulating their production.

We observed that the coordinated production of group 1 and group 2 immune mediators is altered in patients with severe disability (EDSS ≥ 4) (**Figure 4B**) as compared to patients with mild to moderate (EDSS < 4) disability (**Figure 4A**). In fact, part of the correlations between molecules is lost in patients with higher EDSS, despite similar number of patients in the two groups. For instance, correlations between IL-8 and MCP-1, IFN-γ, MIP-1α, and MIP-1β were not significant, as well as those between IP-10 and MIP1-α, and TNF-α. Other correlations such as those between IFN-γ and MIP-1β or between MCP-1 and MIP-1β were weaker in patients scoring high on the EDSS compared to those scoring low on the EDSS. However, in patients with severe disability, we observed a significant correlation between TNF-α and IL-8 which was not present in patients with mild to moderate disability. Among soluble factors in group 2, we found that IL-9 and IL-15 are the unique cytokines, whose correlation is conserved in progressive MS patients with mild to moderate and severe disabilities. These results suggest that neurodegenerative mechanisms associated with a higher degree of disability may interfere with the global expression of immune soluble factors in the CSF of patients with progressive MS.

Interestingly, we found that soluble factors belonging to group 2 (IL-9, IL-15, VEGF, and IL-1ra) are strongly correlated in patients with RR-MS patients (**Figure 4C**). Moreover, in those patients, we found that IL-9, IL-15, VEGF, and IL-1ra correlate with soluble factors belonging to group 1, such as IFN-γ, IL-8, MIP-1β, and TNF-α (**Figure 4C**). These results suggest that, during the onset of the RR-MS disease, there is a simultaneous production of anti-inflammatory and pro-inflammatory soluble factors that could compensate the pathogenic effects of the immune response, thus contrasting the irreversible neurodegeneration typical of the progressive MS forms.

DISCUSSION

Our study investigates the profile of immune mediators released in the CSF of a large cohort of patients with progressive MS and reveals that CSF microenvironment is characterized by chemokines involved in the recruitment and cytokines involved in the activation of other immune cells. In particular, we found that chemokines, MCP-1 (CCL2), MIP-1 α (CCL3), MIP-1 β

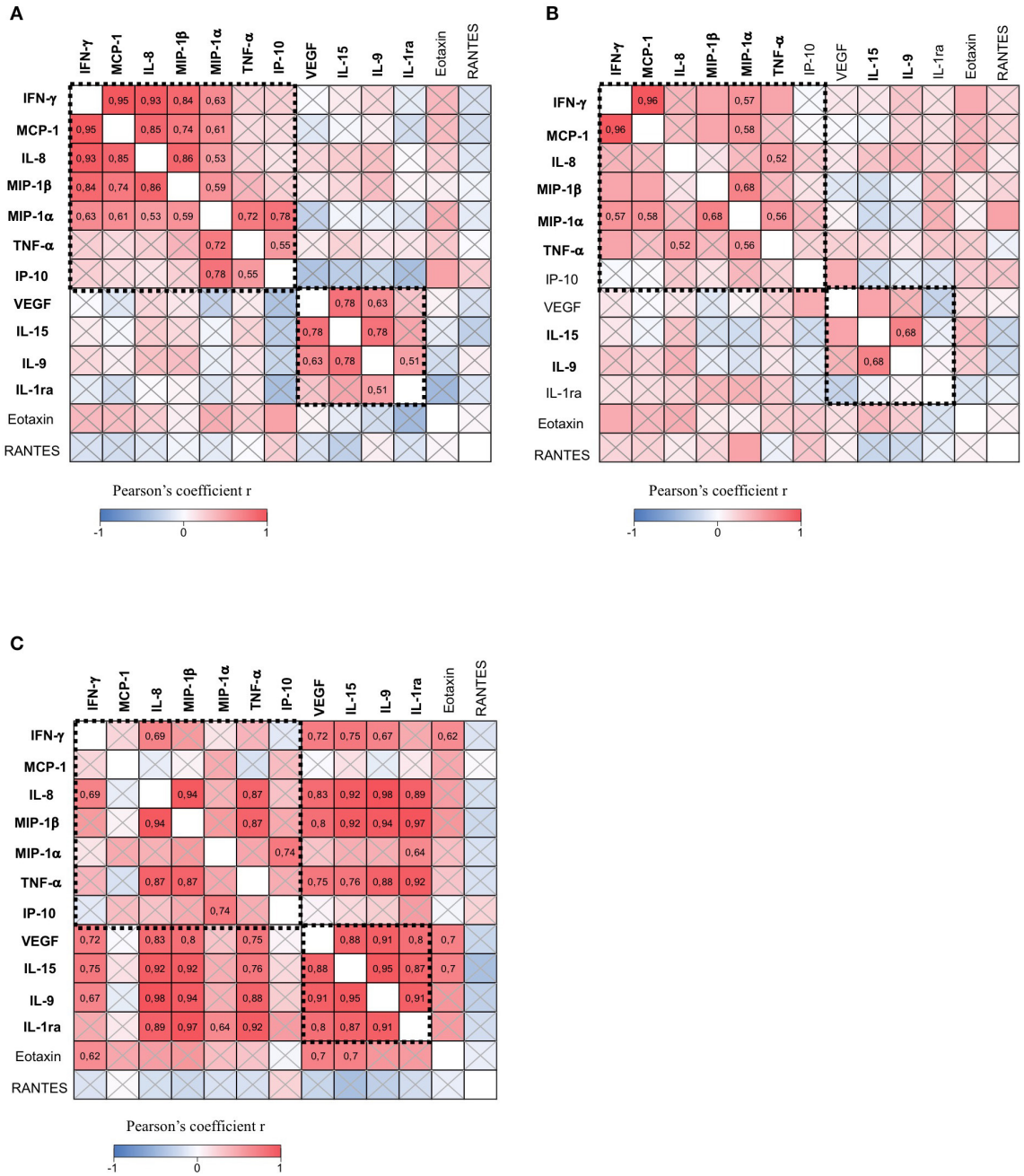

FIGURE 4 | The CSF of patients with progressive MS contains two clusters of immune soluble factors. The correlation matrix among all soluble factors expressed in the CSF of patients with progressive MS with minimal-to-moderate disability (EDSS < 4) ($n = 17$) **(A)**, patients with progressive MS with high disability (EDSS ≥ 4) ($n = 21$) **(B)**, and patients with relapsing-remitting MS (RR-MS) ($n = 11$) **(C)**. Pearson's correlation coefficients $r \geq 0.5$ or ≤ 0.5 are shown. Crossed squares lack statistical significance ($p > 0.05$). The color in each square indicates the Pearson's correlation coefficient r among the variables reported in the two coordinates, as indicated by colored scale bar. The dashed boxes showed two clusters of cytokines and chemokines reciprocally correlated in A: IFN-γ, MCP-1, MIP-1α, MIP-1β, IL-8, IP-10, and TNF-α (group 1), and IL-9, IL-15, VEGF, and IL-1ra (group 2).

(CCL4), RANTES (CCL5), eotaxin (CCL11), IP-10 (CXCL10), and IL-8 (CXCL8), were expressed in the CSF of patients with both PP-MS and SP-MS. In addition, the cytokines IL-15, IL-9, IL-1ra, TNF-α, IFN-γ, and the growth factor, VEGF, were expressed in the CSF of the same patients. These results are consistent with previous data characterizing the immune profile of the CSF of patients with progressive MS (3, 16–21). Moreover, a recent meta-analysis, including 226 studies on patients with

progressive and RR-MS, revealed that MIP-1α, eotaxin, IL-8, and IL-15 are significantly increased in the CSF of volunteers with MS compared to the CSF of non-MS (13). However, few studies used a large number of patients with progressive MS, and these validated only the expression of IL-8 (19, 20, 26) and IL-15 (24) as significantly increased compared to controls. Studying the CSF from 38 patients with progressive MS, we found the expression of 13 immune mediators, including IL-8 and IL-15.

Our analysis revealed that PP-MS and SP-MS do not differ in the composition of CSF environment, whereas MRI disease activity was associated with a weak increase of IL-15, VEGF, IL-1ra, MIP-1β, and TNF-α in progressive MS. Interestingly, we further investigated the CSF of those patients by correlative studies and we found that two groups of soluble factors are reciprocally correlated. Group 1 contains IFN-γ, MCP-1, IL-8, MIP-1β, MIP-1α, TNF-α, and IP-10, while group 2 contains VEGF, IL-15, IL-9, and IL-1ra. Notably, group 1 contains chemokines favoring the infiltration and activation of T lymphocytes, neutrophils, monocytes, and macrophages but also of resident CNS cells, thus ultimately contributing to a compartmentalized inflammation in the CNS (22, 23, 27, 28). Moreover, two cytokines of group 1, IFN-γ and TNF-α, also associated with the CSF of patients with progressive MS with a high degree of meningeal inflammation and a high number of cortical lesions (29), play a crucial role in enhancing excitatory synaptic transmission (30, 31), thus favoring neurodegeneration (17). On the other side, group 2 contains soluble factors with potential protective functions. In fact, IL-1ra is a known anti-inflammatory endogenous molecule acting as a competitive inhibitor of IL-1β (32) and demonstrated as effector molecule in reducing disease severity in the murine models of MS (33–35). IL-1ra significantly correlates with IL-9, which has been recently associated with reduced inflammation and reduced neurodegeneration in MS (36, 37). Additionally, we observed a strong correlation between IL-9 and IL-15, whose role in MS is still unclear but could exert a protective role by attenuating the cytotoxicity of CD8-positive T cells (38). Interestingly, the correlation between IL-9 and IL-15 was already reported in patients with RR-MS, and this association was related to the increased levels of both cytokines in patients receiving prednisolone treatment than those without immunotherapy during the relapse (22). These results indicate that IL-9 and IL-15 could share a common mechanism of production and that they are induced by immune-suppressive therapies.

Another factor associated with IL-9 and IL-15 in group 2 is VEGF, which is produced not only by immune cells but also by endothelial cells, astrocytes, and neurons, and that acts as neuroprotective agent for neurons and neural progenitors in the late MS phase, such as the progressive forms of MS (39). Thus, the presence of a specific cluster of immune soluble factors in the CSF composed by potential protective factors as IL-9, IL-1ra, IL-15, and VEGF in progressive MS could represent an attempt of the immune system to counteract the pro-inflammatory environment regulated by the factors of group 1. Importantly, the majority of the correlations between factors belonging to group 1 and those of group 2 were lost in patients characterized by more severe disability (EDSS $\geq$ 4) compared to patients with mild to moderate disability (EDSS $<$ 4), suggesting that the pro-inflammatory and anti-inflammatory networks generated by the immune system in the CNS is affected by advanced neurodegenerative mechanisms typical of high disability. Indeed, in patients with low disability, the coordinated production of soluble factors with pro-inflammatory and anti-inflammatory functions generates a balanced environment. In contrast, in patients with high disability, the uncoupled production of pro-inflammatory and anti-inflammatory immune mediators in the CSF might interfere with proper resolution of inflammation.

Finally, using a classical approach, this study revealed that the CSF from patients with PP-MS and SP-MS does not significantly differ. Correlative studies, which reflect the coordinated and simultaneous expression of molecules, indicate that the global pattern of immune soluble factors released in the CSF of patients with progressive MS differ according to the level of disability. Moreover, our study revealed that in patients with RR-MS, where the level of disability is consistently lower in comparison to progressive MS, the expression of immune soluble factors in the CSF is even more different. In fact, we found a coordinated expression of molecules with both pro-inflammatory and anti-inflammatory properties that could contribute to the immune compensatory mechanisms and to a better clinical prognosis.

These matrices generated by correlative studies could be a useful tool to globally explore the CSF environment, at different disease stages, during disease activity or disease-modifying therapies, or in other neurodegenerative diseases.

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 studies involving human participants were reviewed and approved by Ethical committee of the Istituto Neurologico Carlo Besta and Regione Liguria. The patients/participants provided their written informed consent to participate in this study.

AUTHOR'S NOTE

Multiple sclerosis (MS) is an immune-mediated disease of the central nervous system (CNS). Approximately 85% of MS patients show a relapsing-remitting disease that may turn to a progressive course termed secondary progressive MS (SP-MS). The 10–15% of patients suffer from a primary progressive MS (PP-MS). Both progressive forms of MS are characterized by accumulation of disability measured by the Expanded Disability Status Scale (EDSS). A complex interaction between immune cells and CNS resident cells is involved in the pathology of MS. However, the mechanisms regulating MS progression are still unknown. Since immune soluble

factors released in the cerebrospinal fluid (CSF) are critical mediators of the intercellular communication between resident and infiltrating cells in the CNS, their characterization could help understanding the mechanisms regulating the disease. Our study provides a comprehensive characterization of immune soluble factors expressed in progressive MS patients. We found that soluble factors clustered in two groups with pro-inflammatory and anti-inflammatory functions, respectively, and such segregation is weaker in patients with high EDSS ($\geq$ 4)than in those with mild to moderate EDSS (<4). These data indicate that in patients with more severe disability the production of soluble factors is less coordinated, likely due to advanced neurodegenerative mechanisms that interfere with the immune response.

AUTHOR CONTRIBUTIONS

GD performed research, analyzed data, and drafted the paper. VS and LB provided samples and the clinical data of patients with progressive MS and performed research. CZ provided samples and clinical data of patients with progressive MS. DP performed statistical analysis. AL and DM provided samples and clinical data of patients with RR-MS. RM and PC coordinated the recruitment of patients with progressive MS. EV designed research, analyzed data, and wrote the paper. 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/fimmu.2021.633167/full#supplementary-material

Supplementary Figure 1 | The expression levels of immune soluble factors released in the CSF of progressive MS patients are not associated with disease duration. Levels of soluble factors in the CSF of patients with progressive MS were correlated to their disease duration (years) at the time of CSF sample collection, using the Pearson's correlation coefficient. None of the correlations were statistically significant.

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frontiers in Neuroscience

REVIEW
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The Interaction Between Microglia and Macroglia in Glaucoma

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Glaucoma, a neurodegenerative disease that leads to irreversible vision loss, is characterized by progressive loss of retinal ganglion cells (RGCs) and optic axons. To date, elevated intraocular pressure (IOP) has been recognized as the main phenotypic factor associated with glaucoma. However, some patients with normal IOP also have glaucomatous visual impairment and RGC loss. Unfortunately, the underlying mechanisms behind such cases remain unclear. Recent studies have suggested that retinal glia play significant roles in the initiation and progression of glaucoma. Multiple types of glial cells are activated in glaucoma. Microglia, for example, act as critical mediators that orchestrate the progression of neuroinflammation through pro-inflammatory cytokines. In contrast, macroglia (astrocytes and Müller cells) participate in retinal inflammatory responses as modulators and contribute to neuroprotection through the secretion of neurotrophic factors. Notably, research results have indicated that intricate interactions between microglia and macroglia might provide potential therapeutic targets for the prevention and treatment of glaucoma. In this review, we examine the specific roles of microglia and macroglia in open-angle glaucoma, including glaucoma in animal models, and analyze the interaction between these two cell types. In addition, we discuss potential treatment options based on the relationship between glial cells and neurons.

Keywords: microglia, macroglia, astrocytes, Müller cells, glaucoma, neuroinflammation

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BACKGROUND

Glaucoma is one of the main causes of irreversible vision and visual field loss. As a progressive optic nerve disorder, its main pathological manifestations are loss of retinal ganglion cells (RGCs) and degenerative lesions of the optic nerve (Weinreb et al., 2014; Gordon and Kass, 2018; Calkins, 2021). Elevated intraocular pressure (IOP) has been recognized as a key risk factor for glaucoma.

Abbreviations: ATP, adenosine triphosphate; bFGF, basic fibroblast growth factor; CNTF, ciliary neurotrophic factor; Cx3cr1, C-X3-C motif chemokine receptor 1; C3ar1, C3a receptor 1; EAE, experimental autoimmune encephalomyelitis; EAG, experimental autoimmune glaucoma; GDNF, glial-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GLAST, glutamate/aspartate transporter; IL-1β, interleukin 1β; IL-6, interleukin 6; IOP, intraocular pressure; LC, lamina cribrosa; LIF, leukemia inhibitory factor; MiRNAs, microRNAs; NF-κB, nuclear factor kappa-B; ONA, optic nerve antigen; ONH, optic nerve head; RGCs, retinal ganglion cells; TANK, TNF receptor-associated factor NF-κB activator; TBK1, TANK-binding kinase 1; TGF-β, transforming growth factor-β; TGF-β2, transforming growth factor-β2;TLR, Toll-like receptor; TNFα, tumor necrosis factor α; TSPO, translocator protein.

Therefore, the main prevention and treatment measure for high-tension glaucoma is adequate control of IOP (Weinreb and Khaw, 2004; Weinreb et al., 2014; Conlon et al., 2017; Gordon and Kass, 2018). However, some patients with normal IOP also have glaucomatous visual impairment and RGC loss. This suggests that in addition to high IOP, there may be other factors that cause pathological glaucomatous changes. For example, the duplication of TBK1 [tumor necrosis factor (TNF) receptor-associated nuclear factor kappa-B (NF-κB) activator (TANK)-binding kinase 1], a gatekeeper of neuroinflammation (Ahmad et al., 2016), can be detected in hereditary normal tension glaucoma patients (Fingert et al., 2011). Wild-type mice could acquire glaucoma-like characteristics, such as loss of RGCs, by receiving T cells from Sh3pxd2b^{nee} mice, a hereditary glaucoma model with elevated IOP (Gramlich et al., 2015). This indicates that neuroinflammation and the immune system can initiate the occurrence and progression of glaucoma.

In glaucoma, the cells involved in immunoregulation in the retina mainly include microglia, astrocytes, and Müller cells, the latter two being macroglia (Adornetto et al., 2019; Reichenbach and Bringmann, 2020). In a normal retina, these cells provide nutrition and structural support, participate in metabolism, and regulate homeostasis while coordinating with each other in the regulation of the state of neurons through phagocytosis and the secretion of inflammatory cytokines and neurotrophic factors (Vecino et al., 2016). Thus, microglia and macroglia do not function in isolation, and a delicate balance always exists between them. Glial cells work together to maintain the stability of the retinal microenvironment. However, once there is a deviation in this delicate balance, effects opposite to what is expected may occur. Therefore, understanding the interaction between glial cells plays an essential role in better glaucoma prevention and treatment.

In this review, we discuss the role and importance of microglia and macroglia in open-angle glaucoma, including glaucoma in animal models. The animal models discussed in this article are RGC loss models, most of which are high IOP models; models with RGC loss caused by other reasons are also mentioned. We analyze the contribution of the interactions between microglia and macroglia to the survival of RGCs and optic nerves with the hopes of providing additional insights into the relationship between glial cells and neurons while also providing some foundations and directions for future research. Based on the functions and characteristics of different glial cells, we propose future research directions for glial cells for better prevention and control of glaucoma.

MICROGLIA IN GLAUCOMA

Microglia, which are full-time phagocytes, play an important role in innate immune responses. Microglia are involved in neural circuits and angiogenesis in a developing retina, whereas they regulate retinal neuron activity and synaptic integrity in a mature retina (Silverman and Wong, 2018; Chen et al., 2019; Rathnasamy et al., 2019). In a healthy retina, perivascular microglia control the substances entering the retina from the circulatory system, whereas other scattered retinal microglia patrol the microenvironment to remove metabolites and cell debris and mediate synaptic remodeling (Provis et al., 1996). Microglia are distributed horizontally in the plexiform layer of the inner retina and make regular short-term contact with neuronal synapses at rest (Wake et al., 2009). They respond quickly and sensitively to pathological stimuli, such as lipopolysaccharides, complements, thrombins, inflammatory cytokines, and chemokines, migrating to an injury site within 24 h (Okunuki et al., 2018).

In normal human eyes, quiescent microglia, with thin ramified processes, are found around the optic nerve head (ONH) in the walls of large blood vessels and in surrounding capillaries in glial columns and the cribriform plate. In human glaucomatous eyes, microglia are activated as clusters of large ameboids in the compressed lamina cribrosa (LC) and as formations of concentric circles surrounding blood vessels. Microglia are also redistributed to the parapapillary chorioretinal region and are present as single cells or clusters at the termination of Bruch's membrane (Neufeld, 1999). In the ONH of glaucomatous eyes, microglia express different cytokines and mediators in addition to their morphology and distribution. Research has shown that abundant transforming growth factor-β2 (TGF-β2), TNF-α, and proliferating cell nuclear antigens are present in the microglia of glaucomatous ONH, whereas no positive signal of these factors is detected in the microglia of normal ONH (Yuan and Neufeld, 2001).

Microglial activation is considered one of the early alterations that occur in glaucoma. For example, in chronic hereditary glaucoma model DBA/2J mice, the aggregation, activation, and redistribution of microglia can be detected before RGC injury (Bosco et al., 2011). In DBA/2 J mice, the transcriptome of ONH microglia changes significantly in the metabolic, phagocytosis, inflammatory, and sensome pathways (Tribble et al., 2020). Among these, microglial surveillance and phagocytosis are downregulated, whereas metabolism-related transcripts are upregulated. These findings suggest that chronic ocular hypertension inhibits the homeostasis and related functions of microglia, and that ONH microglia increase the capacity to metabolize energy (Tribble et al., 2020). Retinal microglial reactions are regulated by many factors, such as microRNAs (miRNAs). Previous investigations have indicated that miRNAs are associated with microglial activation and polarization (Yan et al., 2015; Drewry et al., 2018; Wang et al., 2021). In a study of an acute ocular hypertension rat model, overexpression of microRNA-93-5p reduced microglial proliferation, migration, and cytokine release; these findings were accompanied by reduced loss of RGCs (Wang et al., 2021).

Despite these reported findings, the role of microglia in glaucoma remains controversial. Some researchers have suggested that microglia can aggravate RGC damage and neurodegeneration in multiple ways, including through the release of inflammatory cytokines, such as TNF-α, interleukin 1β (IL-1β), IL-6, matrix metalloproteinases, Fas ligands, and reactive oxygen species (Hanisch and Kettenmann, 2007; Langmann, 2007). In a study of an S100B-induced glaucoma-like animal model, microglia were activated and increased in the optic nerve

on day 14, and RGC apoptosis was observed. However, on day 21, the microglial response was not as prominent, whereas RGC damage was still present (Grotegut et al., 2020). Therefore, the deletion of genes that affect microglial activation or other normal functions can contribute to the survival of RGCs or the integrity of the optic nerve. Microglia express high levels of complement peptide C3a receptor 1 (C3ar1), which has been identified as a damaging neuroinflammatory factor. Deficiency in C3ar1 lowers the risk of retinal degeneration independent of IOP in DBA/2J mice (Harder et al., 2020). The neutralization or genetic deletion of TNF-α significantly reduces RGC loss in a glaucoma mouse model with angle closure induced *via* argon laser irradiation (Nakazawa et al., 2006). Deletion of the CD11b/CD18 gene, which mediates the recruitment and activation of leukocytes, inhibits the loss of RGCs and combats neurotoxicity (Nakazawa et al., 2006). Meanwhile, a deficiency in C-X3-C motif chemokine receptor 1 (CX3CR1), which is expressed by neurons, can inhibit microglial reactivity, aggravate neurotoxicity, and induce extensive damage to RGCs in an experimental mouse glaucoma model with transient elevation of IOP (Wang K. et al., 2014). Some drug interventions can also exert beneficial effects on RGC survival by inhibiting microglial reactivity. Minocycline, a drug that reduces microglial activation, improves the integrity of RGC axons and the function of the optic nerve in DBA/2J mice (Bosco et al., 2008). Intravitreal injection of macrophage inhibitory factor in a rat optic nerve axotomy model (Thanos et al., 1993) and high-dose irradiation of the head of a DBA/2J mouse (Bosco et al., 2012) inhibit the degradation of neurons and promote the development of axons. Furthermore, the proliferation and activation of microglia has shown a significant correlation with the severity of optic neuropathy in DBA/2J mice (Bosco et al., 2015).

However, some studies have suggested that microglia can protect RGCs from damage. Microglia help to prevent secondary retinal damage by phagocytosing damaged or dead RGCs in the later stages of optic nerve damage (Sierra et al., 2013). It is well known that DNA can act as danger-associated molecular patterns, inducing tissue toxicity and organ damage in a mouse glaucoma model with transient elevation of IOP (Supinski et al., 2020). As major phagocytes, microglia can prevent DNA release from dying cells through phagocytosis (Egensperger et al., 1996). Once activated, microglia can clear multiple apoptotic RGCs for at least 14 days after reaxotomy of the RGCs (Schuetz and Thanos, 2004); however, inappropriate phagocytosis by microglia can accelerate the loss of RGCs (Brown and Neher, 2014). Therefore, regulation of phagocytosis enables microglia to function better as guardians during retinal injury. In addition, microglia produce neurotrophic factors (Harada et al., 2002), remove excess glutamate (Basilico et al., 2019), and exert antioxidant effects through antioxidant enzymes in the cells (Vilhardt et al., 2017) when the retina is damaged.

In summary, microglia exert neuroprotective or neurotoxic effects in glaucomatous eyes, depending on the secreted factors. Microglia are activated in the early stages of glaucoma and rapidly migrate to an injury site. They stabilize the retinal microenvironment through phagocytosis and secretion of anti-inflammatory cytokines, thus protecting the optic nerve and RGCs. In the late stage of glaucoma, however, overactivated microglia produce pro-inflammatory cytokines, complement, and other toxic factors that directly affect RGC apoptosis. There is no doubt that microglia are an important target for the prevention of glaucoma, and due to their complexity, more accurate and precise intervention and regulation involving them will be a challenge in future research.

MACROGLIA IN GLAUCOMA

Macroglia, which mainly include astrocytes and Müller cells, possess similar transcription profiles and functions (Xue et al., 2011). Their cell bodies surround the axons of neurons and form glial sheaths around neurons that protect and buffer them. Müller cells span the entire retinal layer, whereas astrocytes are confined to the innermost layer of the retina. The difference between their distribution indicates that their monitoring and reacting areas are different; Müller cells are more global, whereas astrocytes are local. Their close contact with neurons also allows macroglia to sensitively perceive changes in the microenvironment, such as hypoxia, and respond in a timely manner. Macroglia transport most nutrients, wastes, ions, water, and other molecules between blood vessels and neurons (Reichenbach and Bringmann, 2020). In addition, they contribute to various glial homeostatic functions, such as the regulation of extracellular pH and potassium balance (Agte et al., 2011).

When the retina is stimulated or injured, macroglia can be activated and can express glial fibrillary acidic protein (GFAP) and other extracellular matrix signals through enlarged cell bodies and thickened foot processes (Varela and Hernandez, 1997; Tezel et al., 2003; Lukowski et al., 2019), a process known as retinal gliosis. In a human glaucomatous retina, astrocytes and Müller cells show a hypertrophic morphology with increased immunostaining of GFAP, which suggests that retinal gliosis is exhibited in glaucoma (Tezel et al., 2003).

Astrocytes in Glaucoma

As the main component of glial cells in the central nervous system, astrocytes play a variety of essential roles, including the regulation of ion balance, metabolic supply and structural support, neurotransmitter transmission, and synaptic plasticity (Halassa et al., 2007). Astrocytes not only play the role of maintaining retinal homeostasis but also directly act on the pathophysiology of RGCs and the optic nerve through a variety of factors.

In clinical practice, optic disk cupping can be easily discernible in glaucomatous eyes. ONH cupping can be induced by structural changes in the LC, which is enriched with collagens (Elkington et al., 1990). Optic nerve axons in the LC are unmyelinated and surrounded by astrocytes (Shinozaki and Koizumi, 2021). Activated astrocytes have been found in the human glaucomatous retina (Minckler and Spaeth, 1981; Tezel et al., 2003). Recently, intravitreal injection of S100B, which is mainly expressed by astrocytes, was found to cause glaucoma-like neuronal degeneration in the retina and optic nerve (Grotegut et al., 2020).

In glaucoma, astrocytes change from a resting state to an activated state in a process known as astrogliosis and act as early responders to induce inflammatory responses. Astrocytes have been shown to mediate the activation of multiple inflammatory pathways, including TNF-α signaling, NF-κB activation, autophagy, and inflammasome-associated regulators in high IOP rats after administration of hypertonic saline injections into their episcleral veins (Tezel et al., 2012). In patients with glaucoma and glaucoma animal models, astrocytes at the ONH can be observed to upregulate the expression of tenascin-C (Pena et al., 1999; Howell et al., 2011; Johnson et al., 2011), playing a pro-inflammatory role through the Toll-like receptor (TLR) 4 signaling pathway (Midwood et al., 2009). Astrocytes also induce the adhesion and migration of immune cells by secreting cell adhesion proteins (Johnson et al., 2011; Tanigami et al., 2012). They participate in the remodeling of the extracellular matrix of the LC, which is related to a variety of molecular pathways, including those for TGF-β, endothelins, bone morphogenetic proteins, and gremlin (Schneider and Fuchshofer, 2016).

In response to an increase in IOP, astrocytes expand and redistribute metabolites, donating resources from unstressed projections to stressed projections in DBA/2J mice (Cooper et al., 2020). Furthermore, once IOP is increased, activated astrocytes accompanied by upregulated cytoskeletal protein concentrate and tightly surround the ONH; however, long-term high IOP causes the astrocytes around the optic nerve to decrease or even disappear after magnetic microsphere injection (Dai et al., 2012). In the early stage of glaucoma progression in DBA/2J mice models, astrocytes become more parallel with migration to the edge of the nerve; however, the astrocytes' parallelism diminishes as axons degenerate and glial coverage increases (Cooper et al., 2018). Moreover, some studies have shown that ONH astrocytes play a role in the engulfment of optic axons and promotion of axon degradation, which is one of the possible mechanisms for the sectorial nature of RGC loss in glaucoma (Nguyen et al., 2011; Mills et al., 2015).

Astrogliosis is beneficial to the retina, and its morphological remodeling is reversible in the early stages of the disease. However, in the late stage, it can cause excessive scar hyperplasia and degrade optic axons, which are detrimental to the repair of the body (Sun et al., 2013).

Müller Cells in Glaucoma

Müller cells are the most abundant glial cells in the retina and are closely related to RGCs in anatomy and structure. They play a crucial role in neuronal development, survival, and related information processing (Bringmann et al., 2006). The cell body of a Müller cell is located in the inner nuclear layer, and its processes extend to the inner and outer layers of the retina. Müller cells tightly contact synapses and blood vessels in the inner and outer plexiform layers of the retina. Müller cells are rich in different ion channels, ligand receptors, transmembrane transport molecules, and enzymes (Bringmann et al., 2006). Their structural richness determines the diversity of their functions. Regarding physiological conditions, Müller cells are involved in retinal glucose metabolism, regulation of retinal blood flow and neurotransmitter transmission, and regulation of the balance of ions, water, and amino acids (Bringmann et al., 2006). Regarding pathological conditions, Müller cells regulate immunity and phagocytic cells or foreign substances. In particular, Müller cells undergo gliosis to cope with various types of retinopathy (Harada et al., 2002).

Müller cell reactions are detectable in the human glaucomatous retina through immunohistochemistry (Tezel et al., 2003). Müller cells regulate intracellular and extracellular glutamate levels through the expression of glutamate/aspartate transporter (GLAST) (Rauen, 2000). A study of GLAST knockout mice indicated that they showed spontaneous RGC loss and glaucomatous optic nerve degeneration without elevated IOP (Harada et al., 2007).

Müller cell gliosis mainly plays a protective role in the early stages of glaucoma. Investigation using scanning force microscopy has shown that Müller cells have very soft and flexible bodies with strong plasticity and powerful compliance (Lu et al., 2006). Furthermore, their hypertrophic cell bodies and increased intermediate filaments, such as GFAP, enhance resistance to mechanical forces, including elevated IOP (Lu et al., 2006). The expression of neurotrophins and their receptors can be detected in Müller cells after retinal ischemia–reperfusion (Vecino et al., 1998). In addition, Müller cells secrete neurotrophic factors, such as ciliary neurotrophic factor (CNTF), and the antioxidants glutathione (Pease et al., 2009) and ghrelin (Zhu et al., 2017), protecting RGCs from damage in glaucoma.

In the later stages of glaucoma, however, over-gliosis of Müller cells is dysfunctional, leading to disturbances in retinal homeostasis and neuronal death. Müller cells show excitotoxic damage to RGCs because their degradation of glutamic acid is weakened during hypoxia (Evangelho et al., 2019). Owing to the damage of the Müller cell membrane under high pressure, K^+ siphoning is impaired, inducing cation dyshomeostasis in the microbead occlusion model (Fischer et al., 2019). They are not sufficient to rebalance microenvironmental changes and even activate neuronal pro-apoptotic pathways. Müller cell activation also aggravates the damage of RGCs through NF-κB-dependent TNF-α production (Lebrun-Julien et al., 2009), indicating that Müller cells indirectly contribute to neuroinflammation.

INTERACTION BETWEEN MACROGLIA AND MICROGLIA IN GLAUCOMA

Although the origins and main functions of microglia and macroglia are different, they coordinate with each other to complete the nutrition, support, and protection of neurons (**Figure 1**). In a mature retina, macroglia, microglia, and pericytes inhibit the proliferation of endothelial cells and maintain the stability of blood vessels and the inner blood–retinal barrier (Gardner et al., 1997; West et al., 2005; Klaassen et al., 2013). In a healthy retina, the state of microglial activation depends directly on extracellular adenosine triphosphate (ATP). The main source of ATP is Müller cells, which means that Müller cells can indirectly serve as an energy source for microglia (Stout et al., 2002; Pankratov et al., 2006; Uckermann et al., 2006; Li et al., 2012).

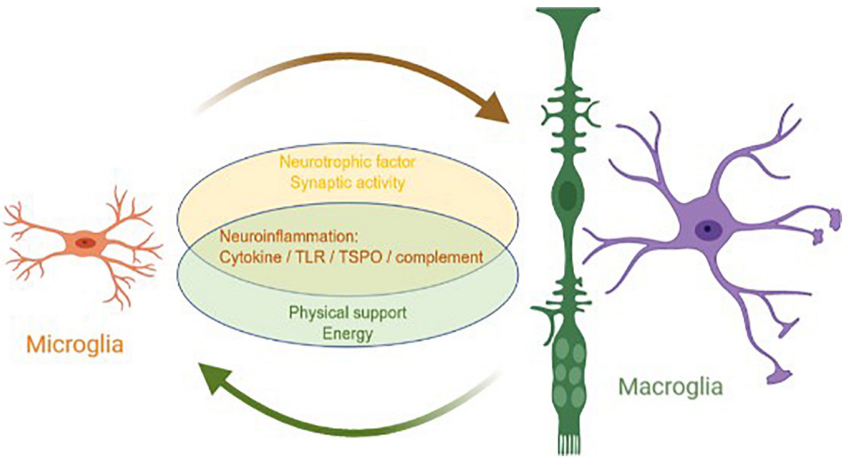

FIGURE 1 | Interaction between macroglia and microglia in glaucoma. Microglia and macroglia are involved in the progression of neuroinflammation in glaucoma, including the inflammatory cytokine, TLR, TSPO, and complement pathways. In addition, microglia prompt macroglia to produce neurotrophic factors and help regulate synaptic activity, whereas macroglia provide microglia with the physical scaffold support and energy required for activities.

After the retina is mechanically damaged or becomes degenerative, the interaction between microglia and macroglia begins immediately, and then the timing and extent of microglial activation is closely regulated (Wang M. et al., 2014). There are some similarities in the changes that occur in microglia and macroglia in glaucoma. In a study of IOP-independent experimental autoimmune glaucoma (EAG) mice models, the mice were immunized with an optic nerve antigen (ONA). Compared with the control group, the EAG mice displayed fewer Brna+ RGCs and SMI-31+ optic nerve neurofilaments, with a significantly higher number of retinal Iba1 + microglia and GFAP + astrocytes (Wiemann et al., 2020).

In a resting state, microglia mainly exhibit horizontal movement. However, with retinal injury or other stress, they begin vertical transretinal movement, which is closer to the behavior of Müller cells. Injection of lipopolysaccharides into the vitreous cavity of mice has shown that the horizontal branches of the microglia are in close contact with the cell bodies of Müller cells, suggesting that microglia may use Müller cells as scaffolds for transretinal migration (Sánchez-López et al., 2004; Wang et al., 2011). Müller cells co-cultured with activated microglia show more elongated spindles and multipolar shapes than their flat, thick lamellipodia when cultured alone (Wang and Wong, 2014). In addition, under stress, gliosis in retinal glia can be triggered by microglial activation through increased cytokine levels (Dharmarajan et al., 2017). Moreover, IL-6 (Fischer et al., 2014; Zhao et al., 2014) and interferon-γ (IFN-γ) (Cotinet et al., 1997) cause morphological changes and promote the production of inflammatory factors in Müller cells. In zebrafish, the pharmacological ablation of microglia results in a lack of reactivity in Müller cells with less GFAP expression; however, it does not affect the migration of Müller cells (Conedera et al., 2019). It has also been found that progesterone can reduce Müller cell gliosis by inhibiting the expression of IFN-γ from microglia in a retinitis pigmentosa mouse model (Roche et al., 2018). Activated microglia also regulate Müller cells to produce and release more trophic factors, such as basic fibroblast growth factor (bFGF) (Harada et al., 2002), glial-derived neurotrophic factor (GDNF), and leukemia inhibitory factor (LIF), thereby amplifying the protective effects of Müller cells. This response is independent of the expression of the typical glial markers of Müller cells (Pease et al., 2009). Activated microglia also participate in the maintenance of the synaptic activity microenvironment of macroglia, and they jointly regulate the content of ions and neurotransmitters (Kimelberg, 2010; Chong and Martin, 2015; Ma et al., 2019). Glial cells also contribute to the activation of the complement pathway in glaucoma (Pauly et al., 2019). The level of complements in Müller cells and microglia increases with transient retinal ischemia (Pauly et al., 2019). In addition, astrocytes activate C3 convertase, thereby amplifying complement signals and alerting microglia to respond to RGC damage (Howell et al., 2011; Astafurov et al., 2014; Williams et al., 2017).

Traditionally, microglia and astrocytes are considered to have two different phenotypes. Microglia are classified into "M1" and "M2" phenotypes. Simply put, M1 is an inflammation-promoting phenotype, whereas M2 is an anti-inflammatory phenotype (Varnum and Ikezu, 2012; Tang and Le, 2016). In animal models of laser-induced glaucoma, most microglial cells exhibit the M1 phenotype (Rojas et al., 2014). Similarly, astrocytes are categorized into "A1" and "A2" phenotypes. The A1 phenotype aggravates neuroinflammation, whereas A2 is neuroprotective (Liddelow et al., 2017). M1 and M2 microglia can stimulate reactive astrocyte changes by secreting different cytokines (Wei et al., 2019). M1 microglia, which secrete pro-inflammatory cytokines, can promote astrocytes to switch to the A1 phenotype, thereby increasing neurotoxicity and inducing neuronal death (Liddelow et al., 2017). Recently, single-cell RNA sequencing, which provides an unbiased analysis of cells, has been used to uncover more information about the heterogeneity of astrocytes and microglia. In studies of experimental autoimmune encephalomyelitis (EAE), astrocytes

were identified as several subpopulations, and pro-inflammatory and neurotoxic subpopulations were the most expanded (Linnerbauer et al., 2020; Wheeler et al., 2020). Microglia show a variable spatial and temporal distribution during development and can gain a discrete context- and time-dependent disease-specific signature in the central nervous system pathology of mice. Corresponding clusters of microglia have also been identified in the brains of patients with multiple sclerosis (Masuda et al., 2019).

In addition to the aforementioned unidirectional coordination between glial cells, microglia and Müller cells are likely to have bidirectional feedback signals in response to injury or other stress. For example, activated microglia induce Müller cells to secrete more adhesion molecules and chemokines so that more microglia can adhere to the Müller cells and express higher chemotaxis, which also promotes microglial migration and recruits other immune cells to the injured areas (Evangelho et al., 2019). The upregulation of TLR signals in both microglia and astrocytes has been found in the retina of patients with glaucoma (Luo et al., 2010), and together they may mediate the initial immunity initiated by glaucoma stimulation signals. Both phagocytic microglia (Yuan and Neufeld, 2001) and reactive astrocytes (Zhang et al., 2004) express metalloproteinases, which increase during optic neuropathy, altering the amount of extracellular matrix to mediate retinal degeneration. Translocator protein (TSPO) and its ligand XBD173 (Mages et al., 2019) may be key mediators of the inflammatory response between microglia and macroglia (Scholz et al., 2015; Cooper et al., 2020). As initiators and amplifiers of retinal inflammation, activated microglia and Müller cells (Scholz et al., 2015) can mutually and reciprocally promote each other to produce more inflammatory cytokines, thereby creating a positive feedback loop and exacerbating the inflammatory response (Wang et al., 2011).

Generally, microglia are the main executors of neuroinflammation in a glaucomatous retina, whereas macroglia are supporters. Both microglia and macroglia undergo changes in morphology and physical location when the retina is under stress. As the only antigen-presenting cells of the retina, microglia are activated when an injury occurs and quickly migrate to the injury site. As the main supporting cells of the retina, macroglia also become mobilized and move parallel to the edge of the nerve, redistributing resources in response to the injury. These two types of glial cells coordinate to mediate neuroinflammation, including neuroinflammation from the cytokine, TLR, TSPO, and complement signaling pathways. Microglia are mainly involved in neuroinflammation, whereas macroglia regulate neuroinflammation in various ways. Furthermore, microglia prompt macroglia to produce neurotrophic factors and help regulate synaptic activity, whereas macroglia provide microglia with the physical scaffold support and ATP required for activities.

GLIA-TARGETED THERAPEUTIC APPROACHES

Overactivation of microglia is considered detrimental to the survival of RGCs. Therefore, protecting RGC by inhibiting the activity of microglia is an effective treatment for glaucoma. Some drug interventions exert beneficial effects on RGC survival by blocking microglial-relevant pathways. Microglia inhibitors have been shown to effectively protect RGCs in animal models (Drewry et al., 2018; Grotegut et al., 2020; Supinski et al., 2020). At present, microglia inhibitors have not been applied to the treatment of glaucoma. However, they have been used for the management of other diseases caused by retinal inflammation, an important aggravating factor for glaucoma. Treatment with the microglial inhibitory agent minocycline is associated with improved visual function, central macular edema, and vascular leakage, targeting the inflammatory etiology of diabetic macular edema in humans (Cukras et al., 2012).

In recent studies, macroglia have shown the potential for reprogramming and re-differentiation, which brings new hope for the treatment of retinal diseases. Glial cells and neurons are derived from the same pluripotent stem cells, and the influence of extracellular signals on them leads to different outcomes (Janowska et al., 2019). In theory, by regulating the extracellular signals of glial cells, their fate can be altered, and they can re-differentiate into glial cells or neurons. Targeting glia for neurorestorative therapy is a safe, effective, and efficient strategy. In the early stage of glaucoma, astrocytes and Müller cells limit the spread of inflammation and the progression of glaucoma. In the late stage of the disease, the overactive glial cells would form glial scars, which aggravate the progression of glaucoma (Reichenbach and Bringmann, 2020). Judging and selecting the appropriate intervention period may be the direction for the exploration of the astrocyte-based treatment in the future. In zebrafish, Müller cells enter a reprogramming state relatively easily, whereas in mammals, they enter a reactive state after injury; however, they stagnate before becoming progenitor cells (Hoang et al., 2020). How to regulate the entrance of mammalian Müller cells into the reprogramming state will be a future research direction.

CONCLUSION

Many studies of patients with glaucoma and glaucoma animal models have shown that optic nerve damage or high IOP can cause the production of inflammatory cytokines and chemokines, as well as the activation of ocular immune cells, such as microglia and macroglia. A delicate balance between glial cells is critical for the development and maturation of the retina under normal conditions and during the pathological process of disease conditions. The neuroinflammation mediated by these cells is one of the key factors in the onset and progression of glaucoma. There is no doubt that IOP reduction is an effective prevention and treatment measure for patients with high-tension glaucoma. However, the roles of controlled neuroinflammation and promotion of neurotrophy in the treatment of glaucoma are also worth considering. However, microglia and macroglia do not function in isolation in glaucoma. Furthermore, an understanding of the interactions between glial cells is necessary for the development of new interventions to reduce neuroinflammation and prevent

glaucoma. More research is needed to better understand the complex molecular spatiotemporal regulatory network in glaucoma. Further elucidation of the mechanism and response of glial cells in glaucoma will have a significant impact on the development of new cell-based therapies for retinal diseases.

AUTHOR CONTRIBUTIONS

All authors read and approved the final manuscript.

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MINI REVIEW
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Involvement of Mast Cells in the Pathophysiology of Pain

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Mast cells (MCs) are immune cells and are widely distributed throughout the body. MCs are not only classically viewed as effector cells of some allergic diseases but also participate in host defense, innate and acquired immunity, homeostatic responses, and immunoregulation. Mounting evidence indicates that activation of MCs releasing numerous vasoactive and inflammatory mediators has effects on the nervous system and has been involved in different pain conditions. Here, we review the latest advances made about the implication of MCs in pain. Possible cellular and molecular mechanisms regarding the crosstalk between MC and the nervous system in the initiation and maintenance of pain are also discussed.

Keywords: mast cells, pain, hyperalgesia, inflammation, immunoregulation

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INTRODUCTION

Pain is a hallmark of inflammation that can be either protective or detrimental during acute or chronic stages. The development and maintenance of chronic pain are involved in neuronal sensitization (Ji et al., 2016). It has long been postulated that interactions between the nervous system and immune system contribute to the pathophysiology of pain. Following intense noxious stimulation, neuropeptides and neurotransmitters released by nociceptors result in neurogenic inflammation and the recruitment of immune cells, whereas infiltrated immune cells release mediators to enhanced responsiveness of sensory neurons. Such positive feedback loops may underlie pain induction (Liu et al., 2021).

Considerable evidence suggests that mast cells (MCs), effectors of innate immunity and local inflammation, regulate pain signaling, for example, by secreting mediators that activate nearby nerves based on their histological proximity (Chompunud Na Ayudhya et al., 2020; Aguilera-Lizarraga et al., 2021). Here, we discuss the role of MCs in pain initiation and maintenance *via* MC-neuron crosstalk. Possible molecular mechanisms and resolution of pain associated with MC are demonstrated. Importantly, the identification of the pathological role of MCs in neuroimmune interactions will provide us novel strategies operative in pain.

MAST CELL BASICS

MCs derive from CD34/CD117-expressing multipotent hematopoietic precursor cells in the bone marrow, which circulate in the bloodstream and are transited out of the circulation to the peripheral tissues where they attain their maturity (Metcalfe et al., 1997; **Figure 1**).

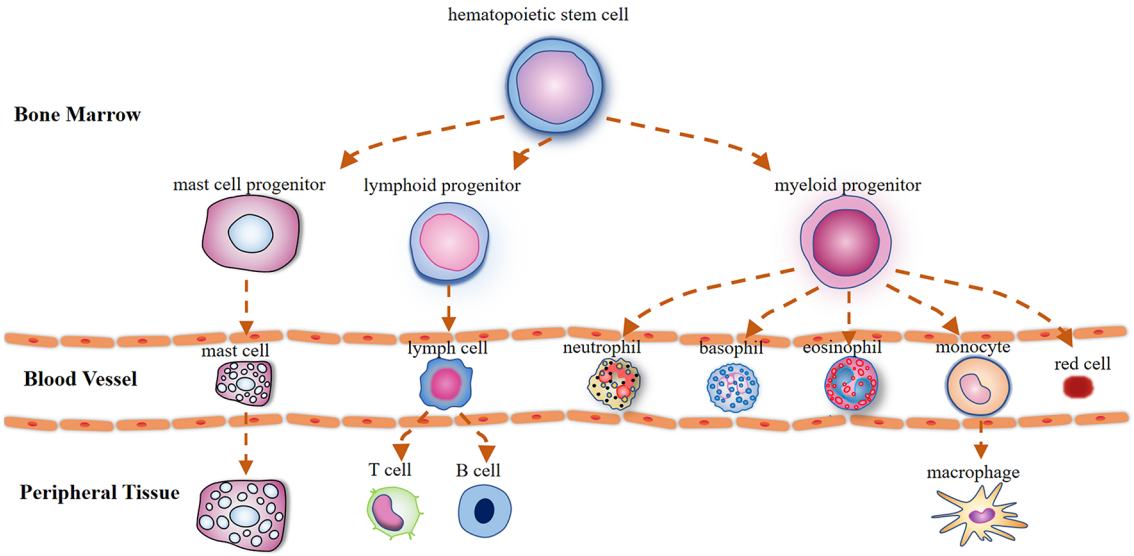

FIGURE 1 | Illustration outlining the mast cells (MCs) differentiation trajectories. Mast cells, lymphocytes, and myeloid cells are derived from pluripotent hematopoietic progenitors in the bone marrow. Unlike basophils that attain their maturity in the circulation, mast cell precursors circulate in the bloodstream as immature cells and are transited out to the peripheral tissues where they mature under the influence of growth factors.

Mature MCs can exert instant effects on vascular function (Albert-Bayo et al., 2019) and sensory neurons as they are in close proximity to vasculature and nerve fibers innervating derma (Morellini et al., 2018), visceral organs (Barbara et al., 2004), meninges (Levy et al., 2007; Hassler et al., 2019), brain parenchyma (Ocak et al., 2019), and hypothalamus (Edvinsson et al., 1977).

MCs can be activated through a variety of mechanisms. Of these, allergens and pathogens acting on their respective receptors expressed on MCs, such as the high-affinity immunoglobulin E receptor and toll-like receptor, represents the classical model of MC activation (González-de-Olano and Álvarez-Twose, 2018). Notably, MCs can be activated by membrane receptors that can not only detect thermal and physical stimuli [e.g., the transient receptor potential vanilloid (TRPV) family] (Zhang D. et al., 2012; Solís-López et al., 2017), but also detect a variety of endogenous mediators, including neuropeptides and neurotransmitters released by nociceptive neurons [e.g., Mrgprb2/X2, a G protein-coupled receptor responsive to substance P (SP)] (Green et al., 2019).

Following activation, MCs release their granule-stored mediators and then secret re-synthesized granules as a late response, called "*de novo* synthesis" (Vukman et al., 2017). The former process is termed "degranulation", in which MCs release pre-formed granules within minutes. These mediators include biogenic amines (e.g., histamine and serotonin), proteases (e.g., tryptase and chymase), proteoglycans (e.g., heparin) tumor necrosis factor alpha (TNFα), leukotrienes, cytokines, and chemokines that facilitate the migration of other immune cells (González-de-Olano and Álvarez-Twose, 2018). They can be recognized in tissues with toluidine blue staining due to the large cytoplasmic granules (mainly heparin) in cells (Eady, 1976).

MAST CELL INVOLVED IN PAINFUL CONDITIONS

MCs are located in the vicinity of nociceptive C-fibers and may interact with nerve endings through the "synapse like" connection (Suzuki et al., 2004). Increased MCs were observed in patients with headaches (Friesen et al., 2018), non-cardiac chest pain (Lee et al., 2014), and self-injurious behavior-associated pain (Symons et al., 2009). Pain-like behaviors have been found to be MC-associated, including models of post-fracture nociception (Li et al., 2012), cancer pain (Lam and Schmidt, 2010; Yu et al., 2019), postoperative pain (Oliveira et al., 2013), fibromyalgia (muscle pain; Theoharides et al., 2019), sickle cell anemia-associated pain (Vang et al., 2015) and visceral hypersensitivity, as is indicated in irritable bowel syndromes (Di Nardo et al., 2014), chronic pelvic pain (Done et al., 2012), interstitial cystitis (IC; Wang et al., 2016; Martin Jensen et al., 2018), and neonatal maternal separation (Chen et al., 2021). Mastocytosis, characterized by constitutive hyperactivity of MC, is often accompanied by pain syndromes (Giannetti and Filice, 2021). Additionally, MC stabilizers significantly attenuate hyperalgesia in inflammatory pain models induced by formalin (Nakajima et al., 2009), potamotrygon venom (Kimura et al., 2018), nerve growth factor (NGF), and dynorphin (Kissel et al., 2017).

Taken as a whole, the results indicate that MCs play an important role in different painful conditions, although some studies showed that depletion or stabilization of MC did not display pain-relieving effect in models induced by

complete Freund's adjuvant, carrageenan, formalin, NGF, or nociceptin/orphanin (McDougall and Larson, 2006; Xanthos et al., 2011; Lopes et al., 2017; Magnusdottir et al., 2018). Whether MC plays a critical role in nociceptive processing remains to be elucidated.

MECHANISTIC INSIGHTS INTO THE DIALOG BETWEEN NEURON AND MAST CELL

MCs are well recognized for their sufficient role in inflammation but much less is known about their contributions to pain pathways. MC may increase the excitability of nociceptors by releasing pro-nociceptive molecules, whose receptors are expressed on sensory neurons (Loewendorf et al., 2016). Mediators released by nociceptive sensory neurons, in turn, regulate the maturation, recruitment, and degranulation of MCs through the activation of their respective membrane receptors on MCs (Serhan and Basso, 2019; Koyuncu Irmak et al., 2019; **Figure 2**).

Neuropeptides

Neuropeptides, critical inducers of neurogenic inflammation, are primarily released from nociceptors following intense noxious stimulation and/or activation of different molecular sensors, such as TRP channels (e.g., TRPV1; Sousa-Valente and Brain, 2018; Yakubova and Davidyuk, 2021), G protein-coupled receptors [proteinase-activated receptor 2 (PAR2) and Mrgprb2] (Wei et al., 2016), sodium channels (Nav1.9; Bonnet et al., 2019), and mechanosensitive Piezo receptors (Mikhailov et al., 2019).

SP and calcitonin gene-related peptide (CGRP) are two of the pivotal neuropeptides implicated in neurogenic inflammation and pain. Recent evidence suggests that SP promotes the recruitment of innate immune cells and the release of pro-inflammatory mediators *via* activation of the Mrgprb2 receptor expressed by MCs (Green et al., 2019). A recent report reveals a regulatory effect of CGRP in MCs using RNA-sequencing, in which differentially expressed genes are enriched in biological processes associated with transcription, MC activation, and proliferation after CGRP treatment (Sun et al., 2020). Although MCs abundantly express receptors for neuropeptides (Le et al., 2016), however, many neuropeptides have less well-defined roles in MC-mediated pain.

In turn, MCs may exacerbate inflammation and pain signals *via* modulating SP production. MCs reside in the microenvironment where SP- immunoreactive nerve fibers are located and modify the degradation of SP by releasing tryptase and chymase (Caughey et al., 1988). Pharmacological inhibition on MCs significantly reduces the release of SP and ameliorates hyperalgesia in sickle mice (Vincent et al., 2013). Identification of the modulatory effects of MCs on SP and CGRP may provide insights into the neuro-immune interaction, but not exclusively, pain hypersensitivity.

Serotonin

Serotonin, or 5-hydroxytryptamine (5-HT), is a neurotransmitter that distributes mainly in the central nervous system and it is involved in the regulation of numerous behavioral and physiological processes, such as perception, memory, and mood (Bamalan and Al Khalili, 2020). Recent studies suggest that serotonin can be released from peripheral MC and promote pain during tissue injury (Sommer, 2004).

The expression level of 5-HT was upregulated in pain models induced by acute inflammation (Nakajima et al., 2009), surgery (Oliveira et al., 2011), and migraine (Koroleva et al., 2019), which can be significantly attenuated by MC stabilizer or MC deficiency. Patients with abdominal pain showed a significantly increased release of 5-HT, which has a significant correlation with MCs counts and the severity of pain (Taylor et al., 2010; Cremon et al., 2011).

5-HT is also a powerful neuromodulator with a receptor-dependent effect. Several subtypes of serotonin receptors, such as 5-HT(1)A (Coelho et al., 1998), 5-HT(3) (Yan et al., 2014), and 5-HT(2A) receptors (Oliveira et al., 2011), have been found associated with nociceptive responses mediated by MC. Selected tricyclic antidepressants, capable of inhibiting 5-HT secretion from MCs, are well introduced in chronic pain treatment, which expand our understanding of mechanisms underlying the pathophysiology of pain (Ferjan and Lipnik-Stangelj, 2013).

Histamine

Histamine is present within all bodily tissues, stored in secretory vesicles that are released by MCs and basophils. Histamine regulates various physiological and pathological processes, such as autoimmune conditions, vasodilation, hematopoiesis, and neurotransmission (Obara et al., 2020), which are facilitated by binding to histamine H_1, H_2, H_3, and H_4 receptors that differ in their tissue expression patterns and functions (Obara et al., 2020; Patel and Mohiuddin, 2020).

Accumulating evidence indicates that MC-derived histamine serves as mediator to pain. Treatment with MC stabilizers and/or histamine antagonists significantly ameliorates vincristine/paclitaxel-induced hyperalgesia (Gao et al., 2016; Schneider, 2017). Blockade of H1 receptor in pain models with increased MCs infiltration inhibits or reduces prostatitis-associated pelvic pain (Done et al., 2012), visceral hypersensitivity (Barbara et al., 2007), venom-induced mechanical allodynia (Lauria et al., 2018), and post-operative nociception (Oliveira et al., 2011). H_2 receptors also indicated in hyperalgesia and allodynia mediated by MC histamine in inflammatory pain (Massaad et al., 2004), vincristine-induced neuropathic pain (Jaggi et al., 2017), and IC pain (Rudick et al., 2008). Given the efficacy of histamine antagonists in treating hyperalgesia, inhibition on MC degranulation may provide a promising target in pain control (Obara et al., 2020).

Tryptase

Tryptase is a trypsin-like serine protease produced by MCs. It serves as a marker of MC activation. The release of tryptase has been proven to be attributed to activation of Kit receptor in MCs (Grimbaldeston et al., 2005; Ammendola et al., 2013; Chen et al., 2021).

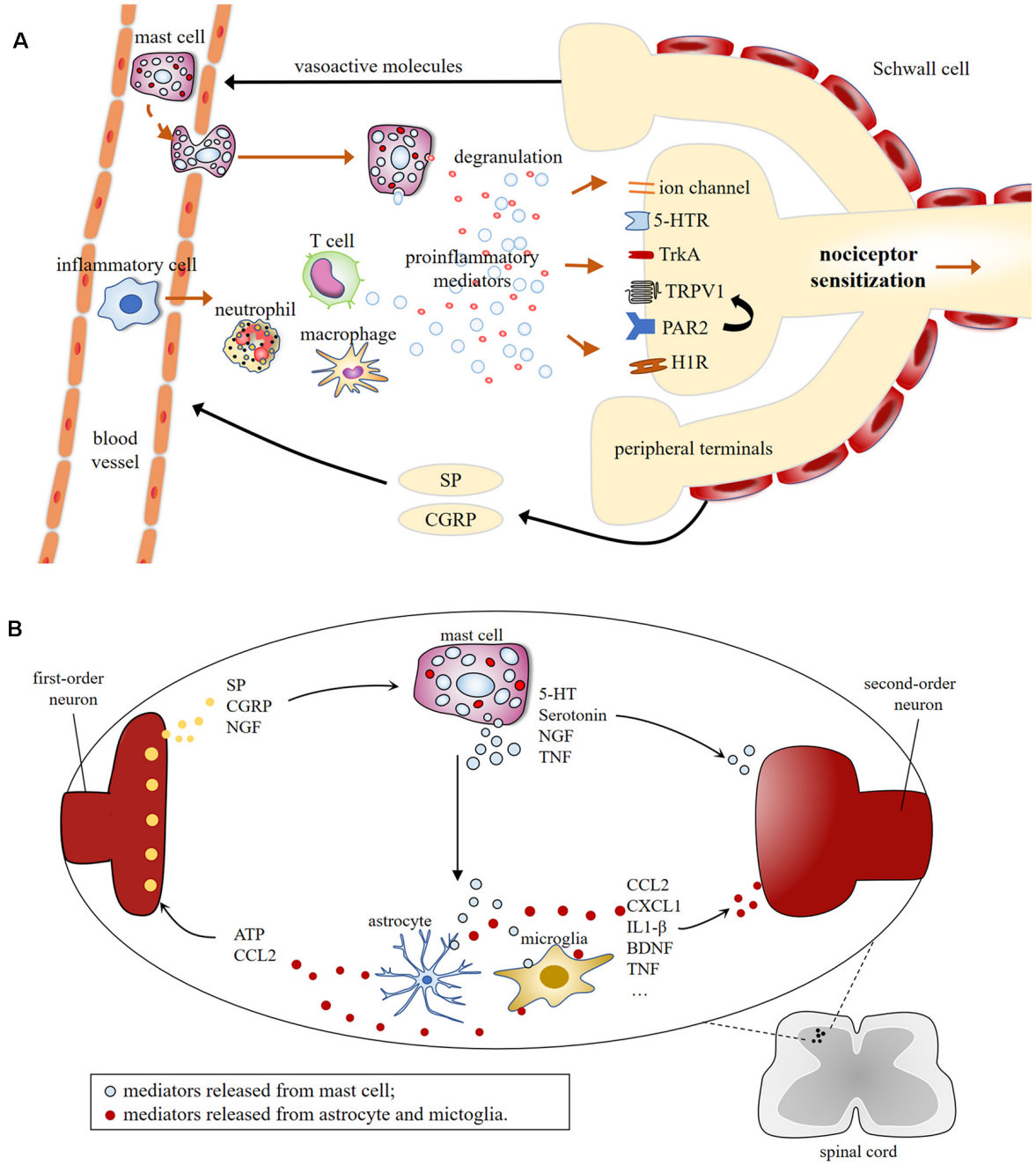

FIGURE 2 | (A) Schematic illustration of mast cell involvement in peripheral sensitization in the terminals of nociceptive primary afferents. Mast cell degranulation induces the production of proinflammatory mediators [e.g., 5-HT, TNF, nerve growth factor (NGF), histamine, tryptase], resulting in nociceptive neurons release vasoactive neuropeptides, which in turn, leads to the recruitment of immune cells, including mast cells, macrophage, neutrophil, T cell, etc. This leads to the possibility of positive feedback loop, which could lead to chronic pain. **(B)** Molecular mechanisms of central sensitization induced by mast cells degranulation in first-order excitatory synapses, where communications between neuronal and non-neuronal cells occur. Central terminals of nociceptors release inflammatory factors that activate the second-order neurons and non-neuronal cells including mast cells, which induces neuronal activation *via* producing proinflammatory cytokines and chemokines [e.g., TNF, interleukins (IL)-1β, CCL2, CXCL1], and granular components, such as 5-HT and serotonin.

MC tryptases are essential for inflammation and nociceptive responses (Hoffmeister et al., 2011; Borbély et al., 2016). Clinically, there was a significant correlation between the intensity of pain and tryptase levels in patients who are with the complex regional pain syndrome (Huygen et al., 2004). Increased level of tryptase in the incised tissue was detected in most patients who were undergoing moderate-to-severe pain for up to 1 month (Pepper et al., 2013). Tryptase may be

involved in pain through cleaving and activating its receptor PAR2 expressed on sensory neurons (Anaf et al., 2006; Bunnett, 2006). As pretreatment with PAR2 antagonist was capable of attenuating chronic visceral hyperalgesia (Roman et al., 2014), preventing postoperative nociception (Oliveira et al., 2013), and abolishing cancer-dependent allodynia (Lam and Schmidt, 2010).

Some studies revealed that tryptase-PAR2 may affect neurogenic inflammation and pain transmission *via* regulating the activity of TRP ankyrin 1 and TRPV1, TRPV4 channels of sensory neurons (Dai et al., 2004, 2007; Zhao et al., 2014), by phospholipase C, protein kinase A, and protein kinase C-dependent mechanisms (Chen et al., 2011). Moreover, MC tryptase activates neutrophil (de Almeida et al., 2020) and microglia (Zhang S. et al., 2012), which are important culprits for inflammation and exerts an active role in pain (Tsuda, 2018). MC tryptase has been implicated in peripheral and central sensitization, albeit there remain large gaps in our knowledge about the tryptase-mediated mechanism of nociception.

Cytokines

Cytokines are synthesized mainly by the immune and nervous system and are responsible for the regulation of differentiation, inflammation, immune responses, cell apoptosis, and necrosis *via* transmitting signals between cells (Totsch and Sorge, 2017; Zahari et al., 2017). Additionally, cytokines contribute significantly to pain arising from nociceptor activation. A range of cytokines, including TNFα, interleukins (IL)-1beta, IL-6, IL-17, granulocyte macrophage colony-stimulating factor (GM-CSF), have been shown to play prominent roles in sensitizing neuronal cells *via* their specific receptors (Cook et al., 2018).

Non-neuronal cells, such as MCs, monocytes, lymphocytes, are producers of TNF (Grivennikov et al., 2005). A previous finding has identified MCs as an important source of both preformed and immunologically inducible TNF implicated in different biological responses (Gordon and Galli, 1990). After being activated, MCs rapidly secret granule-stored TNF through degranulation and then release the *de novo* synthesized TNF 24 h later (Zhang B. et al., 2012). TNFα, as a neuro-sensitizing molecule, causes neurogenic inflammation and a lowering of the threshold to stimulation (Wheeler et al., 2014), which may be attributed to activation of cyclooxygenase and the p38 MAP kinase (Zhang et al., 2011). TNFα binds to its receptors and initiates the generation and release of inflammatory mediators produced by immune cells, including MCs (Yang et al., 2018). However, a study of IC pain models that displays an increased number of MCs fails to suggest a role for TNFα in initiating nociception (Rudick et al., 2008).

IL-33 (Martin Jensen et al., 2018) and IL-1beta that secreted from MCs in response to inflammatory molecules, such as lipopolysaccharide and SP, may involve in the processing of local inflammation and hypersensitivity (Coelho et al., 2000; Ebenezer et al., 2018; Taracanova et al., 2018). The neuro-sensitizing effects of some inflammatory cytokines generated and secreted from MCs, such as IL-2, IL-5, IL-6, IL-9, IL-10, IL-11, IL-16, IL-37 and platelet-derived growth factor (Mukai et al., 2018; Conti et al., 2019), need to be validated.

NGF

NGF is believed to be an important mediator in peripheral hyperalgesia (Pezet and McMahon, 2006). NGF is stored and released by a range of cell types, such as MCs, macrophages, and the sensory and sympathetic neurons (Bandtlow et al., 1987; Liu et al., 2021).

A vitro study reveals that MCs can synthesize, store, and release NGF in response to antigen/IgE stimulation (Leon et al., 1994), while NGF induces human MCs differentiation, maturation, and degranulation (Skaper, 2017). On the one hand, NGF released from MC have profound implications in pain-associated pathobiology, such as osteoarthritis pain (Sousa-Valente et al., 2018) and visceral hypersensitivity (Li et al., 2019). MC-derived NGF may participate in long–lasting peripheral sensitization by governing the enteric synaptic plasticity (Zhang et al., 2018). On the other hand, as MCs express receptors for NGF (Tam et al., 1997), endogenous NGF can elicit the degranulation of MCs, which may be relevant to the early stages of peripheral sensitization and inflammation (Marshall et al., 1990; Groneberg et al., 2005; Sousa-Valente et al., 2018) as well as central sensitization (Kissel et al., 2017).

From the foregoing, it can be concluded that the crosstalk between NGF and MCs may contribute to tissue inflammation and hyperalgesia *via* amplifying each other's effects. However, the detailed mechanisms of their interaction warrant further research.

CONCLUSION

The recent flood of evidence demonstrates the involvement of MCs in painful conditions and suggests a possible mechanism of MCs to pain pathobiology. Noxious stimuli can rapidly activate resident MCs at the injured site, where they release neuro-sensitizing molecules that induce peripheral sensitization, local inflammation, and the recruitment of other immune cells. Meanwhile, MCs interact with mediators that are critical for the maintenance of pain. MCs also modulate nociception centrally *via* enhancing neuronal sensitivity and altering the permeability of the blood-brain barrier (Esposito et al., 2001), allowing the infiltration of additional cells (**Figure 2**).

The involvement of the immune system in pain appears to be more common than once thought, as common analgesics are often not sufficient to control pain associated with MC activation (Butterfield, 2009; Aich et al., 2015). Systemic MC activation disease (MCAD) is characterized by the accumulation of genetically altered dysfunctional MCs with the abnormal release of these cells' mediators. Although therapeutic alternatives in MCAD patients with pain are drugs that profoundly stabilize MCs, it remains a challenge considering its adverse effects on human beings (Wirz and Molderings, 2017). Based on the demonstrated efficacy in pain, analgesics that can significantly mitigate MC degranulation, such as morphine (Vincent et al., 2016), *Palmitoylethanolamide* (D'Amico and Impellizzeri, 2020), and ketotifen (Klooker et al., 2010),

are promising for treating all those painful conditions in which MC activation is the main cause. Pharmacological targeting of MC proliferation, specific surface antigens, and downstream signaling pathways, in addition to stabilizing MCs, may improve analgesics therapy (Molderings et al., 2016).

Given that MC serves as important source of proinflammatory mediators in sustained nociceptive sensitization, new strategies to manipulate crosstalk between neurons and MC hold considerable promise. However, mechanisms of pain are still emerging, and the molecular mechanisms of MC-mediated pain are worth exploring.

AUTHOR CONTRIBUTIONS

WF and LM designed and drafted the manuscript and figures. QL analyzed the data. QL, FH, and HH revised the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Molecular Neuroscience

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Harnessing the Activation of RIG-I Like Receptors to Inhibit Glioblastoma Tumorigenesis

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Glioblastoma (GB) is an incurable form of brain malignancy in an adult with a median survival of less than 15 months. The current standard of care, which consists of surgical resection, radiotherapy, and chemotherapy with temozolomide, has been unsuccessful due to an extensive inter- and intra-tumoral genetic and molecular heterogeneity. This aspect represents a serious obstacle for developing alternative therapeutic options for GB. In the last years, immunotherapy has emerged as an effective treatment for a wide range of cancers and several trials have evaluated its effects in GB patients. Unfortunately, clinical outcomes were disappointing particularly because of the presence of tumor immunosuppressive microenvironment. Recently, anti-cancer approaches aimed to improve the expression and the activity of RIG-I-like receptors (RLRs) have emerged. These innovative therapeutic strategies attempt to stimulate both innate and adaptive immune responses against tumor antigens and to promote the apoptosis of cancer cells. Indeed, RLRs are important mediators of the innate immune system by triggering the type I interferon (IFN) response upon recognition of immunostimulatory RNAs. In this mini-review, we discuss the functions of RLRs family members in the control of immune response and we focus on the potential clinical application of RLRs agonists as a promising strategy for GB therapy.

Keywords: glioblastoma, immunotherapy, RIG-I like receptors (RLRs), RIG-I, MDA5, LGP2, RLRs agonists

INTRODUCTION

Glioblastoma (GB) is the most frequent and aggressive primary adult brain tumor, with a median overall survival (OS) of approximately 1 year (Ostrom et al., 2014). First-line therapy for newly diagnosed GB consists of maximal surgical resection of the tumor, followed by radiotherapy (RT) and concomitant adjuvant chemotherapy with the alkylating agent Temozolomide (TMZ; Stupp et al., 2005, 2009). Unfortunately, the disease remains incurable and often returns as recurrent GB (Lieberman, 2017). Limited progress in the development of more effective therapeutic approaches for GB is mostly due to its heterogeneous genetic, molecular landscape, and cell plasticity (Brennan et al., 2013; Meyer et al., 2015; Gangoso et al., 2021).

GB can be subdivided into primary and secondary subtypes on the basis of their clinical presentation (Dunn et al., 2012). Despite these GB subtypes being morphologically and clinically indistinguishable and sharing the same devastating prognosis, they revealed differences in genetic alterations, such as gene copy number aberrations, changes in chromosome structure, and genetic instability (Cancer Genome Atlas Research Network, 2008). Multiple genetic drivers are involved in the onset of GB, including amplification of epidermal growth factor receptor (*EGFR*) gene and mutations in telomerase reverse transcriptase (*TERT*), isocitrate dehydrogenase (*IDH*), phosphatase and tensin homolog (*PTEN*), platelet-derived growth factor receptor alpha (*PDGFR*α), and neurofibromatosis type 1 (*NF1*) genes (Stoyanov and Dzhenkov, 2018). Based on this evidence, several targeted therapies for GB have been tested, such as against growth factor receptors (i.e., EGFR) and downstream pathways (i.e., PI3K/AKT/mTOR and MAPK; Le Rhun et al., 2019). However, these approaches have shown only occasional responses in patients, and none of them has been formally validated as effective in clinical trials (Touat et al., 2017). Furthermore, current clinical immunotherapy strategies have largely been disappointing (Weenink et al., 2020; Medikonda et al., 2021), due to the presence of tumor immunosuppressive microenvironment. For this reason, the activation of an anticancer innate immunity in the tumor microenvironment (TME) stands as an emerging option to increase immunogenicity also in GB (Elion and Cook, 2018).

Host innate immunity represents the first line of defense and mediates the detection of danger signals through pattern recognition receptors (PRRs), thereby modulating the expression of cytokines and chemokines that recruit T-lymphocytes to the affected tissue, increasing antigen presentation and cross-priming to antigen-specific T-cells (Takeuchi and Akira, 2010; Shalapour and Karin, 2015).

Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) belong to the PRRs family and they are key sensors of both viral and host-derived RNAs, thus mediating the activation of the innate immune system through the type I interferon (IFN) response (Rehwinkel and Gack, 2020).

Numerous findings have underlined that the activation of RLRs signaling in the tumor triggers several effects: (i) tumor cell death; (ii) activation of innate immune cells in the TME; (iii) increased recruitment and cross-priming of adaptive immune effectors, especially in poorly immunogenic, non-T-cell inflamed tumors. These evidences have brought out the possibility to target RLRs for anti-cancer therapy. To date, synthetic RLRs mimetics are under investigation in pre-clinical and early clinical studies for the treatment of gliomas, multiple myeloma, breast, pancreatic, and ovarian cancers (Sabbatini et al., 2012; Okada et al., 2015; Dillon et al., 2017; Mehrotra et al., 2017).

In this mini-review we provide an overview of immunotherapeutic approaches tested in GB patients and discuss the role of RLR family members in the control of the immune response, focusing on the recent developments of RLRs mimetics as new therapeutic perspectives for GB therapy.

BRIEF OVERVIEW OF IMMUNOTHERAPEUTIC APPROACHES IN GLIOBLASTOMA

Clinical use of immunotherapies for brain tumors represents a great challenge particularly due to the blood–brain barrier and the unique immune tumor microenvironment afforded by the central nervous system (CNS)-specific cells. Recently, the discovery of the CNS lymphatic system eroded the concept of CNS as an "immune privileged site", thus prompting the translation of immunotherapy to brain malignancies (Aspelund et al., 2015; Louveau et al., 2015). In GB, four main immunotherapeutic approaches are under investigation in clinical trials: (i) immune checkpoint inhibitors (ICIs); (ii) vaccination with peptides or dendritic cells (DCs); (iii) adoptive transfer of effector lymphocytes; and (iv) oncolytic virotherapy (Weenink et al., 2020; Majc et al., 2021).

ICIs strategy is based on the use of monoclonal antibodies able to block receptors (i.e., PD-1) or their ligands (i.e., PD-L1) expressed by immune cells and tumor cells to elicit an effective antitumor immune $CD8^+$ T cell response (Darvin et al., 2018). ICIs have been extensively studied for GB, used either as monotherapy or in combination with RT and TMZ. CheckMate 143 (NCT02017717) was the first phase III trial to evaluate the efficacy of anti-PD1 nivolumab vs. Vascular Endothelial Growth Factor (VEGF)-inhibitor bevacizumab in patients with recurrent GB. Overall survival (OS) of patients was not improved after treatment with nivolumab compared to bevacizumab, with a median survival time of 9.8 and 10.0 months, respectively (Reardon et al., 2020a).

Currently, two ongoing phase III clinical trials, Checkmate 498 (NCT02617589) and Checkmate 548 (NCT02667587), are evaluating the effectiveness of anti-PD-1 and RT combined therapy, with and without TMZ, in patients with newly diagnosed O-6-methylguanine DNA methyltransferase (MGMT)-unmethylated GB or MGMT-methylated GB, respectively (Lim et al., 2017). Safety analysis from these trials showed that these combinations are well-tolerated, but OS does not appear to increase in treated patients.

Interestingly, two recent Phase II clinical trials based respectively on the administration of the anti-PD-1 antibody nivolumab before and after surgery (NCT02550249), and on the use of the anti-PD-L1 antibody durvalumab in combination with RT (NCT02336165), have shown promising results prolonging the OS of GB patients (Reardon et al., 2019; Schalper et al., 2019). These results prompted to focus attention on the development of combinatorial therapy of ICIs with RT and/or chemotherapy as well as on alternative immunotherapeutic strategies for GB. Therapeutic cancer vaccines aim to induce an anti-tumor immune response through the exogenous administration of selected tumor-associated antigens, combined with adjuvants that activate DCs, or even DCs themselves (Saxena et al., 2021).

The variant III of EGFR (EGFRvIII) is a constitutively active mutant of EGFR expressed on GB cells in 25–30% of patients and it represents a recognized target in many peptide vaccination studies (Congdon et al., 2014). A peptide vaccine

targeting EGFRvIII (rindopepimut) was evaluated in GB patients following gross total resection and chemo-radiotherapy, showing a modest improvement of OS (David et al., 2015). However, the multicenter phase III trial ACT IV failed to confirm this initial result and was terminated after interim analysis (Weller et al., 2017).

A phase II trial known as ReACT found that the treatment with rindopepimut plus bevacizumab of patients with recurrent GB increased the OS to 12.0 months compared to 8.8 months with bevacizumab plus vaccine control treatment (Reardon et al., 2020b). While rindopepimut has elicited immune responses and may have some activity in a small and selected cohort of recurrent GB patients, further studies are required to determine the optimal patient population and treatment regimen.

Dendritic cells (DCs) vaccines are based on the use of engineered DCs loaded with tumor-specific antigen(s) with the aim of activating antigen-specific T-cells that selectively eliminate antigen-bearing tumor cells (Van Willigen et al., 2018). A randomized phase-III trial based on the use of autologous tumor lysate-pulsed DC vaccine (DCVax-L) in addition to Stupp protocol in patients with newly diagnosed glioblastoma led to the approval of this vaccine in Switzerland (Liau et al., 2018).

The high heterogeneity of GB has highlighted the need to identify personalized treatments. In this regard, particular interest was given to chimeric antigen receptor (CAR) T cell therapy, based on the use of genetically modifying T cells harvested from the patients. A phase I trial (NCT01109095) of Human Epidermal Growth Factor Receptor 2 (HER-2)-targeted CAR-T cell therapy has shown an acceptable safety profile and some patients have demonstrated stable disease for 8 weeks–29 months (Ahmed et al., 2017). However, one of the biggest pitfalls of this approach remains the difficulty to develop a CAR-T cell therapy that can target all of the clonal populations of GB (Fecci and Sampson, 2019). Pre-clinical results suggest that the use of tri-valent CAR-T cells able to target multiple tumor-specific antigens (i.e., HER2, IL13Rα2, and EphA2) may be more efficacious than mono- or bi-valent CAR-T cells therapy (Bielamowicz et al., 2017).

Further clinical studies are also needed to confirm the efficacy of oncolytic virus therapy in GB. This strategy is based on the intratumoral administration of genetically modified viruses that usually have the ability to selectively replicate inside malignant infected cells. The replication of lytic viruses leads to the destruction of the target cell and further propagation of viral progeny, which can induce an anti-tumor immune response. Some tumor-selective lytic viruses, such as herpes simplex virus, adenovirus, and poliovirus have shown the ability to replicate in GB cells, but they are currently being studied in early phase clinical trials (Martikainen and Essand, 2019).

An urgent aspect that needs to be considered in the further progression of immunotherapy for GB is the tumor microenvironment (TME), which has an influence on tumor initiation, response, and therapy. Immunotherapeutic strategies, including ICIs or CAR-T cells, are specifically aimed at enhancing adaptive anti-tumor immunity. However, these approaches appear less effective in cancers that are poorly immunogenic, showing low levels of tumor-infiltrating lymphocytes (TILs), minimal cross-presentation of tumor neo-antigens, or high levels of immune-suppressive leukocytes tumor-associated macrophages (TAMs; Marincola et al., 2000; Garrido and Algarra, 2001; Woroniecka et al., 2018).

All these findings recommend future treatment strategies that sensitize GB to immunotherapies, through the activation of either adaptive or innate immune response, or combinatorial immunotherapeutic approaches able to hit this tumor at multiple levels.

RLRs: MEMBERS AND MECHANISMS OF ACTIVATION

The RIG-I like receptors (RLRs) are a protein family of cytoplasmic viral RNA detectors composed of three members: RIG-I (Retinoic acid Inducible Gene 1), MDA5 (melanoma differentiation associated factor 5), and LGP2 (laboratory of genetics and physiology 2; Loo and Gale, 2011; Onoguchi et al., 2011).

All RLRs are characterized by a conserved structure, consisting of a central DExD/H box RNA helicase domain with ATPase activity and a carboxy-terminal domain (CTD), which plays a crucial role in detecting immunostimulatory RNAs (**Figure 1**). In addition, the CTD of RIG-I and LGP2 acts as a repressor domain (RD), keeping the two receptors in an inactive form in the absence of stimuli (Loo and Gale, 2011; Onoguchi et al., 2011; Agier et al., 2018). Further, both RIG-I and MDA5 have additional amino-terminal caspase activation and recruitment domains (CARDs) that mediate downstream signaling (**Figure 1**). LGP2 lacks the CARDs and it is widely considered a regulator of RLRs signaling rather than an active receptor, exerting co-stimulatory and inhibitory functions on MDA5 and RIG-I, respectively (Gack, 2014; Reikine et al., 2014; Rehwinkel and Gack, 2020).

RLRs are expressed in a wide range of tissues and they commonly mediate the activation of the innate immune system by triggering type I interferon (IFN) response and induce apoptosis upon recognition of RNAs not usually present in healthy cells (Loo and Gale, 2011; Reikine et al., 2014; Rehwinkel and Gack, 2020).

RIG-I and LGP2 are physiologically found in an auto-repressed state and are activated by the presence of immunostimulatory RNAs that induce their conformational change leading to the binding with ATP (Kowalinski et al., 2011; Bruns et al., 2013). Compared to RIG-I and LGP2, MDA5 shows a more open structural conformation even in the absence of RNA ligands (Berke and Modis, 2012; Brisse and Ly, 2019).

Although RNAs activating RLRs are generally of viral origin, RNAs that are unusual, mislocalized, or misprocessed can activate these receptors (Rehwinkel and Gack, 2020).

RIG-I recognizes short 5′ tri-phosphorylated double-strand RNAs (dsRNAs), single-strand RNAs (ssRNAs) forming secondary structure (i.e., hairpin or panhandle conformations), and RNAs with uncapped diphosphate (PP) groups at the 5′ (Hornung et al., 2006; Pichlmair et al., 2006; Schmidt et al., 2009; Schlee, 2013; Goubau et al., 2014). In order to allow the

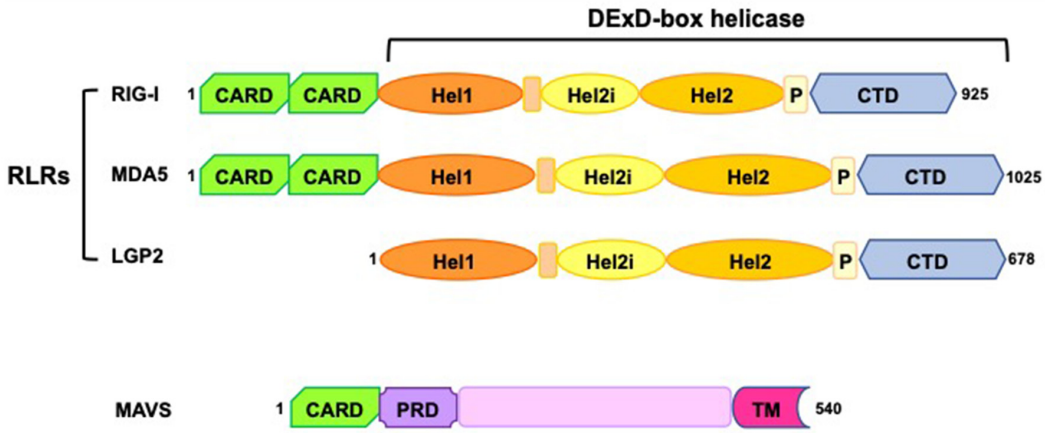

FIGURE 1 | Schematic representation of RIG-I-like receptors (RLRs) and mitochondrial antiviral-signaling protein (MAVS) domains. RLRs have a central DExD-box helicase containing: (i) two conserved helicase domains (Hel1 and Hel2); (ii) a helicase insertion domain (Hel2i) with ATPase activity; (iii) a pincer domain (P); (iv) a C-terminal domain (CTD). Both the helicase domains and the CTD have RNA binding ability. RIG-I and MDA5 have two N-terminal caspase activation and recruitment domains (CARDs), essential for the interaction with MAVS and the induction of downstream signaling. MAVS consists of a single CARD, a proline-rich region (PRD), and a C-terminus transmembrane domain (TM) required for its tethering to mitochondria, mitochondrial-associated membranes (MAM), and peroxisomes.

recognition by RIG-I, the 5′-terminal nucleotide of activating RNAs must not be methylated at the 2′-O position. Indeed, this modification is a crucial hallmark of endogenous RNAs. The steric exclusion of N1–2′O-methylated RNA mediated by the conserved amino acid H830 in the RIG-I RNA binding pocket prevents RIG-I stimulation by self-RNAs (Schuberth-Wagner et al., 2015).

MDA5 and LGP2 bind to long dsRNAs, however, their activating RNAs are less characterized (Hornung et al., 2006; Pichlmair et al., 2009; Schlee, 2013).

Overall, the activation of RLRs is a multi-step process that can be triggered not only by viral RNAs, but also by different regulatory mechanisms that modulate the amount and the activity of these receptors, as well as other components of the RLRs signaling cascade, such as the mitochondrial antiviral-signaling protein (MAVS, also named IPS-1, VISA, or CARDIF; Gack, 2014; Rehwinkel and Gack, 2020).

RLRs: DOWNSTREAM SIGNALING AND FUNCTIONS

Upon ligand recognition, RIG-I and MDA5 interact through their CARDs with MAVS, an adaptor protein needed to initiate the RLRs signaling cascade. Given that LGP2 does not have a CARD, it does neither recruit MAVS nor induce MAVS signaling (Reikine et al., 2014).

MAVS protein belongs to the 14–3–3 protein family, containing a transmembrane domain by which it tethers the intracellular membrane of mitochondria, mitochondrial-associated membranes, and peroxisomes, thereby regulating the RLRs signaling transduction from the cytosol to mitochondria (Gack, 2014; Rehwinkel and Gack, 2020). Importantly, MAVS signaling is dependent on cellular localization, inducing a different antiviral response depending on whether its activation occurs at the peroxisomal or mitochondrial membrane (Dixit et al., 2010; Yu et al., 2010).

At an early time of viral infection, MAVS activates the cytosolic TANK-binding kinase 1 (TBK1) and IκB kinase-ε (IKKε). This event leads to the activation of the transcriptional factors IFN regulatory factor 1 (IRF1) and 3 (IRF3) triggering an immediate IFN-independent signaling that induces a rapid expression of several antiviral or immunostimulatory genes (i.e., IFN-stimulated genes, ISGs).

Conversely, at a later time from infection, mitochondrial MAVS promotes an IFN-dependent signaling pathway activating IRF3/7, which together with nuclear factor-κB (NF- κB) induce the transcription of type I IFNs and ISGs (Dixit et al., 2010; Gack, 2014; Rehwinkel and Gack, 2020; **Figure 2**).

Interestingly, studies from different research groups have reported that the activation of RLRs, triggered by the cytosolic delivery of RNA ligands, requires the function of MAVS and IRF3 leading either to type I IFN production or to intrinsic apoptosis pathway through the expression of the pro-apoptotic genes *Noxa* and *Puma* (Poeck et al., 2008; Rintahaka et al., 2008; Besch et al., 2009; Maelfait et al., 2020; **Figure 2**).

The complexity of the RLRs signaling is finely orchestrated by several combinatorial mechanisms that ensure an appropriate immune response in presence of immunostimulatory RNAs and preserve immune homeostasis under normal physiological conditions. Among these regulatory mechanisms, both non-degradative and degradative ubiquitylation events, deubiquitylation and phosphorylation processes are the most studied post-translational modifications of the RLRs (Reikine et al., 2014; Chan and Gack, 2015; Rehwinkel and Gack, 2020).

Over the past few years, RLRs activation has also been observed in several autoinflammatory and autoimmune diseases, as well as in cancer regardless of viral infection (Rehwinkel and Gack, 2020).

Despite this activation stimulating the immune response, cancer cells are able to escape the immunosurveillance by the selection of non-immunogenic tumor cell variants or by active immunosuppression (Zitvogel et al., 2008). In this regard, the RLRs stimulation could represent an alternative therapeutic approach to overcome tumor-mediated immunosuppression in relation to their role in stimulating type I IFN production and apoptosis (Zitvogel et al., 2015). Recently, promising data have been obtained with the use of specific RIG-I and MDA5 agonists as vaccine adjuvant or potentiator in cancer immunotherapies, giving the possibility to exploit the patient's immune defenses (Kasumba and Grandvaux, 2019).

AGONISTS OF RLRs AS A PROMISING THERAPEUTIC STRATEGY FOR GLIOBLASTOMA

Over the past decade, the scientific community's interest in the understanding of the multiple biological functions of RLRs has particularly been focused on the study of their non-infectious activation as a possible cancer treatment option. These innovative therapeutic approaches aim to improve the expression and the activity of RLRs in order to stimulate innate and adaptive immune responses against tumor cells. In this regard, the use of replication incompetent (oncolytic) viruses and synthetic RLR agonists as anticancer agents are being investigated in a wide range of tumors, including GB (Wu et al., 2017; Elion and Cook, 2018; Iurescia et al., 2020).

The effectiveness of the standard treatments in GB is limited due to the remarkable tumor heterogeneity and the difficulty of eradicating GSCs, which contribute to a large extent to the rapid proliferation of cancer cells, therapeutic resistance, and immune attenuation (DeCordova et al., 2020; Majc et al., 2021). For this reason, the use of immunotherapeutic approaches based on the activation of RLRs could be a good strategy for the treatment of GB, with the aim to increase tumor cell death *via* mitochondrial apoptosis and to overcome the obstacle of immunosuppression.

Glas et al. (2013) provided the first *in vitro* evidence on the potential benefit of the use of RLRs agonists to counteract tumor growth in GB, demonstrating that the induction of the immune response through the activation of RIG-I and MDA5 targets different populations of GB cells. Specifically, the stimulation of these receptors by polyinosinic-polycytidylic acid [p(I:C)] and 5′-triphosphate RNA (3pRNA) in human primary GB (pGB) leads to the activation of the innate immune system [evaluated by the secretion of the in C-X-C motif chemokine ligand-10 (CXCL10) and type I IFN], and to the induction of apoptosis. Interestingly, the authors found that the treatment with p(I:C) and 3pRNA target tumor cells with and without stem cell feature to the same extent, having only mild toxicity in human non-malignant neural cells (Glas et al., 2013).

The expression level of RIG-I or MDA5 in GB is another important aspect to be considered for the clinical application of this therapeutic option. Glas et al. (2013) reported that pGB CSCs have low baseline expression levels of these receptors, which can be increased through the treatment with IFN-β.

Accordingly, a recent study has shown very low protein levels of RIG-I in GB specimens as well as in different human GB cell lines when compared to healthy brain tissue and non-tumor brain cell lines, respectively (Bufalieri et al., 2020). In this study, the authors have demonstrated that RIG-I protein levels are inversely associated with the expression of the RNA-binding Ubiquitin Ligase MEX3A, known to play an oncogenic role in

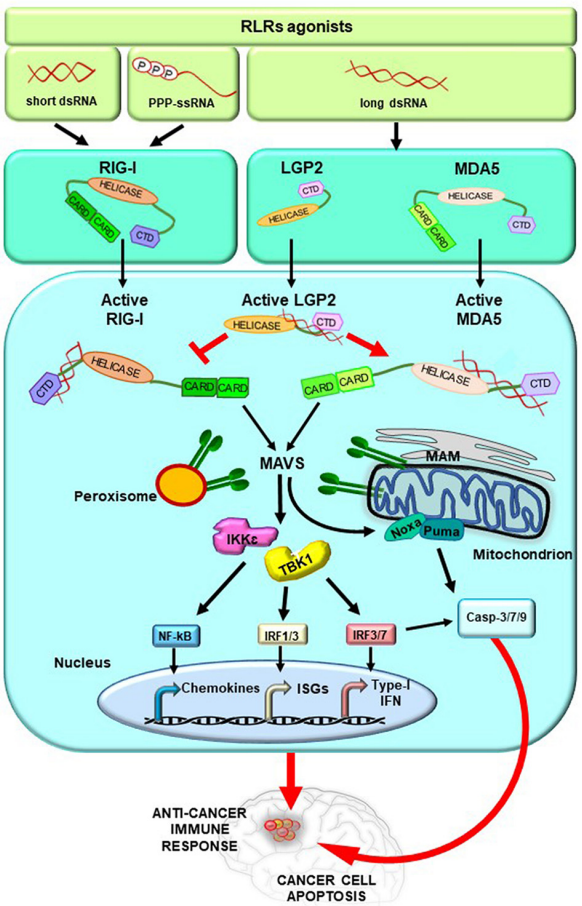

FIGURE 2 | RLRs signaling pathway activation. Upon binding of immunostimulatory RNAs, RLRs undergo conformational changes leading them to an active state. These events are tightly regulated by ubiquitylation and phosphorylation processes (not shown for simplicity) and by the action of LGP2, which stimulates MDA5 and inhibits RIG-I. The activation of RIG-I and MDA5 induces the exposition of their CARDs for the interaction with MAVS. MAVS is located on mitochondria, mitochondrial-associated membranes (MAM), and peroxisomes and transduces the signal to TANK-binding kinase 1 (TANK1) and IkB Kinase ε (IKKε). Subsequently, the interferon regulatory factors 1, 3, and 7 (IRF1, 3, and 7) are activated together with the transcription factor nuclear factor-κB (NF-κB), leading to the expression of type I interferon (IFN) and other genes, such as IFN-stimulated genes (ISGs). In addition, activated MAVS promotes intrinsic/mitochondrial apoptosis through the expression of the pro-apoptotic genes *Noxa* and *Puma*, whose encoded proteins finally induce the cleavage of the caspases 3, 7, and 9 into the active forms. Overall, these molecular events stimulate anticancer immune response and lead to cancer cell apoptosis.

several tumors (Jiang et al., 2012; Huang et al., 2017; Liang et al., 2020; Wang et al., 2020, 2021; Wei et al., 2020).

Remarkably, it has been found that MEX3A binds and ubiquitylates RIG-I, thus promoting its proteasomal degradation. Of note, the genetic depletion of MEX3A leads to an increase of RIG-I protein levels in GB and to a reduction of tumor growth, although it is still under investigation whether this effect is mediated by a consequent activation of RIG-I (Bufalieri et al., 2020).

These findings suggest that targeting these receptors with specific agonists could lead to strong activation of the immune response and induction of apoptosis in tumor cells. Particularly, the anticancer effects promoted by the stimulation of RIG-I/MDA5/MAVS signaling are triggered by the release of type I IFNs, chemokines, and pro-inflammatory cytokines. This event results in cancer cell apoptosis either by IFN-dependent or IFN-independent manner. In addition, the production of chemokines and cytokines by RIG-I/MDA5 in TME activates several innate immune effectors, such as NK cells and macrophages, and increases the recruitment and the cross-priming of adaptive immune effectors (e.g., $CD8^+$ T-lymphocytes), while reducing the T-regulatory cell differentiation. The maturation and activation of antigens-presenting cells (e.g., macrophages and DCs) result in an increased presentation of cancer-associated antigens to $CD8^+$ T cells, which leads to cancer antigen-specific cytotoxicity (Wu et al., 2017; Elion and Cook, 2018; Iurescia et al., 2020). In the light of these evidence, RLRs mimetics for the treatment of GB could be also used to enhance the efficacy of other immunomodulatory drugs. In this regard, new therapeutic approaches that combine the use of RLRs agonists and ICIs have already shown good results in pre-clinical and clinical studies (i.e., NCT03065023; NCT03739138; NCT03203005; NCT03291002) for the treatment of different tumors (Elion et al., 2018; Middleton et al., 2018; Elion and Cook, 2019; Meister et al., 2019).

Several studies have reported that radiotherapy activates type I IFN production, indicating that the IFN signaling plays an important role in the tumor cytotoxicity and/or the activation of the immune response induced by the ionizing radiation (IR) treatment (Tsai et al., 2007; Chajon et al., 2017; Van Limbergen et al., 2017; Turgeon et al., 2019). In particular, RIG-I is essential for the cytotoxic IFN-β response and apoptosis induced by IR in human D54 GB cell line both *in vitro* and *in vivo*, demonstrating the role of this receptor in mediating the RLR tumor cells response to IR (Ranoa et al., 2016). On the contrary, LGP2 enhanced by IR protects the human D54 GB cell line from the cytotoxic effect induced by radiotherapy. Indeed, high LGP2 expression levels are associated with poor clinical outcome in GB patients (Widau et al., 2014). Unlike RIG-I, which represents a powerful resource for the induction of both innate immune response and apoptosis after IR treatment, MDA5 does not significantly contribute to the promotion of these processes (Ranoa et al., 2016).

To date, several early clinical trials based on the use of the synthetic RLRs agonist p(I:C) stabilized with poly-l-lysine and carboxymethylcellulose [p(I:C-LC)] in combination with radiation and TMZ in adult GB patients show an impressive increase of OS (Butowski et al., 2009; Rosenfeld et al., 2010). Further, p(I:C-LC) has also been used as a tumor peptide-based vaccine adjuvant in low-grade glioma patients achieving promising results (Okada et al., 2015).

Overall, these findings suggest the potential of integrating radiotherapy and/or chemotherapy and immunotherapy based on the activation of RIG-I for the development of new clinical perspectives for GB therapy.

CONCLUSION

The development of novel and more effective treatments represents a dramatic therapeutic emergency for GB patients. In the last years, many clinical trials testing alternative therapeutic approaches for GB, including immuno- and targeted molecular-therapy have been launched. Unfortunately, the clinical response has been mild to moderate at best and observed on a very limited number of patients. The presence of dysfunctional and deregulated immune cell subpopulations constitutes a major challenge in the development of alternative immunotherapies in the treatment of GB.

GB patients and preclinical models have reported several mechanisms of systemic immunosuppression: the sequestration of T cells in the bone marrow, the expansion of T-regulatory (Treg) cells which are responsible for immune tolerance and promotion of tumor growth, the suppression of natural killer (NK) cells, and the increased expression of tumor-associated macrophages (TAMs; Wiendl et al., 2002; Gabrilovich et al., 2012; Chen and Hambardzumyan, 2018). The high heterogeneity of GB appears to reflect distinct GB immune subsets based on the molecular signature (Luoto et al., 2018). A number of evidence has underlined that specific genetic alterations or epigenetic signatures can be associated with a better response to immunotherapy, and could help for selecting subgroups of GB patients (Parsa et al., 2007; Rutledge et al., 2013; Berghoff et al., 2017; Gangoso et al., 2021).

Recently, approaches aimed to activate intrinsic cellular immunity in the TME have acquired great interest, based on the capability of the RLRs signaling to induce cancer cell apoptosis while orchestrating innate and adaptive immune responses against tumor antigens. In particular, the use of synthetic RLRs mimetics is being investigated in preclinical and early clinical studies of several hematological and solid tumors (Sabbatini et al., 2012; Okada et al., 2015; Dillon et al., 2017; Mehrotra et al., 2017). However, the potential success of RLR agonists for the treatment of GB requires that some issues have to be warmly considered, such as the possible on-target induction of autoimmunity or the induction of a cytokine "storm" that could pose a threat to patient safety (Trinchieri, 2010; Buers et al., 2016; Lee-Kirsch, 2017). Another major obstacle to the widespread use of RLRs agonists in cancer treatment is their delivery to tumor cells. Palmer et al. (2018) focused on this aspect with the aim to generate stable, specific, and potent RIG-I ligands that retain functionality *in vivo*.

Although future works are needed for the translation of RLRs agonists in clinical practice, the multifaceted mechanisms by

which they eliminate tumor cells represent a promising weapon to fight this devastating and incurable tumor.

AUTHOR CONTRIBUTIONS

FB and IB performed the literature research and drafted a first version of the manuscript. LDM and PI supervised and coordinated the work as well as wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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The Role of T Cell Senescence in Neurological Diseases and Its Regulation by Cellular Metabolism

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Immunosenescence is a state of dysregulated leukocyte function characterised by arrested cell cycle, telomere shortening, expression of markers of cellular stress, and secretion of pro-inflammatory mediators. Immunosenescence principally develops during aging, but it may also be induced in other pathological settings, such as chronic viral infections and autoimmune diseases. Appearance of senescent immune cells has been shown to potentially cause chronic inflammation and tissue damage, suggesting an important role for this process in organismal homeostasis. In particular, the presence of senescent T lymphocytes has been reported in neurological diseases, with some works pointing towards a direct connection between T cell senescence, inflammation and neuronal damage. In this minireview, we provide an overview on the role of T cell senescence in neurological disorders, in particular in multiple sclerosis and Alzheimer disease. We also discuss recent literature investigating how metabolic remodelling controls the development of a senescence phenotype in T cells. Targeting metabolic pathways involved in the induction of senescent T cells may indeed represent a novel approach to limit their inflammatory activity and prevent neuroinflammation and neurodegeneration.

Keywords: immunosenescence, T cell, neuroinflammation, neurodegeneration, immunometabolism

INTRODUCTION

Aging is a natural process that has to deal with a multitude of challenges, and loss of organismal homeostasis during aging is frequently associated with increased susceptibility to infection, cancer, cardiovascular disease and autoimmunity in the elderly (1–4). A dysregulation of the immune system, 'termed immunosenescence', is a hallmark of the aging process, and is thought to play a central role in the higher likelihood of developing such pathologies. Senescent immune cells display a dysfunctional immune profile, including altered cytokine production, limited proliferative capacity and reduced chemotactic and phagocytic potential (5). Immunosenescence is typically associated with chronological age, and it is tightly connected to the process of 'inflammaging', i.e. the chronic and systemic low-grade inflammation observed in the elderly (6). However, premature immunosenescence, in particular in T lymphocytes, has also been observed in patients with chronic

viral infections and in autoimmune diseases (7–10). Thus, it is essential to appreciate those influences in the context of immunosenescent manifestations.

Age represents the main risk factor for the development of several neurological disorders, in particular chronic neurodegenerative pathologies such as Alzheimer disease (AD) and Parkinson disease (PD), where inflammaging is proposed to play a relevant role in the disease course (11). Blood-borne immune cells infiltrating the central nervous system (CNS) and causing direct or indirect neuronal damage also have a central role in CNS pathologies such as multiple sclerosis (MS), traumatic brain injury (TBI), ischemic stroke, AD and PD (12–16). Among immune cells, T lymphocytes represent key players in neurological diseases, where they cause detrimental inflammation taking place in the CNS, but also participate in regulatory mechanisms aimed at protecting neurons from the inflammatory damage (12–16). Intriguingly, an increasing number of studies reported the presence of T cell subsets with a senescent-like phenotype in patients with neurological disorders (senescent T cells, sTC). Here, we summarise our knowledge on the role of sTC, in particular $CD4^+$ and $CD8^+$ conventional T cells, in neuroinflammation and neurodegeneration. We also provide an overview of recent works showing how intracellular metabolic reprogramming may modulate the development of a senescent phenotype in T lymphocytes.

T CELL SENESCENCE IN NEUROLOGICAL DISEASES

T cell senescence is characterised by a decline in naïve T cell number and clonal diversity, which are mainly caused by age-associated thymic atrophy and reduced homeostatic proliferation of naïve-resting T cells. sTC also show loss of their proliferative capacity upon T cell receptor (TCR) reactivation, accelerated telomeric erosion, and accumulation of DNA damage. Throughout the development of a senescent phenotype, T cells downregulate co-stimulatory markers like CD28, while up-regulating natural killer cell-associated molecules, including the killer lectin receptor G1 (KLRG1) (8, 17, 18). These alterations induce a refractoriness of sTC to TCR-mediated activation, but, in parallel, they potentially augment antigen-independent effector functions. Finally, sTC display a pathogenic phenotype characterised by the secretion of several pro-inflammatory mediators, such as the cytokines tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), collectively known as senescence-associated secretory phenotype (SASP) (8, 17, 18) (**Figure 1A**). sTC may thus support the systemic low-grade inflammation observed in the elderly, i.e. inflammaging. However, to what extent sTC are involved in neurological diseases is still uncertain.

Development of a senescent phenotype in T cells may be relevant in MS, an autoimmune disease characterised by CNS

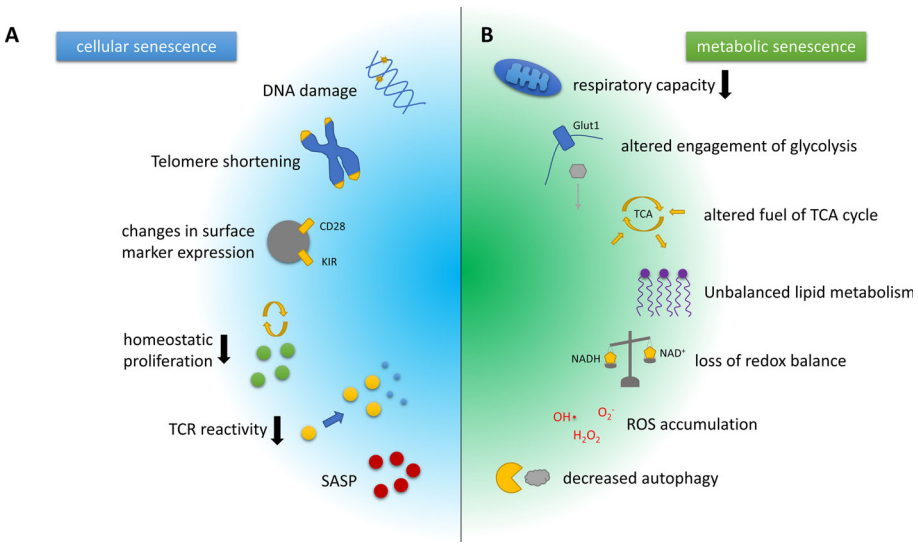

FIGURE 1 | Hallmarks of cellular and metabolic senescence and/or aging in T cells. **(A)** Key characteristics of cellular senescence include DNA damage and telomere erosion that contribute to genomic instability and dysregulation of the epigenome. Phenotypically, T cell senescence is associated with a loss of surface CD28 expression and the upregulation of innate T cell markers such as the killer cell immunoglobulin-like receptors (KIRs). The involution of the thymus increases the homeostatic pressure on T cells. Senescent T cells, however, show a diminished capacity for homeostatic proliferation, and additionally feature reduced T cell receptor (TCR) reactivity. Another hallmark of T cell senescence is the production of pro-inflammatory mediators, collectively known as senescence-associated secretory phenotype (SASP). **(B)** Key metabolic characteristics associated with T cell senescence and aging often closely interwoven and include a decline in the respiratory capacity or efficacy and substantially altered engagement of glycolysis, as well as alterations in the pathways used to fuel the tricarboxylic acid (TCA) cycle, which are likely context- and cell subset-dependent. The same holds true for the dysbalanced lipid metabolism observed in senescent/aged T cells. In addition, ratios of coenzymes for metabolic reactions such as NAD/NADH that are crucial for balancing the cellular redox state are shifted in senescent cells. Furthermore, reactive oxygen species (ROS) accumulate and eventually cause DNA and protein damage. Damaged organelles, cell membranes and proteins are usually degraded by autophagy, another pivotal mechanism that is attenuated by the aging or the senescent process.

infiltration of peripherally activated immune cells that cause neuroinflammation, neuronal death and disability (19). In particular, myelin-specific T cells are key players in the disease, and recent works highlighted the potential involvement of sTC, in particular $CD4^+$ T cells, in MS (**Table 1**). Accelerated thymic involution was reported in MS patients with either relapsing-remitting (RRMS) or primary progressive (PPMS) MS forms (20), and initial works also found an increased percentage of $CD4^+CD28^-$ T cells in a subset of MS patients (mainly RRMS), preferentially producing IFN-γ (21). $CD4^+CD28^-$ T cells were subsequently detected in MS brain biopsies, where they displayed a cytotoxic phenotype and expressed CX3C chemokine receptor 1 (CX_3CR1), which binds to chemokine (C-X3-C motif) ligand 1 (CX3CL1) (22). As CX3CL1 is upregulated in the cerebrospinal fluid (CSF) and brain of MS patients, compared to healthy controls, the authors speculated a CX3CL1-mediated recruitment of these highly inflammatory cells in the MS brain. Importantly, some $CD4^+CX3CL1^+$ brain cells where in close proximity to cleaved caspase-3 $(cCASP3)^+$ oligodendrocytes, suggesting that they may cause direct oligodendrocyte death and demyelination (22). Another recent work performed a longitudinal analysis of several markers in T cells from blood, CSF and brain samples obtained post-mortem from patients with advanced disease (mainly progressive MS) (23). Authors found that $CD8^+$ T cells in white matter lesions displayed a more chronically activated, effector memory-like profile, compared to their blood counterparts. They also proposed that chronic reactivation in the brain, confirmed by limited TCR diversity in the $CD8^+$ cell population, was caused, at least in part, by specific reactivity against Epstein Barr virus-infected B cells (23). These T cells also presented a cytotoxic phenotype, and in some cases they co-localised with $cCASP3^+$ cells in the brain, indicating putative cytotoxic activity *in situ*. However, the expression of the senescent marker CD57 was comparable between blood-derived T cells and brain T cells (23), which might exclude induction of cell senescence in such chronically activated, brain-infiltrating T cells.

T cell senescence was also suggested to play a role in AD, a multifactorial neurodegenerative disorder characterised by progressive neuronal death and development of dementia (**Table 1**). AD has long been viewed as a 'pure' neurodegenerative disease, but recent work highlighted the importance of both local and peripheral immune system, including T cells, in disease onset and progression (29, 30). Telomere shortening in T cells, a marker of proliferative senescence, was initially associated with signs of mild cognitive impairment and dementia in Down syndrome patients, which develop premature AD-like dementia (25, 26). Shorter telomere length in T cells was also reported in AD patients, where it directly correlated with disease severity, higher plasma TNF-α levels, lower CD28 expression by $CD8^+$ T cells, and increased sensitivity to apoptosis in T cells (27). These data suggest that dysfunctional, senescent T cells may associate with higher disease burden and systemic inflammation in AD. Supporting this view, another work showed decreased percentage of naïve $CD4^+$ T cells and increased percentage of terminally differentiated memory $CD4^+$ T cells expressing KLRG1 in AD patients, compared to aged-matched controls, while $CD8^+$ T cell phenotype was unchanged (28). Lack of $CD8^+$ sTC was lately confirmed by another study (31), suggesting that the senescent phenotype might be restricted to $CD4^+$ T cells in AD individuals. However, T cell senescence was recently not confirmed in patients with AD or vascular dementia (32), and conflicting results have also been reported in other forms of dementia and neurodegeneration. Indeed, while a trend towards an increased number of circulating senescent-like $CD8^+CD45RA^+CCR7^-$ T cells was observed in dementia with Lewy bodies (33), a recent work found reduced numbers of senescent/terminally differentiated $CD8^+$ T cells in the blood of PD patients (34). Thus, the role of sTC in neurodegeneration and dementia is still unclear.

The involvement of senescent T cells in other neurological conditions is still unknown. Several studies associated signs of leukocyte senescence like telomere shortening, oxidative stress, and reduced lymphoproliferative potential with onset or severity of other neurological disorders, such as PD (35), ischemic stroke (36) and amyotrophic lateral sclerosis (37). However, such works didn't discriminate between different immune cell populations, and whether T cell senescence plays a role in these diseases has

TABLE 1 | Summary of previous works suggesting the presence of senescent T cells in MS and AD patients.

DISEASE	EVIDENCES OF T CELL SENESCENCE	REFERENCES
Multiple sclerosis	- Accelerated thymic involution in RRMS and PPMS patients	Reviewed in Haegert DG, Mult Scler Int 2011 (20)
	- Increased percentage of circulating IFN-γ-producing $CD4^+CD28^-$ T cells in a subset of RRMS patients	Reviewed in Broux et al., Trends Mol Med. 2012 (21)
	- CX3CL1-mediated infiltration of potentially cytotoxic $CD4^+CD28^-$ T cells in MS brain	Broux et al., J Autoimmun. 2012 (22)
	- Presence of chronically activated, effector memory-like $CD8^+$ T cells with putative cytotoxic activity in white matter lesions of progressive MS patients	van Nierop et al., Acta Neuropathol 2017 (23)
	- Reduced thymic output of naïve T cells and increased percentage of circulating memory-like T cells in paediatric MS patients	Balint et al., Neurology 2013 (24)
Alzheimer disease	- Association of telomere shortening in T cells with mild cognitive impairment and dementia in Down syndrome patients	Jenkins et al., Neurobiol Aging. 2006 (25); Jenkins et al., Neurobiol Aging. 2010 (26)
	- Positive correlation of shorter telomere length in T cells with disease severity, plasma TNF-α levels, lower CD28 expression by $CD8^+$ T cells, and increased sensitivity to apoptosis in T cells in AD patients	Panossian et al., Neurobiol Aging. 2003 (27)
	- Decreased percentage of naïve $CD4^+$ T cells and increased percentage of terminally differentiated memory $CD4^+$ T cells expressing KLRG1 in AD patients	Pellicanò et al., J Neuroimmunol. 2012 (28)

not been investigated. Interestingly, reversed CD4:CD8 ratio, increased percentage of effector memory and reduced numbers of naïve $CD4^+$ T cells, all potential signs of T cell senescence, were correlated with the presence of chronic viral infections and cognitive dysfunctions in old individuals (38–40). Similarly, expansion of $CD8^+CD28^-$ T cells was associated with worse cognitive performances in patients with rheumatoid arthritis (41), and higher numbers of memory $CD4^+$ T cells and $CD8^+$ sTC negatively correlated with cognitive impairment in systemic lupus erythematous patients (42). These works support the idea that persistent T cell activation and maturation observed during chronic viral infections and autoimmune diseases may generate detrimental sTC that cause or sustain cognitive impairment, most likely through pro-inflammatory mechanisms. Additionally, a shift towards a memory/effector-like phenotype in T cells was detected in patients after spinal cord injury (43), possibly caused by the sustained inflammation accompanying the acute damage. Similarly, in a mouse model of TBI, the concussion injury induced an acute lymphopenic response, with reduced thymic size and reduced number of circulating T cells (44). Noteworthy, some of these effects were maintained chronically (60 days after trauma), with T cells also developing into a more pro-inflammatory phenotype and $CD4^+$ T cells displaying an effector/memory-like polarisation (44). These studies suggest that acute CNS injury may cause premature T cell senescence, which would eventually sustain the detrimental systemic inflammatory response.

METABOLIC REGULATION OF T CELL AGING AND SENESCENCE

It is now well established that intracellular metabolic remodelling plays a key role in the activation and engagement of effector functions in T cells (45). T cell metabolism has been extensively investigated in the last few years, including the metabolic profile of T cells from elderly individuals, but relatively little is known about the metabolic remodelling controlling T cell senescence (**Figure 1B**). Also, it remains unclear if such changes are a cause of T cell dysfunction or are rather a by-product of the aging process.

Several studies found mitochondrial alterations in senescent lymphocyte subsets or aged T cells (46–50). Ron-Harel and colleagues reported declined mitochondrial mass and reduced basal and maximal respiratory capacity in T cells from old mice (47). Interestingly, aged naïve T cells also showed lower glycolytic activity, as well as low levels of central carbon intermediates in glycolysis, pentose phosphate pathway, and tricarboxylic acid (TCA) cycle, suggesting and overall slower metabolism, respiration and protein synthesis (47). Upon activation, a specific deficit in the induction of enzymes of one-carbon metabolism in aged cells was shown, that potentially accounts for impaired T cell activation, such as the diminished response to vaccination observed in the elderly (47, 51). Another study showed that mitochondrial proteins involved in the electron transport chain were elevated, but at the same time mitochondrial respiration was impaired in total $CD4^+$ T cells from older individuals. The authors also noted a significantly higher number of autophagosomes containing undegraded mitochondria, and thus suggested a defective mitochondrial turnover by autophagy, which may trigger chronic inflammation (49). Defective autophagy and mitochondrial bioenergetics in $CD4^+$ T cells from older individuals were recently confirmed, and associated with redox imbalance (50). These metabolic alterations furthermore led to a specific proinflammatory profile of IL-17 producing (Th17) cells in older individuals, suggesting that Th17 cells are a pivotal driver of inflammaging. Noteworthy, treatment with the drug metformin enhanced autophagy and normalised mitochondrial function to attenuate age-associated inflammation (50).

Aged, resting cells feature other major metabolic alterations, such as enhanced basal activation of the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway. Lymphocytes of patients carrying dominant-activating mutations in PI3K exhibited an aged phenotype (accumulation of senescent or terminally differentiated cells), in combination with augmented mTOR signalling and glycolysis, and cellular malfunction (52). Intriguingly, treatment with the mTOR inhibitor rapamycin reversed the immunosenescent and dysfunctional effects in these patients (52). Chronic PI3K/Akt/mTOR pathway activation may also be caused by chronic infections in humans, where T cells upregulate glucose transporter 1 (Glut1) and increase their glycolytic activity, which ultimately leads to $CD4^+$ T cell depletion *in vivo* (53, 54). Given that chronic viral infections are known to induce sTC (7), this signalling axis may be relevant in infection-induced T cell senescence. A recent work also reported that T cells from old mice relied heavily on glutaminolysis, and are potentially involved in Th1 and Th17-driven alloimmune responses (55). Treatment with an inhibitor of glutaminolysis prolonged allograft survival specifically in old recipients, whereas in young animals, additional inhibition of glycolysis and oxidative phosphorylation (OXPHOS) was needed to achieve the same effect. Of note, immunosuppressive capacities of glutaminolysis inhibition were specific to $CD4^+$ T cells, and depletion of $CD8^+$ T cells did not alter transplant outcome (55). Memory $CD4^+$ T cells from aged individuals were also shown to upregulate fatty acid β-oxidation (FAO)-coupled mitochondrial respiration, with this process being mediated *via* upregulation of sirtuin-1, a nicotinamide adenine dinucleotide $(NAD)^+$-dependent protein deacetylase, that leads to increased carnitine palmitoyl transferase (CPT1a) transcription and maintains a more lipid-catabolic state (56). However, another study showed an age-related loss of sirtuin-1 in human $CD8^+CD28^-$ T cells, that potentially contributes to metabolic reprogramming towards an enhanced glycolytic capacity (57). Interestingly, constitutive activation of glycolytic flux was reported to limit memory development in $CD8^+$ T cells. Accordingly, blocking glycolytic metabolism promoted the generation of long-lived, functional memory cells. Enforcing glycolysis, on the other hand, drives $CD8^+$ T-cells towards a terminally differentiated state (58). These findings suggest that high glycolytic rates may be involved in the induction of terminally differentiated/sTC.

In an elegant study, Lanna et al. observed that senescent $CD4^+CD27^-CD28^-$ T lymphocytes exhibit endogenously elevated

p38 phosphorylation, triggered by the intracellular metabolic sensor 5' adenosine monophosphate-activated protein kinase (AMPK) (59). In this context, AMPK responds to endogenous DNA damage and also to a fall of intracellular energy levels, thus highlighting an 'intra-sensory' pathway for p38 activation that senses intracellular changes such as glucose deprivation and genotoxic stress. Triggering this pathway leads to inhibition of T cell proliferation and telomerase activity, two typical features of senescent $CD4^+$ T cells (59). The same group also found that terminally differentiated human $CD8^+$ T cells (T_{EMRA}) have decreased numbers of mitochondria and fail to efficiently upregulate glycolysis or OXPHOS following TCR activation, although showing high production of reactive oxygen species (48). These T_{EMRA} cells also showed elevated levels of p38 MAPK, and inhibition of p38 MAPK signalling elevated mitochondrial biogenesis and fitness. In addition, p38 MAPK blockade also induced an increase in autophagy through enhanced interactions between p38 interacting protein (p38IP) and autophagy protein 9 (ATG9) to compensate for the heightened energy demand (48). Of note, the polyamine spermidine is capable of inducing autophagy and undergoes an age-dependent decline (60). Recently, it was shown that spermidine modulates T cell differentiation towards a regulatory phenotype and dietary supplementation with spermidine reduced pathology in a mouse model of T cell transfer-induced colitis (61). Moreover, in a study in humans, spermidine supplementation was able to recover the autophagic flux and cellular functionality in T cells from old donors (62). Interestingly, spermidine was reported to reverse cellular senescence of B cells (63), an effect that has not yet been addressed in T cells.

DISCUSSION AND FUTURE PERSPECTIVES

Despite recent evidence suggesting an involvement of sTC in neurological diseases, many questions are still open. First, most studies analysing parameters such as telomere length and distribution of specific T cell subset are mainly observational, and do not investigate in detail the functional consequences of the observed changes. Second, identification of *bona fide* sTC may be tricky, due to overlapping expression of some senescence markers in effector memory *vs* terminally differentiated *vs* sTC (17, 18). Third, it is still not completely clear why patients with neurological disorders would accumulate sTC, compared to aged-matched individuals. Apart from the age-related reduction in the naïve T cells compartment, the main hypothesis is that circulating T cells undergo continuous antigen-specific or cytokine-induced re-activation, due to low grade systemic inflammation and/or to the presence of persistent CNS-derived antigens. This process would eventually reduce the extent of T cell receptor diversity, and increase the amount of terminally differentiated and potentially exhausted and senescent lymphocytes. Nonetheless, how immunosenescence develops and contributes to neuroinflammation and neurodegeneration remains unclear. Strikingly, a study showed that paediatric MS patients displayed signs of premature T cell senescence, with reduced number of circulating naïve T cells recently emigrated from the thymus, and increased percentage of memory-like cells, resembling the profile of adult individuals (24). This work may support the presence of an early antigen-specific (autoimmune) response inducing T cell maturation/activation, but might also suggest that appearance of a senescent/aged phenotype in T cells could predispose to MS development. Another aspect to consider is the recently hypothesised effect of disease-modifying therapies (DMT) on immunosenescence. Some anti-inflammatory DMT used to treat neuroinflammatory conditions, for example MS, may indeed not only limit the activation capacity of the immune system, but also induce signs of premature senescence of the immune system, such as reduced T cell output from the thymus (64, 65). These effects may differ between different DMT, potentially due to their intrinsic mechanisms of action (65). Thus, these DMT may be partially responsible for the appearance of severe side effects in aged individuals, such as viral reactivation and CNS inflammation and demyelination in MS patients (64, 65), again highlighting the importance of finely balancing aging of the immune system for optimal organismal homeostasis.

An increasing amount of studies showed metabolic alterations in circulating leukocytes in diseases of the CNS, with such metabolic modulation potentially playing a role in the pathogenic activity of different immune cell populations (66). However, direct comparisons of immunosenescence and immunometabolism in neurological diseases is lacking, even though the two features may be strongly interwoven. As an example, a recent work showed that T cells with dysfunctional mitochondria due to mitochondrial transcription factor A (TFAM) deficiency act as accelerators of senescence, and furthermore incite multiple aging-related features including neurological inflammation (67). This supports the notion that alterations of metabolic pathways in immune cells may directly cause inflammation-induced neurodegeneration. Importantly, there is a multitude of potential approaches to counteract age-associated immune cell malfunctions by metabolic intervention, including supplementation of age-limited nutrients such as formate and glycine, and reinforcement of autophagy with spermidine (47, 62). Of note, a recent study reported that mTOR inhibitor therapy in elderly humans decreased the incidence of infections, improved influenza vaccination responses and up-regulated antiviral immunity (68), thus highlighting the potential clinical relevance of such metabolic approaches in age-related immune dysfunctions. Given the therapeutic potential of immunometabolic intervention in neurological diseases (66), this strategy may indeed represent a brand-new approach to limit T cell senescence and dampen T cell-induced inflammation in CNS disorders and aging.

AUTHOR CONTRIBUTIONS

Both authors equally contributed to literature search and manuscript writing, and approved the final version of the manuscript.

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The Role of Platelets in the Stimulation of Neuronal Synaptic Plasticity, Electric Activity, and Oxidative Phosphorylation: Possibilities for New Therapy of Neurodegenerative Diseases

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The central nervous system (CNS) is highly vascularized where neuronal cells are located in proximity to endothelial cells, astroglial limitans, and neuronal processes constituting integrated neurovascular units. In contrast to many other organs, the CNS has a blood-brain barrier (BBB), which becomes compromised due to infection, neuroinflammation, neurodegeneration, traumatic brain injury, and other reasons. BBB disruption is presumably involved in neuronal injury during epilepsy and psychiatric disorders. Therefore, many types of neuropsychological disorders are accompanied by an increase in BBB permeability leading to direct contact of circulating blood cells in the capillaries with neuronal cells in the CNS. The second most abundant type of blood cells are platelets, which come after erythrocytes and outnumber ∼100-fold circulating leukocytes. When BBB becomes compromised, platelets swiftly respond to the vascular injury and become engaged in thrombosis and hemostasis. However, more recent studies demonstrated that platelets could also enter CNS parenchyma and directly interact with neuronal cells. Within CNS, platelets become activated by recognizing major brain gangliosides on the surface of astrocytes and neurons and releasing a milieu of pro-inflammatory mediators, neurotrophic factors, and neurotransmitters. Platelet-derived factors directly stimulate neuronal electric and synaptic activity and promote the formation of new synapses and axonal regrowth near the site of damage. Despite such active involvement in response to CNS damage, the role of platelets in neurological disorders was not extensively studied, which will be the focus of this review.

Keywords: platelets, neurons, neurodegeneration, major brain gangliosides, serotonin, CNS repair

INTRODUCTION

Platelet biology recently gains a particular interest in neuroscience. Quite intriguing that platelet granules, which are organized like vesicles of presynaptic neurons, store key neurotransmitters: dopamine, glutamate, histamine, serotonin, ATP along with proper receptors and transporters for these molecules (Rainesalo et al., 2005). Moreover, platelet granules contain a

significant set of neurotrophic factors such as EGF, NGF and, BDNF (Yamamoto and Gurney, 1990; Au et al., 2014; Kniewallner et al., 2014). Finally, platelets and the CNS might be linked via specific structural similarity of platelets with neurons. Both cell types have complex granule organization with diverse content and regulated secretion, as well as the presence of mitochondria and extensive oxidative phosphorylation. Platelet granule secretion and neuronal synaptic release work virtually identically engaging the same triggers and downstream signaling cascades (Ponomarev, 2018). Platelets also contain a variety of pro-inflammatory factors that could strongly influence the pathology of neurodegenerative diseases (Reed et al., 2000). Nevertheless, the platelet role in neuroscience remains neglected. This review aims to elucidate an important role of platelets in most common neurologic disorders: epilepsy, traumatic brain injury, Alzheimer's disease, multiple sclerosis, and Parkinson's disease. Future perspectives to use platelets and antiplatelet drugs for diagnostics and treatment of neurologic disorders will be also discussed.

EPILEPSY AND TRAUMATIC BRAIN INJURY

Platelets were found in our very recent study (Kopeikina et al., 2020) to actively participate in the development of several epilepsy-associated pathological events in the CNS such as the swift release of serotonin, direct stimulation of neuronal electric activity, blood-brain barrier (BBB) permeabilization, release, and induction of expression of pro-inflammatory mediators, and induction of neuronal oxidative stress (Kopeikina et al., 2020). Previously we found that platelets were also actively involved in TBI pathology (Dukhinova et al., 2018). These findings support the hypothesis that platelets play a significant role in epilepsy development, especially in epilepsy that is associated with TBI.

Epilepsy is a neurological disorder that affects more than 70 million globally and is manifested by the occurrence of seizures due to global or focal abnormally high brain electric activity (Thijs et al., 2019). This disorder is also associated with motor, cognitive, and psychological abnormalities (Devinsky et al., 2018). Although the real cause of epilepsy is unknown, some factors create a predisposition to this disease. Previous studies on epilepsy have mostly focused on genetic factors and pathological events related to neuronal functions in the central nervous system (CNS). Such studies reported that the imbalance between activating and inhibitory circuits in the area of abnormal neuronal electric activity plays a major role in seizure development (Staley, 2015). Blocking inhibitory $GABA_A$ receptors with pharmacological antagonists, such as pentylenetetrazole (PTZ), results in seizure development in several species, including humans and mice. The acute PTZ-induced seizure model is widely used to study the effects of new antiepileptic drugs (Löscher, 2011). Stimulation of activating glutamate receptors with specific agonists also results in epileptic seizures (McKhann et al., 2003). Available antiepileptic drugs mostly change the balance toward inhibitory pathways. Yet more than 30% of epilepsy patients do not respond to these drugs (Thijs et al., 2019). Thus, it is critical to find new drugs to treat epilepsy based on other mechanisms and possibly targeting other cell types besides neurons.

Although the main cause for epilepsy is known, a predisposition to this disorder comes from both genetic and environmental factors such as abnormal CNS development and CNS injuries such as stroke and traumatic brain injury (TBI; Devinsky et al., 2018; Thijs et al., 2019). As many as ∼50% of all epilepsy cases are triggered by initial neuronal injury and are classified as acquired epilepsy (AE). The three stages of AE include: (1) initial neuronal injury, (2) epileptogenesis, and (3) chronic epilepsy (period of spontaneous recurrent seizures). TBI and stroke are the most frequent brain injuries that often result in the development of AE. AE seizures are classified as acute (hours or days post-TBI) and chronic (from weeks to months). The occurrence of seizures following TBI or other insult is classified as immediate (less than 24 h), early (1–7 days), or late (more than 1 week; Tomkins et al., 2008; Lucke-Wold et al., 2015; Glushakov et al., 2016) AE.

Neuronal functions are substantially influenced by a milieu of platelet-derived factors. One of our key results of whole-genome transcriptome profiling followed by real-time RT PCR validation is that platelets induced expression of many genes related to neuronal electric/synaptic activity, neuroinflammation, and oxidative phosphorylation in TBI and epilepsy models (Dukhinova et al., 2018; Kopeikina et al., 2020). Platelets induced the expression of mRNA for pro-inflammatory cytokines *IL-1B*, *IL-6*, and *TNF* (Kopeikina et al., 2020). The oxidative phosphorylation pathway (e.g., expression of mRNA for mitochondrial genes *MT-CO1*, *MT-ATP6*, and *MT-ND6*) was also significantly upregulated, while the glycolysis pathway is downregulated (Kopeikina et al., 2020). Platelets also upregulated the expression of mRNA for several early response genes responsible for neuronal synaptic plasticity, such as *PSD95, TrkB, Syn1, FOSB*, *EGR1*, *ARC*. We also found that platelets stimulated the formation of dendritic spines and new synapses both *in vitro* and *in vivo* (Dukhinova et al., 2018; Kopeikina et al., 2020).

Serotonin (5HT) is known to contribute to thrombosis, but at the same time, this neurotransmitter is involved in the regulation of innate and adaptive immune responses, neuroinflammation, anaphylaxis, and CNS tissue repair (Sotnikov et al., 2013; Starossom et al., 2015; Dukhinova et al., 2018; Kopeikina et al., 2020). During TBI, we showed that platelet-derived 5HT enhances neuronal axonal growth and the formation of new synapses (Dukhinova et al., 2018). We also demonstrated the significant role of platelets and platelet-derived 5HT in the development of PTZ-induced seizures in mice (Gharedaghi et al., 2014; Carhart-Harris and Nutt, 2017). Our *in vitro* and *in vivo* experiments where we did the co-incubation of platelets with brain slices, or with cultured neurons, or adoptive transfer of serotonin-depleted platelets strongly proved that platelet-derived 5HT was required to increase neuronal electric activity (Dukhinova et al., 2018; Kopeikina et al., 2020).

Quite interesting that in both PTZ-induced epilepsy and TBI models, we observed increased neuronal electric activity,

and expression of pro-inflammatory and synaptic plasticity genes (Dukhinova et al., 2018; Kopeikina et al., 2020). Remarkable, intracranial injection of a small volume of platelets, but not saline or platelet-poor plasma, induced severe seizures and epilepsy-like elevated neuronal electrical activity (Kopeikina et al., 2020), which bridge together TBI and epilepsy and may imply the mechanisms of development of acute AE such as post-traumatic or post-stroke epilepsy. An elevated level of oxidative phosphorylation often results in ROS formation leading to oxidative stress in the CNS (Pearson-Smith and Patel, 2017), which was confirmed in our studies where platelets upregulated a large number of mitochondria oxidative phosphorylation genes leading to reactive oxygen species (ROS) formation in neurons leading to neuronal oxidative stress *in vitro* and *in vivo* (Kopeikina et al., 2020).

Thus, we hypothesize that platelets could promote the development of immediate (less than 24 h) AE by directly stimulating neuronal electric activity by robust 5HT release (Kopeikina et al., 2020). Platelets could also stimulate the development of early AE (1–7 days) possibly by upregulation of the number of genes related to neuronal activity and synaptic plasticity, neuroinflammation, and oxidative phosphorylation (Dukhinova et al., 2018; Kopeikina et al., 2020). Finally, platelets could contribute to late AE (more than 1 week) by stimulating axonal regrowth and formation of new synapses in the area around brain injury (Dukhinova et al., 2018), which might lead to the imbalance of excitatory vs. inhibitory circuits (Musto et al., 2016; Pfisterer et al., 2020).

Our studies indicated a new concept that during epilepsy and TBI platelets could enter CNS due to increased BBB permeability and could interact with neuronal cells via cell-cell contacts and/or via secretion of soluble factors. In **Table 1**, we summarized the role of platelets in epilepsy and TBI. During initial CNS insult such as epileptic seizures or TBI, BBB permeability increases and platelets enter CNS perivascular space where they interact with astroglial and neuronal lipid rafts (Sotnikov et al., 2013) and secrete neurotransmitters (serotonin; Kopeikina et al., 2020), cytokines (IL-1α; Sotnikov et al., 2013; Starossom et al., 2015), chemokines (platelet factor 4, PF4; Starossom et al., 2015) and lipid mediators (platelet-activating factor, PAF; thromboxane, etc.; Dukhinova et al., 2018). At this stage, platelets contribute to the development of neuroinflammation leading to further increase in BBB permeability (Kopeikina et al., 2020) and stimulating macroglia activation and leukocyte infiltration from the periphery (Sotnikov et al., 2013; Starossom et al., 2015; Dukhinova et al., 2018; Ponomarev, 2018). Increased levels of neuroinflammation result in a further increase in BBB permeability. At this stage platelets enter CNS parenchyma

TABLE 1 | Platelet-neural crosstalk and possible mechanisms involved in the development of neurodegenerative diseases and central nervous system (CNS) repair[1].

Stage of neuro-pathology	Blood-brain barrier (BBB) permeability	Recognition of major brain gangliosides on astroglial and neuronal lipid rafts and secretion of platelet-derived factors	Effect of platelets on CNS function (Role of platelet-derived factors)
Normal CNS	Intact	No	No effect
Primary CNS damage	Mild BBB permeability	Platelets enter CNS perivascular space, recognize major brain gangliosides on astroglial lipid rafts, and secrete serotonin/5-HT, BDNF, PAF, PF4, and IL-1[2, 3]	Platelets enhance BBB permeability (unknown platelet-derived factor)[6]
			Platelets initiate neuroinflammation by stimulating perivascular macrophages and microglia (PAF, PF4, IL-1)[2–6]
			Platelets promote blood coagulation (5-HT, ATP)[4, 6]
Secondary CNS damage	Severe BBB permeability	Platelets enter CNS parenchyma, recognize major brain gangliosides on neuronal lipid rafts, and continue to secrete serotonin/5-HT, BDNF, PAF, PF4, and IL-1[2–6]	Platelets stimulate neuronal synaptic activity (5-HT, BDNF)[4, 6]
			Platelets stimulate neuronal electric activity (5-HT)[4, 6]
			Platelets induce mitochondrial oxidative phosphorylation and oxidative stress in neurons (unknown platelet-derived factor)[6]
CNS repair	Decreased BBB permeability	Platelet-derived factors result in marked changes in CNS gene expression profile[4, 6]	Platelets induce expression of neuronal early response genes (5-HT, BDNF)[4, 6]
			Platelets induce the formation of dendritic spines and new synapses (5-HT, BDNF)[4]
			Platelets induce axonal outgrowth (5-HT)[4]

[1] *Proposed scheme based on cited below literature data.* [2] *Sotnikov et al. (2013).* [3] *Starossom et al. (2015).* [4] *Dukhinova et al. (2018).* [5] *Ponomarev (2018).* [6] *Kopeikina et al. (2020).*

and directly interact with neurons by stimulating their electric activity via secretion of serotonin (Kopeikina et al., 2020) and stimulating neuronal gene expression related to synaptic plasticity and oxidative phosphorylation (Dukhinova et al., 2018; Kopeikina et al., 2020). Finally increased expression of neuronal synaptic plasticity genes stimulated the formation of new synapses around the area of injury promoting CNS repair and recovery from the disease (Dukhinova et al., 2018; **Table 1**). We believe that this concept could be extended to other neurodegenerative diseases, as discussed below.

ALZHEIMER'S DISEASE

It became quite clear after our recent studies that platelets can stimulate neuronal electric and synaptic activity during traumatic brain injury and epilepsy (Dukhinova et al., 2018; Kopeikina et al., 2020). We believe that similar processes of interaction of platelets with neurons could be found in other types of neurological disorders, such as Alzheimer's disease (AD). AD is a neurodegenerative disorder, which is the common cause of dementia in elderly. Neuropathology includes extracellular β-Amyloid (Aβ) plaques co-localized with ganglioside containing neuronal lipid rafts, neuronal intracellular neurofibrillary tau protein tangles, and neuroinflammation (Masters et al., 2015; Dukhinova et al., 2019). Recent studies demonstrated regulation of BBB permeability by circadian rhythms and sleep (Cuddapah et al., 2019), indicating possibilities for platelet migration into CNS during even early stages of various pathological conditions such as AD. To support this hypothesis, it was shown that Aβ undergoes daily oscillation in interstitial fluid in the vicinity of brain blood vessels, suggesting a possible influx of Aβ from the periphery into CNS (Kress et al., 2018). Very recent studies indicate that platelets can contribute to BBB disruption (Kopeikina et al., 2020; Wu et al., 2021) and can transfer Aβ from blood vessels into CNS (Wu et al., 2021; **Table 1**). Moreover, it was demonstrated that activated platelets from APP/PS1 transgenic mice invade brain parenchyma and are closely associated with astrocytes (Kniewallner et al., 2020) that are enriched with major brain gangliosides in lipid rafts of astroglial limitations and efficiently activate platelets (Sotnikov et al., 2013). Platelets and their secreted factors could affect many cell types involved in the regulation of BBB integrity including endothelial cells, astroglia, and pericytes (Fang et al., 2011; Gonzales et al., 2020; Kniewallner et al., 2020).

Indeed, outside of CNS, platelets are known as the main source of Aβ (Veitinger et al., 2014; Inyushin et al., 2020). Thus, the cellular mechanisms of AD can be effectively studied using platelet sample preparations, due to dramatically increased levels of the Aβ precursor protein (APP) in them in comparison to all peripheral tissues, and all three isoforms of APP (130, 110, and 106 kDa) being detectable within platelets. During platelet activation, full-length APP is cleaved by a Ca^{2+}-dependent cysteine protease, while APP processing is altered in AD patients when compared to healthy individuals. This results in a decreased ratio between the 130 kDa and 106–110 kDa of cleaved APP isoforms, which implies that APP isoform ratios in platelets might act as a biomarker for AD (Tobergte and Curtis, 2013). A recent study demonstrated that Aβ fragments could enter CNS from blood vessels in the mouse model of AD (Bu et al., 2018). Using the parabiosis model, where there was connected the blood system of control (wild-type, WT) mice and transgenic mice with human APP overexpression in the CNS, it was revealed that in a previously healthy WT mouse the brain exhibited signs of AD that include Aβ depositions, neurodegeneration, and neuroinflammation (Bu et al., 2018). Thus, platelets can secrete various processed forms of APP and other substances while infiltrating the brain, which results in the growth of Aβ depositions in the brain and increases the permeability of BBB (Espinosa-Parrilla et al., 2019; Wu et al., 2021). We demonstrated that major brain gangliosides within neuronal lipid rafts in post-synaptic membranes induced platelets' granule release (Sotnikov et al., 2013) suggesting a possible mechanism of Aβ secretion by platelets in the CNS. At the same time, brain-specific gangliosides serve as an anchor point for binding of Aβ peptides, which was critical for AD development in 5XFAD mouse model (Ponomarev, 2018; Dukhinova et al., 2019).

Besides secretion of Aβ peptides, platelets could also stimulate neuronal electric activity vis production of serotonin (Dukhinova et al., 2018; Kopeikina et al., 2020; **Table 1**), while neurons with a high level of electric activity were found in close vicinity of Aβ depositions (Busche et al., 2008; Liu et al., 2018). Thus, we could speculate that platelets contribute to elevated neuronal electric activity in AD. The elevated level of electric activity could even result in the development of seizures in some AD patients (Born, 2015; Kitchigina, 2018). Thus, elevated neuronal activity was shown to contribute to AD pathology via multiple mechanisms (Hefter et al., 2020), while our study indicated the critical role of platelets in the stimulation of neurons (Kopeikina et al., 2020).

MULTIPLE SCLEROSIS

Altered platelet function is also suggested in autoimmune neuroinflammatory CNS diseases, such as multiple sclerosis (MS), where the level of activated platelets in the blood of patients is elevated (Morel et al., 2017). MS is an autoimmune disease of the nervous system (CNS) that affects predominantly young adults leading to substantial neurological disability that include upper/lower motor syndrome. MS and experimental autoimmune encephalitis (EAE; an animal model for MS) involve autoimmune Th1 and Th17 cells that recognize myelin self-antigen such as MBP, MOG, and PLP. MS onset usually occurs with the relapsing-remitting type (RRMS), which is characterized by multiple relapses followed by spontaneous remission. Platelets were found to aggravate EAE (Langer et al., 2012; Sotnikov et al., 2013) and induce the development of gray matter damage (Sonia D'Souza et al., 2018). During the early stages of EAE platelets entered the hippocampus, and this is linked with the establishment of a neuroinflammatory environment because of platelet-neuron associations, but not with inflammatory cell infiltration, emphasizing the key role of platelets at the preclinical stages of the disease (Kocovski et al., 2019). This effect along with enhanced anxiety-like behavior observed in mice with EAE was ameliorated after the platelet

depletion in mice. We have previously shown that platelet-derived 5-HT and PAF (**Table 1**) boosts the differentiation of pathogenic Th1 and Th17 cells during the early stages of MS and EAE (Ponomarev, 2018), while at later stages of the disease, platelets become depleted in granule content but upregulate adhesion molecules such as CD62P to form aggregates with lymphocytes (Starossom et al., 2015). This, in turn, implies that platelets act as a target to alleviate MS symptoms during relapses, among which are subsequent neuropsychiatric symptoms present in these patients (Kocovski et al., 2019). Targeting platelets would be especially effective during the remission period for prophylaxis of future relapses in RRMS. Thus, platelet-neuronal interaction plays an important role during the early stages of MS/EAE and neuronal dysfunctions, which could especially be important for the prevention of future relapses.

PARKINSON'S DISEASE

The role of platelets in Parkinson's disease (PD) is currently unknown. However, there is accumulating knowledge that suggests the possible involvement of platelets in PD pathology. PD is a degenerative disorder caused by the loss of dopaminergic neurons in the substantia nigra, which leads to a decrease in motor and cognitive functions. There is no final answer to what causes PD, though mitochondrial dysfunction is implied to be one of the major reasons. A toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is selectively toxic for dopaminergic neurons, was found to act by inhibiting complex I of the electron transport chain in neuronal mitochondria, which is important for oxidative phosphorylation. It is known that MPTP is widely used as a chemical intermediate for herbicide production, that could contaminate agricultural products and predispose them to PD (Fukuda, 2001). In addition to herbicides, it was found that insecticide rotenone is also toxic for neurons in the substantia nigra affecting complex I in neuronal mitochondria leading to the symptoms of PD (Sherer et al., 2003).

Decreased complex I activity was found in the lymphocytes and platelets isolated from PD patients when compared to healthy controls (Haas et al., 1995; Subrahmanian and LaVoie, 2021). Moreover, a hybrid PD model where mitochondrial DNA from PD platelets was expressed in human teratocarcinoma cells demonstrated decreased complex I activity in mitochondria of these cells. Besides, 1-methyl-4-phenyl-pyridinium ion (MPP^+), the metabolite of MPTP, was proved to deplete adenosine triphosphate in platelets and induce attenuated platelet aggregation and activity, which is a probable mechanism of the anti-aggregation effect found in PD patients (Koçer et al., 2013; Leiter and Walker, 2020). Monoamine oxidase B (MAO-B) enzyme, which is abundant in neuronal and platelet mitochondria, also contributes significantly to MPTP toxicity and the etiology of PD as demonstrated by several studies. Enhanced MAO-B activity has been discovered in PD patients, nevertheless, the findings regarding platelet MAO-B activity in PD patients are not straightforward enough, as other works imply that platelet MAO-B activity is unaffected in PD patients (Bonuccelli et al., 1990). Nevertheless, the ability of platelets to modulate the functions of mitochondria in the CNS by enhancing oxidative phosphorylation and oxidative stress (Kopeikina et al., 2020) could play an important role in PD pathology.

Besides modulation of neuronal mitochondrial functions by platelets, these cells could affect dopaminergic neurons by producing several substances. Among these factors are a platelet-activating factor (PAF; **Table 1**), which is produced by activated platelets and plays a pro-inflammatory role in the development of TBI-associated neuroinflammation affecting astrocytes and microglia (Yin et al., 2017; Dukhinova et al., 2018). In addition to glial cells, the PAF receptor (PAFR) is also expressed in neuronal synapses. Stimulation of neurons via PAFR enhanced long-term potentiation and synaptic vesicle release (Hammond et al., 2016). On the other hand, overstimulation of neurons by PAF caused neuronal apoptosis, although the role of PAFR in this process remains controversial (Bennett et al., 1998; Ryan et al., 2007). Mice deficient in PAFR were found to be resistant to the development of disease in a mouse model of MPTP-induced PD. Thus, platelet-derived factors such as PAF could significantly contribute to the development of PD.

PLATELETS AS MARKERS FOR DIAGNOSTICS

Recently, platelets have come to be regarded as substantial indicators for neurologic diseases of various types. Being multifunctional blood anucleated cells, they are now seen as crucial clinical targets for many neurologic disease pathophysiology. Not only do platelets play a key role in normal hemostasis and thrombosis, but they also are first responders in inflammatory processes (Sotnikov et al., 2013; Starossom et al., 2015; Ponomarev, 2018). They are also involved in a vast range of inflammation-associated pathologies, such as atherosclerosis, cardiovascular diseases, cancer metastasis, and neurodegenerative disorders (Koçer et al., 2013; Franco et al., 2015; Pluta et al., 2018).

During many types of neurodegenerative diseases such as TBI, AD, and MS, platelets change their phenotype and content (Kumar, 2013; Koc et al., 2014; Starossom et al., 2015; Ponomarev, 2018). For example, during AD, platelets show an activated phenotype and there are changes in the processing of platelet Aβ, which could serve as an early marker of AD (Di Luca et al., 1998; Koc et al., 2014). In the mouse model of AD when human APP with Swedish mutation is expressed in the CNS, platelets start to express MMP2 and MMP-9 and damage brain blood vessels in the brain slice model (Kniewallner et al., 2018). This data indicates that CNS-derived amyloid could activate platelets that could contribute to increased BBB permeability. Our data in the epilepsy model also demonstrated that platelets contribute to increased BBB permeability (Kopeikina et al., 2020). In MS, platelets decrease their level of serotonin in dense granules and the level of PF4 in α-granules and upregulate adhesion molecules such as CD62P (Starossom et al., 2015). Quite interestingly the exhaustion of platelet granule content and upregulation of CD62P make platelets anti-inflammatory indicating that platelets play a differential role in the early and

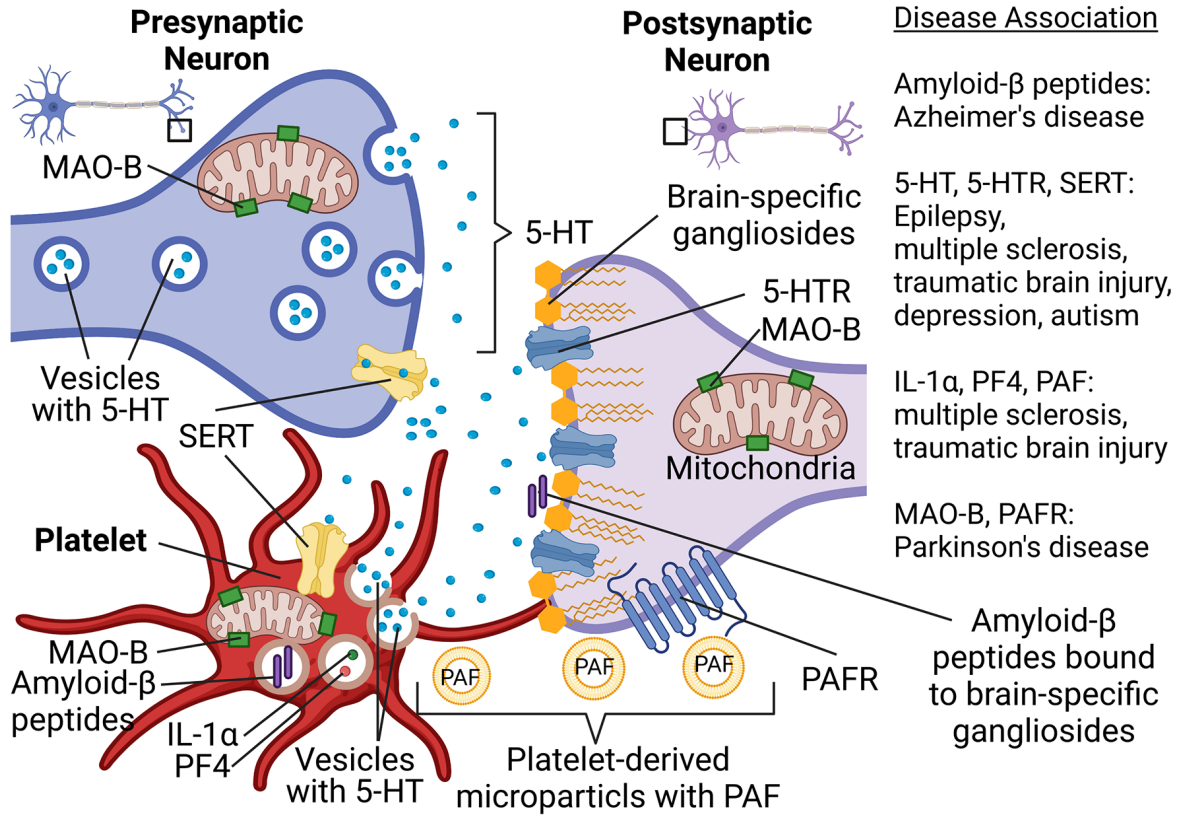

FIGURE 1 | Platelet-neuron similarity and platelet-neuron interactions have implications for a wide range of neuropsychological disorders such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, traumatic brain epilepsy, and depression. Abbreviations: 5-HT, Serotonin; 5-HTR, 5-HT receptor; IL-1α, Interleukin 1α; MAO-B, Monoamine oxidase B; PAF, Platelet-activating factor; PAFR, PAF receptor; PF4, Platelet factor-4; SERT, Serotonin transporter. Created in BioRender.com.

late stages of MS (Starossom et al., 2015). This indicates the importance of looking at various parameters to characterize detrimental and beneficial phenotypes of activated platelets to target pathogenic platelet subsets. Imaging cytometry has great potential to assess platelet content such as microRNA (Ponomarev et al., 2011). However, currently, it is challenging, especially in the case of neurologic disorders.

To better understand the possible involvement of platelets in neurologic disorders, a new concept was proposed by several scientists several years ago (Ponomarev, 2018). According to this concept, platelets can be viewed as "circulating mirrors" of neurons and innate immune cells (Ponomarev, 2018; Canobbio, 2019; **Figure 1**). In the coming years, the analysis of platelet morphology, functionality, metabolism, as well as protein and lipid composition may advance the investigation of neurodegenerative diseases (**Figure 1**). For example, analysis of metabolism and secretion of serotonin could be used for the understanding of predisposition and progression of depression and TBI. Platelets also use serotonin transporter (SERT), which is targeted by selective serotonin reuptake inhibitors, and anti-depressant drugs (**Figure 1**). Moreover, we recently demonstrated an important role of serotonin in epilepsy and the formation of new synapses. The latter also plays a role in the development of autism spectrum disorder. The ability of platelets to store, process, and secrete various forms of Aβ could be used as a marker for AD. Analysis of platelets' mitochondrial function could be used to diagnose predisposition for PD (**Figure 1**). Thus, platelets can become a suitable tool for the analysis of peripheral biomarkers when it comes to diagnosing neuronal dysfunctions in the future (Ehrlich, 2012; Oji et al., 2018; Canobbio, 2019; Padmakumar et al., 2019).

PLATELETS AS TARGETS FOR FUTURE THERAPY

Targeting platelets and their products for therapeutic purposes in the field of neurological pathologies seems to present an exceptional challenge, both because of the blood-brain barrier and due to obstacles for the drugs on their way to the desired locations of action inside the CNS parenchyma. Above all else, there might be demand for various approaches on how to target platelet or platelet-derived microparticles in circulation and CNS parenchyma in the same patients. These interventions will require advanced drug formulations and delivery techniques, along with modern imaging methods to determine drug stability,

accurate targeting, and efficacy. Antiplatelet therapy is broadly applied in the treatment and prevention of patients with thrombotic cerebrovascular or cardiovascular diseases. Platelet aggregation and activation can be decreased by antiplatelet therapy with one or several drugs available on the market with the most common being heparin and aspirin (Gianazza et al., 2020). There are several antiplatelet drugs currently available on the market with the most popular being aspirin (Chandrasekhara et al., 2016). In the 1960s aspirin appeared as the first valid antiplatelet drug and is still applied to prevent cardiovascular pathologies.

Aspirin was found to ameliorate spontaneous recurrent seizures in SWR/J mice with pilocarpine-induced chronic temporal lobe epilepsy, however, the role of platelets was not investigated in this study (Zhu et al., 2017). It was an interesting case study documenting that woman with epilepsy history who constantly took aspirin (75 mg daily for 3 years) had epileptic convulsion 3 weeks after aspirin withdrawal, but not during the period when she took aspirin (Scheepers et al., 2007). A preliminary clinical study also showed decreased seizure frequency for epilepsy patients on day 2 after starting to take aspirin. The authors of this study emphasize that further prospective study is needed towards this direction (Godfred et al., 2013). Thus, the usage of aspirin (and possibly other antiplatelet drugs) is a perspective for the prevention and treatment of epilepsy. At present, the main platelet target which is used as a target in antiplatelet therapy is cyclooxygenase-1/2 (COX-1/2) with more selective targeting of COX-1. COX-1 is responsible for regulating the production of the prostaglandins, which control platelet aggregation and activation, and is affected by irreversible cyclooxygenase inhibitors such as NSAIDs. NSAIDs were found to be promising for prophylaxis of AD; however, these drugs decrease neuroinflammation by targeting endothelial cells and macrophages and it is not entirely platelet specific (Zhang et al., 2018). Another currently approved more specific antiplatelet drug clopidogrel or its analogs ($P2Y_{12}$ inhibitors) target ADP receptors on platelets significantly reducing their activation (Amin et al., 2017). Usage of this drug for the prevention and treatment of neurodegenerative diseases is very limited; however, it was recently reported that clopidogrel reduced pathology in the rat model of AD (Khalaf et al., 2020). New drugs and their platelet targets are still being studied, and some promising targets are at the stage of clinical investigation to discover new possible biomarkers for accurate treatment (Coppinger et al., 2007; Yousuf and Bhatt, 2011; Dovizio et al., 2014).

Knowing the important role of platelets in neurodegenerative diseases, it is time to rethink the mechanism of action of some of the known drugs widely used for the treatment of neurologic conditions. There are several candidates for re-evaluation of their therapeutic mechanisms. A common drug for MS called glatiramer acetate (Copaxone^{TM}) in addition to targeting T cells also targets platelets inhibiting their calcium influx, activation, aggregation, and prolongs bleeding time (Starossom et al., 2014). valproic acid, a common drug for the treatment of epilepsy and bipolar disorder, could also affect platelet functions by decreasing their numbers causing thrombocytopenia. So these two drugs could be also called anti-platelet agents.

Most current platelet-specific drugs non-specifically inhibit platelet activation and aggregation, which may cause dangerous complications such as bleeding. Moreover, in certain neurodegenerative diseases, platelets' granule content becomes exhausted and their ability to get further activated is decreased (Starossom et al., 2015), which may require platelet functions to be restored and pathogenic platelets to be replaced by non-pathogenic platelets. To do so, there is a possibility of using autologous genetically engineered iPSC-derived platelets, or artificial platelet-like microparticles for transfusion of patients with neurological disorders (Brown et al., 2014; Moreau et al., 2016; Lawrence et al., 2019). Yet, a more detailed investigation of detrimental vs. beneficial pathways of platelet activation during neurodegenerative diseases should be performed to understand specific pathways in platelets to be targeted using these novel technologies.

CONCLUDING REMARKS

Recent research results demonstrated that platelets play a crucial role in the pathology of multiple neurologic disorders. During many types of neurodegenerative diseases, the BBB is compromised, which enables platelets to infiltrate the central nervous system (CNS), where they release serotonin and other mediators. Enhanced neuronal electric activity and seizure severity have been greatly contributed to by platelet-derived (but CNS-derived) serotonin and possibly other platelet-derived co-factors. Besides, a whole-genome transcriptome analysis suggested that platelets trigger the expression of many genes associated with neuronal synaptic activity, neuroinflammation, and oxidative phosphorylation in neuronal mitochondria. Based on this, we introduced the model shown in **Table 1**. Our model implies that platelets could provide the "missing link" between TBI and epilepsy, or initial neuronal damage and further development of other neurodegenerative pathologies such as AD, PD, and MS (**Figure 1**). Thus, the main conclusion of our review is that platelets cannot be any further ignored in modern neurobiology.

AUTHOR CONTRIBUTIONS

EK and EP conceived and wrote the manuscript. EP edited the manuscript. EK made **Figure 1**. EP made **Table 1**. All authors contributed to the article and approved the submitted version.

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REVIEW
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Physical Exercise Modulates Brain Physiology Through a Network of Long- and Short-Range Cellular Interactions

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In the last decades, the effects of sedentary lifestyles have emerged as a critical aspect of modern society. Interestingly, recent evidence demonstrated that physical exercise plays an important role not only in maintaining peripheral health but also in the regulation of central nervous system function. Many studies have shown that physical exercise promotes the release of molecules, involved in neuronal survival, differentiation, plasticity and neurogenesis, from several peripheral organs. Thus, aerobic exercise has emerged as an intriguing tool that, on one hand, could serve as a therapeutic protocol for diseases of the nervous system, and on the other hand, could help to unravel potential molecular targets for pharmacological approaches. In the present review, we will summarize the cellular interactions that mediate the effects of physical exercise on brain health, starting from the factors released in myocytes during muscle contraction to the cellular pathways that regulate higher cognitive functions, in both health and disease.

Keywords: physical exercise, brain physiology, brain pathology, myocytes, neurons, microglia, neurodegeneration, neurodevelopmental disorders

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INTRODUCTION

Among the number of different activities that shape lifestyle, physical exercise is known to elicit various beneficial consequences on health.

At systemic level, it has been shown to exert a positive outcome in different systems: it prevents cardiovascular diseases and osteoporosis, improves glucose metabolism, and decreases the risk of developing cancer (Warburton et al., 2006). Moreover, the remarkable impact of voluntary physical activity on glucose metabolism exerts relevant beneficial effects in the insulin-resistant state (James et al., 1984) and in preventing type 2 diabetes in high-risk individuals (Pan et al., 1997; Kelley and Goodpaster, 1999).

Furthermore, physical exercise induces a wide range of beneficial effects on brain health at different levels: regardless of the specific type of aerobic activity, it reduces anxiety, depression (Sharma et al., 2006), and negative mood (Callaghan, 2004). At the cognitive level, a relationship between aerobic capacity, hippocampal plasticity and memory is widely recognised and accepted (Duzel et al., 2016), and physical exercise improves and maintains cognition in older individuals (Duzel et al., 2016). Studies conducted in animal models have shown that running modulates neurotransmitters, neurotrophin level, neuronal morphology and adult neurogenesis

(Voss et al., 2013). Interestingly, running has also been shown to reinstate juvenile-like plasticity in the visual cortex of adult rats through a reduction of the intracortical inhibitory tone (Baroncelli et al., 2012). Very recently, this approach has been successfully applied to restore plasticity also in adult human subjects (Lunghi and Sale, 2015).

It is worth noting that while moderate physical activity is considered a safe practice – World Health Organization suggests 150–300 min of weekly moderate aerobic exercise for adults [World Health Organization (WHO), 2020], the effects of higher intensities on brain physiology are still controversial. Some studies reported that high-dose exercise induces higher increase of neurotrophins than moderate exercise (Marquez et al., 2015); on the other hand, it has also been reported that the exaggerated oxidative stress exerted by exhausting exercise could impair cognitive functionalities in mice (Rosa et al., 2007). In order to avoid possible misunderstandings, all the works here reported deal about moderate aerobic exercise.

Despite the ever-increasing number of studies investigating the effects of physical activity on the brain, the peripheral mechanisms that drive these beneficial events remain unclear. In particular, very little is known about the physiological processes involved in the translation of general muscle activation into the enhancement of brain molecular pathways involved in neural plasticity.

Given the complexity of the cellular and biochemical framework that underlies physical exercise's output on brain physiology, a reductionistic approach to this issue is not possible and the present work is not aimed to suggest the pivotal role of a particular pathway or cellular network over others; it is rather conceived as a map that untangles some of the numerous ropes that span from muscular activation to brain health. Thus, this review focuses on the cellular interactions that regulate brain physiology in response to physical exercise in health and disease.

FROM PERIPHERY TO CNS: DIFFERENT CELLULAR INTERACTIONS MODULATE THE EFFECTS OF PHYSICAL EXERCISE ON THE BRAIN

The journey from muscle contractions to neuronal modulation is assembled by numerous routes: the molecules released by peripheral organs are regulated through the somatotropic axis with feedback and feedforward mechanisms, that eventually contribute to environment perception and cognitive functionalities.

Periphery to CNS

In response to physical exercise, several peripheral organs release a plethora of molecular factors in the bloodstream. Once in the circulatory system, some of these factors can cross the blood-brain barrier (BBB) and enter the brain where they affect the neuronal activity of CNS cells.

Skeletal muscle is one of the major sources of these exercise-induced circulating factors; during prolonged muscle contraction, myocytes produce, and secrete small proteins called myokines that act on various other organs including the heart, liver, pancreas, and the brain (Schnyder and Handschin, 2015). By definition, myokines are cytokines released by skeletal muscles, exerting both an autocrine control on muscle metabolism and a paracrine or endocrine regulation on other body parts (Pedersen et al., 2003). Among them, irisin, a recently discovered myokine (Boström et al., 2012), is getting increasing attention. In skeletal muscle, prolonged physical exercise activates the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) through the 5′ AMP-activated protein kinase (AMPK). In turn, PGC-1α increases the blood concentration of irisin controlling the expression of fibronectin type III domain-containing protein 5 (FNDC5), the transmembrane protein from which irisin is cleaved. In the brain, this myokine exerts an antidepressant-like effect (Wang and Pan, 2016; Siteneski et al., 2018) and confers neuroprotection in animal models (Xia et al., 2017; Noda et al., 2018; Lourenco et al., 2019). Similarly, physical exercise increases the level of cathepsin B that has anti-amyloidogenic and neuroprotective functions (Mueller-Steiner et al., 2006) and whose expression is required for functional spatial memory, proper mood-related behavior, and adult neurogenesis (Moon et al., 2016).

Myocytes are not the only peripheral cell type modulating neuronal activity during locomotion; indeed, hepatocytes produce and secrete molecular factors in response to vigorous exercise as well. The insulin-like growth factor 1 is the earliest identified peripheral factor that mediates the interaction between peripheral and brain cells following physical activity (Carro et al., 2000). IGF-1 belongs to the insulin superfamily, a large group of peptides sharing a highly conserved structural motif consisting of two peptide chains linked by three disulfide bonds (Shabanpoor et al., 2009). Insulin and insulin-like growth factors (IGF-1 and IGF-2) are chiefly known for their role in controlling glucose metabolism peripherally, but they also have various functions in the CNS. In the developing brain, IGF-1 is locally expressed by all cell types; this peptide is indeed necessary for cell proliferation, survival, and differentiation as well as for proper axon guidance and synaptogenesis during CNS formation (Beck et al., 1995; O'Kusky et al., 2000; Cheng et al., 2001; Hodge et al., 2007; Hurtado-Chong et al., 2009; Croci et al., 2011; Fernandez and Torres-Alemán, 2012; for a review). However, the local synthesis of this peptide dramatically drops postnatally and, though IGF-1 is produced by all adult tissues, hepatocytes are the main source of this trophic factor in adulthood (Bartlett et al., 1991; García-Segura et al., 1991; Bondy et al., 1992).

The hepatic expression of IGF-1 is regulated by the growth hormone (GH) synthesized by the somatotropic cells in the pituitary gland (Hartman et al., 1993). Notably, the serum concentration of GH is increased in running trained subjects (Sutton and Lazarus, 1976; Hartman et al., 1993; Kraemer et al., 1993). When released into the serum, IGF-1 is bound by the IGF-binding proteins (IGFBPs), a group of six proteins that modulate the bioavailability of circulating IGF-1 (Allard and Duan, 2018) and transport it to the brain.

Stimulating the release of peripheral factors, physical exercise has a profound influence on both the developing and the adult

CNS. To date, the mechanisms by which these messengers modify neuronal activity are incompletely understood; however, accumulating evidence suggests that they may ultimately promote epigenetic changes on the promoters of Brain-derived neurotrophic factor (BDNF) (Gomez-Pinilla et al., 2011), which is a master regulator of neural function (Kowiański et al., 2018). Consistent with this, a recent study (Sleiman et al., 2016) proved that β-hydroxybutyrate, one of the factors secreted by the liver during physical exercise, can increase the expression of this neurotrophin inactivating the histone deacetylases (HDACs), which are a class of enzymes known to decrease BDNF synthesis (Koppel and Timmusk, 2013).

Brain-Blood Barrier and Brain-Cerebrospinal Fluid Barrier

The neural microenvironment is isolated from plasma by several selective membranes, including the BBB and the blood-cerebrospinal fluid (CSF) barrier (BCSFB). BBB supplies the brain with essential nutrients and mediates the efflux of many waste products (Begley and Brightman, 2003). It plays an important role in the homeostatic regulation of the brain microenvironment necessary for the stable and coordinated activity of neurons (Cserr and Bundgaard, 1984), limits small molecule permeation, regulates large molecular traffic, and also separates peripheral and central neurotransmitters pools (Abbott, 2004). BBB is formed by brain endothelial cells lining the cerebral microvasculature, but neurons, astrocytes, pericytes, extracellular matrix and microvessels are organized into well-structured neurovascular units (Hawkins and Davis, 2005), which are involved in the regulation of cerebral blood flow (Iadecola, 2004). However, the main structures responsible for the barrier properties of the BBB are tight junctions (TJs) (Reese and Karnovsky, 1967), that seal the intercellular cleft between one cell and another.

Blood-brain barrier is not the only barrier dividing the CNS from the periphery, a second interface is formed by the epithelial cells of the choroid plexus (CP) (Wolburg and Paulus, 2010). With the aim of separating blood and CSF compartments, the epithelial cells of the CP and the tanycytes of circumventricular organs (CVO) constitute the BCSFB (Segal, 2000). Similar to BBB, TJs between the CP epithelial cells inhibit paracellular diffusion of hydrophilic substances (Engelhardt and Sorokin, 2009). Compared to BBB, this barrier is leakier (Wolburg and Lippoldt, 2002), but this does not reflect an increase in the bioavailability of substances in the deep brain parenchyma (Spencer et al., 2020). Besides their barrier function, CP epithelial cells are involved in the supply and distribution of peptides into the brain, in the removal of toxic metabolites, in the excretion of xenobiotics (Engelhardt and Sorokin, 2009; Weiss et al., 2009), and in the production of CSF through free access to the blood compartment of the leaky blood vessels (Brown et al., 2004).

TJs act as a "physical barrier" (Abbott et al., 2006, 2010) that precludes the entry into the CNS of a whole series of molecules necessary for its functioning. Only small gaseous molecules, such as O_2 and CO_2, and a wide range of lipid-soluble molecules can passively diffuse across the lipid membranes of the epithelial cells (Liu et al., 2004). Most polar molecules cannot diffuse through the BBB thus, all endothelial cells express a large number of solute carrier (SLC) proteins in the membrane (Zhang et al., 2002) that mediate the influx and efflux of these molecules. Carrier-mediated transport (CMT) allows the entry of essential nutrients (i.e., glucose) into the brain and the elimination of metabolic waste.

Large molecules such as peptides and proteins also require a transport mechanism to cross the BBB: they can pass through the endothelial cell membranes via the specific receptor-mediated transcytosis (RMT), or by the less specific adsorptive-mediated transcytosis (AMT) (Pardridge, 2003; Wolka et al., 2003; Abbott et al., 2006). In particular, IGF-1 and IGF-2, undergo RMT across the BBB via separate type 1 and type 2 IGF receptors (IGF1R and IGF2R) (Pardridge, 2007). In the circulatory system, the great majority of serum IGF-1 forms a 150 kDa complex along with the acid-labile subunit (ALS) and the IGFBP3 (Delafontaine, 1995), i.e., the IGFBP with the highest affinity to IGF-1. Blood released IGF-1 can then enter the CNS via two distinct gateways: it can enter the CSF through the CP or the brain parenchyma through the BBB (Carro et al., 2005; Nishijima et al., 2010). These two structures are indeed well suited to transport serum IGF-1: the CP epithelium, the end-feet glial cells and the brain vessels in the BBB express high amounts of IGF-1Rs (García-Segura et al., 1991; Marks et al., 1991). The CSF concentration of IGF-1 linearly depends on the systemic level of this trophic factor; an observation suggesting that IGF-1 is constitutively transported into the CSF through the CP (Carro et al., 2000, 2005). This direct passage of IGF-1 into the CSF occurs through a mechanism involving the multiligand receptor megalin expressed in the CP epithelium (Carro et al., 2005). In parallel, serum IGF-1 is carried in the brain parenchyma via an activity-dependent mechanism; the entrance of IGF-1 through the BBB is guided by a process initiated by glutamate release at active brain regions (Nishijima et al., 2010). The release of glutamate by active synapses leads to Ca^{2+} influx in astrocytes with the subsequent release of prostaglandin E2, arachidonic acid derivatives, NO and ATP. These diffusible mediators induce local vasodilation increasing the BBB permeability to serum IGF-1, oxygen, and glucose. Furthermore, several of these same mediators activate the matrix metalloproteinase 9 (MMP9) that cleaving IGFBP3 releases bioavailable IGF-1. The MMP9 activity, therefore, raises the amount of serum IGF-1 able to bind the IGF-1Rs, which are locally expressed by the BBB (Nishijima et al., 2010). Crossed the BBB, IGF-1 can directly interact with active neurons or can be indirectly transported to active neurons by the end-feet glial cells. Upon entry in the adult brain, IGF-1 controls cellular homeostasis, modulates synaptic plasticity, promotes adult neurogenesis, and counteracts neurodegeneration (Fernandez and Torres-Alemán, 2012).

A great number of CNS pathologies (including brain injury, stroke, multiple sclerosis, epilepsy, Parkinson's disease, and Alzheimer's disease) cause TJ dysfunctions (Förster, 2008) that, increasing BBB permeability, leading to the entry of neurotoxic substances into the brain. Lifestyle and bad eating habits also influence the functioning and integrity of the BBB. Metabolic syndrome, insulin resistance, type 2 diabetes,

arterial hypertension, dyslipidaemia, and obesity cause low-grade inflammation due to increased blood levels of pro-inflammatory cytokines including Interleukin-1β,-6 (IL-1β,-6) and tumour necrosis factor-α (TNF-α) and the CNS response to inflammation could lead to endothelial cell damage which increases BBB permeability (Kim et al., 2012). It has been shown that moderate physical exercise can maintain endothelial health (D'Alessio, 2004; Middlebrooke et al., 2005). Furthermore, exercise induces a reduction in C-reactive protein (CRP), IL-1, IL-6, interferon-γ (INF-γ) levels in coronary artery disease patients (Goldhammer et al., 2005), demonstrating its anti-inflammatory effects. A reduction of TNF-α and IL-6 blood levels has been observed also in a group of healthy elderly women after 14 weeks of exercise (Chupel et al., 2018). Diabetes mellitus and obesity are frequently associated with oxidative stress (Houstis et al., 2006) that refers to elevated intracellular levels of reactive oxygen species (Schieber and Chandel, 2014) which produce a loss in TJs integrity (Baeten and Akassoglou, 2011) causing a direct damage on BBB. In animal model of obese type 2 diabetes regular and moderate physical exercise reduce oxidative stress (Teixeira-Lemos et al., 2011). Therefore, counteracting obesity, physical exercise indirectly protects the BBB. In short, physical exercise seems to be an important therapeutic resource that protects and repairs the BBB which in turn maintains homeostasis in the cerebral microenvironment.

Hippocampus

The impact exerted on the CNS by exercise has been extensively studied in the hippocampus, a brain region embedded in the medial temporal lobe with a major role in learning and memory (Bird and Burgess, 2008).

In human subjects, moderate-intensity physical activity was correlated to increased hippocampal size, hippocampal blood flow, and memory (Erickson et al., 2011; Steventon et al., 2020). Likewise, exercise has been shown to improve spatial navigation (Ang et al., 2006; Aguiar et al., 2011), object recognition (Bechara and Kelly, 2013) and contextual fear memory (Greenwood et al., 2009) in rodents. These memory improvements appear to be mediated by the genesis of new CNS cells in the subgranular zone (SGZ), a stem-cell niche that lies between the hilus and the granule cell layer in the hippocampal dentate gyrus (Gonçalves et al., 2016). Physical exercise indeed promotes adult neurogenesis and newborn cell survival (Van Praag et al., 1999a,b) through a mechanism that could be mediated by BDNF expression (Liu and Nusslock, 2018). In fact, growing evidence supports the notion that BDNF is required for proper neuronal differentiation and survival in adulthood (Lee et al., 2002; Scharfman et al., 2005; Chan et al., 2008; Waterhouse et al., 2012). Moreover, this enhancement of neurogenesis is an exclusive process to the SGZ; physical exercise influences the production of new cells in the hippocampal formation but not in the subventricular zone, which is one of the two major stem-cell niches in the adult brain along with the SGZ (Brown et al., 2003).

Hippocampal neurogenesis is intimately connected with angiogenesis. Newborn cells cluster in striking proximity to blood vessels (Palmer et al., 2000) that are integral constituents of stem-cell niches since the vasculature serves not only as a conduit for nutrients, but also conveys signaling molecules that modulate stem-cell differentiation (Licht and Keshet, 2015). Physical exercise shapes the neurovascular interface augmenting the vascular density in the granular layer of the dentate gyrus. Vascular endothelial growth factor (VEGF) originating from outside the BBB represents an essential player in this angiogenic response to exercise. Following acute muscle contractions, skeletal myofibers release VEGF in circulation (Fabel et al., 2003; Rich et al., 2017). Once in the cortex, VEGF stimulates both the proliferation of neural cell precursors and the perfusion of blood capillaries in the SGZ (Jin et al., 2002; Fabel et al., 2003; van Praag et al., 2005; Ding et al., 2006; Clark et al., 2009; Rich et al., 2017). In agreement with this hypothesis, the peripheral injection of a VEGF antagonist abolishes exercise-induced SGZ neurogenesis but is ineffective in suppressing baseline neurogenesis (Fabel et al., 2003). However, according to recent lines of research, the local VEGF production by CNS resident cells might itself instruct the SGZ neurovascular interface. During physical exercise, hypoxic conditions favour the pyruvate conversion into L-lactate in skeletal muscles. This metabolite is then secreted in the bloodstream and transported in the CNS through the monocarboxylate carriers (Proia et al., 2016). In the dentate gyrus, L-lactate increases local VEGF release activating the lactate receptor – hydroxycarboxylic acid receptor 1 – (HCAR1) expressed by the perivascular pial and pericyte-like cells (Morland et al., 2017).

By promoting the genesis of granular cells, physical exercise modulates synaptic plasticity in the hippocampus. Adult newborn cells acquire the properties of completely mature granule cells and are functionally integrated into the hippocampal network by 4 weeks (Van Praag et al., 2002). During this process, newborn cells exhibit a narrow time window of enhanced plasticity that depends on the transient expression of the NR2B subunits of N-methyl-D-aspartate receptors (NMDAR) early (4–6 weeks) post-mitosis (Snyder et al., 2001; Ge et al., 2007). Long-term potentiation (LTP) is consistently enhanced in hippocampal slices of running mice as compared to sedentary animals. This increase in synaptic plasticity is tightly connected with running-induce SGZ neurogenesis; changes in LTP properties can be detected only in the dentate gyrus, where new granule cells born, and not in other hippocampal regions (Van Praag et al., 1999b; Farmer et al., 2004). Furthermore, adult-born cells modify not only the functional properties of the dentate gyrus but also rewire the hippocampal circuitry. Indeed, physical exercise reshapes the local innervation to newborn neurons and increases the distal projections coming to these cells from subcortical and cortical regions (Vivar et al., 2016; Sah et al., 2017).

Cortical Areas

Cortical areas are the headquarters of higher cognitive functions that allow us to respond adaptively to a constantly changing environment, for this reason these areas are highly influenced by physical activity, which represents a way to interact with the environment. Exercise increases cortical thickness in older individuals (Colcombe et al., 2006) and modulates neuronal protein expression via epigenetic regulation (Abel and Rissman, 2013; Kashimoto et al., 2016). Interestingly, exercise protects

microglial cells from age-dependent loss of functionality, a fact that, in turn, improves cognitive functions in elderly subjects (Mela et al., 2020).

It is worth noticing that, locomotion is also able to directly modulate specific cortical areas: from an evolutionary point of view, the integration of motor coordination and sensory information -visual, auditory, and tactile (Lee et al., 2013; Dipoppa et al., 2018; Ayaz et al., 2019; Yavorska and Wehr, 2021) – is a process that is necessary for fundamental skills required for surviving such as foraging and threats escape.

The visual cortex represents, since the 1960s, the prime model to study the complex interaction between external cues – such as physical exercise – and cellular reorganization of neural circuits. The proper maturation of the visual system is a phenomenon strictly dictated by the mutual interaction between genetic programs and plasticity processes driven by environmental experience. Postnatal exposure to an enriched environment, a condition of enhanced physical exercise and sensory stimulation, conspicuously accelerates visual system development (Sale et al., 2004). A number of data now support the hypothesis that this acceleration is partly elicited by the increased amount of serum IGF-1 in enriched animals, which experience high levels of motor stimulation. Such hypothesis is supported by experiments showing that the exogenous supply of JB1 (an IGF-1R antagonist) or anti-IGF-I antiserum completely prevents this acceleration (Ciucci et al., 2007; Landi et al., 2009). The faster maturation of the visual system might be caused by the IGF-1 interaction with different pathways. Indeed, the increased presence of IGF-1 enhances BDNF expression, stimulates retinal development, promotes a precocious maturation of the GABAergic interneurons and a precocious decrease in the NKCC/KCC2 ratio (Sale et al., 2007; Baroncelli et al., 2017). Interfering with all these pathways, IGF-1 can eventually prompt the premature visual system development observed at behavioral, electrophysiological, and molecular level.

Number of studies are needed to evaluate whether other exercise-related molecules extend their action to the visual system. Nevertheless, a number of researches provided evidence that physical exercise acts on visual neurons promoting the release of different neuromodulators in the primary visual cortex; in particular, locomotion increases the amount of serotonin and acetylcholine (Jacobs and Fornal, 1999). In the CNS, serotonin is almost exclusively released by the serotoninergic neurons located in the raphe nuclei, which are a cluster of nuclei in the brain stem (Hornung, 2003). Neurotransmission of serotonin is involved in structural and functional remodeling of visual cortical circuits and is considered one of the non-visual neurochemical bases of attention, arousal, and motivation (Gu, 2007). Serotoninergic neurons produce serotonin through a process that is limited by the plasma level of tryptophan, a large neutral amino acid from which serotonin is synthesized (Russo et al., 2007; Höglund et al., 2019). Tryptophan is transported through the BBB via a transporter carrier shared by all the large neutral amino acids; therefore, tryptophan's entry in the CNS does not depend on its plasma concentration *per se*, but on its plasma concentration with respect to other large neutral amino acids since they all compete for the same transporter (Fernstrom, 2005; Markus, 2008; Höglund et al., 2019). During physical exercise, the branched-chain amino acids – a subset of the large neutral amino acids – are transported into the muscles (Patrick and Ames, 2015). Consequently, physical exercise increases tryptophan transport into the brain decreasing the competition for the BBB transporter.

On the other hand, the effect of acetylcholine in shaping the circuitry of the visual cortex has been extensively studied in the last few years (Sugihara et al., 2016). The primary visual cortex receives direct input from the nucleus of the diagonal band of Broca, a cholinergic centre in the basal forebrain (Fu et al., 2014), specifically from the mesencephalic locomotor region (MLR), a midbrain region associated with the ascending reticular activating system described by Moruzzi and Magoun (1949). During locomotion, the enhanced activity of the MLR increases visual cortical responsiveness via a mechanism involving the vasoactive intestinal peptide (VIP) and the somatostatin (SST) positive cells, which are two of the major classes of inhibitory interneurons. To dissect this cellular mechanism, Fu et al. (2014) imaged calcium responses of these interneurons in awake mice free to run on a treadmill. They found that neural activity of VIP neurons is significantly increased during locomotion even in the absence of visual stimulation; SST neurons were instead inhibited by locomotion. These observations suggest the involvement of a circuit in which VIP neurons trigger the inhibition of SST neurons. Therefore, the activation of VIP cells boosts cortical excitation releasing pyramidal cells from the inhibition exerted by SST neurons, a mechanism termed disinhibition. VIP interneurons are activated by the basal forebrain through nicotinic acetylcholine receptors (nAChRs) (Alitto and Dan, 2012). Consistently, local infusion of nAChRs antagonists greatly reduces the response of VIP cells to locomotion, pinpointing acetylcholine as a crucial mediator of physical exercise in the primary visual cortex (Fu et al., 2014).

PHYSICAL EXERCISE AS A POTENTIAL THERAPEUTIC TOOL FOR CNS PATHOLOGIES

Physical exercise has a marked influence on several physiological processes; indeed, general myocyte contraction can, indirectly, control adult neurogenesis, cortical excitability, and the release of different neuromodulators such as BDNF. Given its multifactorial action, exercise, and its downstream cellular pathways, could represent potential targets for preventing or reversing the symptoms of those pathologies in which neuroprotection, plasticity and transmission are defective (**Figure 1**). In the following section, we describe amblyopia, Down syndrome, neurodegenerative diseases, and glioma as explicative cases.

Amblyopia

Amblyopia, with an incidence of 1–5%, is the prevalent monocular impairment in the world human population (Holmes and Clarke, 2006). The central physiological mechanism in amblyopia is considered inter-ocular suppression, which is the empowering of the inputs coming from the apparently healthy

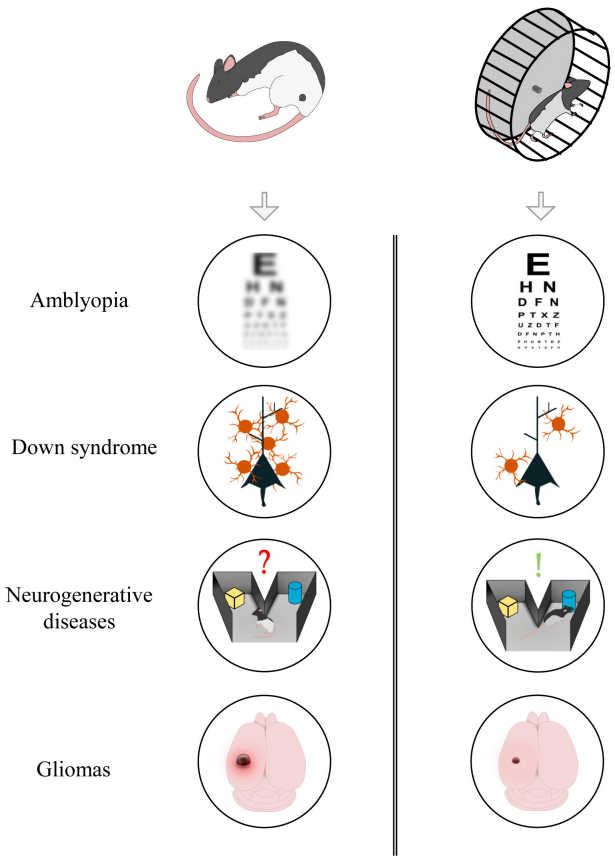

FIGURE 1 | Aerobic exercise could exert beneficial effects in different forms of neuropathology. From top to bottom: exercise induces visual recovery in adult amblyopic subjects; it reduces GABAergic innervation in animal model of Down Syndrome; it improves memory formation and consolidation in neurodegenerative diseases; it contributes in reducing tumoral mass in glioma-bearing mice.

eye at the expense of those coming from the amblyopic eye (Antonini et al., 1999). This suppression is evident at the level of the primary visual cortex and appears to be mediated by the inhibitory circuitry (Sengpiel et al., 2005).

Amblyopia, if not promptly treated during childhood, leads to severe visual impairments including loss of visual acuity and defective stereopsis. Occlusion therapy, i.e., the temporary exclusion of the healthy eye from visual activity by means of an eye patch, completely reverses amblyopia when performed during the critical period for binocular vision (Loudon and Simonsz, 2005). Nevertheless, amblyopia is an almost untreatable disease in adulthood; the occlusion therapy is indeed completely ineffective in adult subjects due to the dramatic decline in cortical plasticity caused by the maturation of different plasticity-limiting factors as the GABAergic and the cholinergic systems (Fagiolini and Hensch, 2000; Morishita et al., 2010).

Amblyopia can be artificially induced in animal models depriving one eye of visual stimuli through a long-term lid suture. Several studies have proved physical exercise as an effective strategy to treat amblyopia in adult rodents. Employing adult amblyopic rats, it has been recently shown that voluntary physical exercise under binocular conditions promotes a full and long-lasting recovery of both visual acuity and depth perception (Sansevero et al., 2020). Moreover, physical exercise can also promote amblyopia recovery in humans; indeed, it has been shown that a short-term occlusion of the amblyopic eye coupled with moderate physical activity improve visual acuity and stereo-sensitivity in adult patients (Lunghi et al., 2019). Notably, the exercise-induced factors may be directly involved in promoting the beneficial effects exerted by physical exercise; pharmacological interventions enhancing IGF-1, serotonin or serotonin transporters can counteract visual impairments in adult amblyopic rodents even in the absence of intense exercise (Vetencourt et al., 2008; Maya Vetencourt et al., 2011; Maya-Vetencourt et al., 2012). However, a thorough analysis is still needed to understand whether these molecular factors promote amblyopia recovery increasing BDNF expression. BDNF itself, indeed, can be successful in eliciting visual function recovery in adult animals (Sansevero et al., 2019).

It is worth noting that short-distance cellular interactions also play a crucial role in promoting amblyopia recovery in physical active animals. Recent studies have indeed demonstrated that activation of the VIP-SST disinhibitory circuit promoted, during locomotion, by cholinergic afferents originating from the mesencephalic locomotor region, induces a complete recovery of responsiveness in both excitatory and inhibitory neurons in the primary visual cortex (Kaneko and Stryker, 2014; Sansevero et al., 2020). In this scenario, physical exercise promotes amblyopia recovery releasing the excitatory neurons from excessive levels of inhibition exerted by SST interneurons.

Based on the above-reported studies, a complex and interactive model could be postulated in which both long- and short-range interactions contribute to visual function recovery in running amblyopic animals; with long-distance cellular interaction controlling the expression of pro-plastic genes – like bdnf – and short-distance cellular interaction increasing cortical responsiveness through the release of pyramidal neurons from the local inhibition.

Down Syndrome

Down syndrome (DS), a developmental disorder elicited by the partial or total triplication of the chromosome 21 (Hsa21), is the most common genetic cause of intellectual disability in humans, with an incidence ranging from 1 in 700 to 1 in 1000 live births (Roizen and Patterson, 2003). People with DS exhibit major cognitive deficits in learning and memory along with a number of moderate to severe impairments in motor function, language, and sensory processing (Contestabile et al., 2010; Dierssen, 2012).

The last two decades have brought the development of several mouse models with DS-related features (Gupta et al., 2016; Herault et al., 2017). To date, Ts65Dn is one of the most commonly used and best-studied model of DS. Ts65Dn is characterized by the triplication of the distal segment of Mm16, the mouse chromosome harbouring the largest syntenic region of homology to Hsa21 (Davisson et al., 1990; Duchon et al., 2011). Ts65Dn mice recapitulate the main hallmarks of DS phenotypes. Ts65Dn indeed displays impairments in learning and memory

(Hunter et al., 2003), motor dysfunction (Costa et al., 1999), visual deficits (Scott-McKean et al., 2010) and several anatomical alterations including craniofacial dysmorphology (Richtsmeier et al., 2002), hippocampal hypocellularity (Guidi et al., 2008), and impaired neurogenesis (Lorenzi and Reeves, 2006). The Ts65Dn phenotype correlates with major functional deficits in synaptic plasticity, particularly in the hippocampus where the possibility to induce LTP is drastically compromised (Siarey et al., 1997, 1999). This LTP failure is largely determined by alterations in the GABAergic system (Kleschevnikov et al., 2004; Costa and Grybko, 2005). Indeed, marked morphological and functional changes have been detected in the GABAergic circuitries of both the cerebral cortex and the hippocampus of Ts65Dn mice (Belichenko et al., 2009; Chakrabarti et al., 2010; Best et al., 2012; Martin et al., 2020). Therefore, this large body of evidence has now led to the notion that the excessive level of cortical inhibition is the major functional impairment in this DS model (Best et al., 2007). Accordingly, pharmacological interventions targeting the GABAergic system can completely reverse the defects displayed by Ts65Dn mice (Fernandez et al., 2007), confirming the prime role of overinhibition in DS pathogenesis (Baroncelli et al., 2011; Contestabile et al., 2017).

Physical exercise emerged as an appealing strategy to ameliorate Ts65Dn mice conditions, notably for its role in restoring the proper excitatory/inhibitory balance in the CNS (Sale et al., 2014); an effect largely ascribed to the activation of an interaction network between specific GABAergic cell subtypes (Stryker, 2014). Complex sensory-motor stimulation, including a high level of voluntary physical exercise, can indeed restore spatial navigation, hippocampal plasticity, and brain development in Ts65Dn promoting a reduction in the GABA transmission paralleled by an increase in BDNF expression (Baroncelli et al., 2011; Begenisic et al., 2015). Strikingly, the overexpression of this neurotrophic factor has been extensively associated with reduced inhibition in the adult brain (Sale et al., 2010; Baroncelli et al., 2011). Recent studies have also shown that physical exercise *per se* might exert a positive effect on the DS phenotype promoting recovery of learning and cognitive performances in Ts65Dn mice (Llorens-Martín et al., 2010; Parrini et al., 2017). In human subjects with DS, exercise has been shown to improve physical fitness (Rimmer et al., 2004; Dodd and Shields, 2005; Mendonca et al., 2011). Nonetheless, little is known about the effects of physical exercise on cognitive functions. Recent studies, however, seem to suggest that exercise might also improve executive functions in DS individuals (Pape et al., 2021). Likewise, the exercise-related factors might represent promising targets for therapeutic application to DS since they could be particularly effective in reducing overinhibition by increasing the neuronal expression of BDNF. Interestingly, a recent work showed that either physical exercise or a BDNF-mimetic pharmacological intervention can rescue cognitive functions and synaptic plasticity in Ts65Dn mice (Parrini et al., 2017). Moreover, it is noteworthy to point out that pharmacological strategies based on exercise-related factors can circumvent the major throwback of BDNF administration, i.e., the impossibility for this neurotrophic factor to efficiently cross the blood-brain barrier when delivered peripherally (Nagahara and Tuszynski, 2011).

Neurodegenerative Diseases

Neurodegenerative diseases (NDs) are a heterogeneous group of pathologies resulting from the progressive loss of selective neuronal types in specific CNS regions. Major NDs include Alzheimer's disease (AD), Parkinson's disease (PD), and dementia with Lewy bodies (DLB) (Erkkinen et al., 2018). Ageing is nowadays considered the primary risk factor for neurodegeneration; most NDs occur after the fourth decade of life and their prevalence increases with increasing age (Wyss-Coray, 2016; Hou et al., 2019).

Although the clinical symptoms are diverse depending on the core population of neurons involved in the neurodegenerative process, NDs can be broadly classified as: (1) diseases causing cognitive decline, dementia, and alterations in higher cortical functions; (2) diseases characterized by motor dysfunctions including hyperkinetic and hypokinetic movements (Kovacs, 2018). However, in spite of their heterogeneity in clinical symptoms, numerous NDs share common pathological features: deposition of misfolded proteins, oxidative stress, apoptosis, and neuroinflammation (Dugger and Dickson, 2017; Marsh, 2019).

In search of candidate molecular and cellular mechanisms underlying neurodegeneration, different mouse strains have been generated to model NDs exploiting either genetically based or pharmacologic-based strategies (Dawson et al., 2018; Fisher and Bannerman, 2019). Accumulating evidence shows that physical exercise and exercise-related factors can confer neuroprotection in mouse models of NDs. In recent years, physical exercise emerged as a promising non-invasive strategy to ameliorate neuronal loss, memory impairments, oxidative stress, neuroinflammation, and motor dysfunctions (Souza et al., 2013; Hüttenrauch et al., 2016; Do et al., 2018; Klemann et al., 2018; Palasz et al., 2019). Remarkably, exercise seems to reverse the genetic pattern of inflammation and apoptotic markers set in motion by the progression of NDs (Hüttenrauch et al., 2016; Do et al., 2018) probably through epigenetic mechanisms (Ferioli et al., 2019). A large number of studies has also analyzed the neuroprotective action of specific exercise-released factors. Lourenco et al. (2019) recently reported that irisin levels are reduced in AD mice. Moreover, these authors showed that the peripheral overexpression of irisin attenuates synaptic and memory impairments and that the blockade of either peripheral or brain irisin conversely hampers the neuroprotective effects of physical exercise in AD model. The effect of another myokine, cathepsin B, on AD is instead controversial. According to some studies, cathepsin B can exert neuroprotective actions and promote learning and memory in AD mice (Mueller-Steiner et al., 2006; Embury et al., 2017); on the contrary, other studies have associated this myokine with AD onset and amyloid plaque accumulation (Cataldo and Nixon, 1990; Hook et al., 2005). In addition to myokines, IGF-1 and β-hydroxybutyrate can mitigate the phenotype of various mouse models of NDs (Carro et al., 2006; Lim et al., 2011). IGF-1 can serve as a protective agent against neuroinflammation modulating the activation of CNS resident cells. This growth factor indeed

attenuates the inflammatory response of astrocytes (Bellini et al., 2011), decreases the expression of pro-inflammatory cytokine (Park et al., 2011), and it might also repress neurotoxic microglia (Grinberg et al., 2013; Rodriguez-Perez et al., 2016).

The results obtained in mouse models of NDs encourage stronger efforts in the application of physical exercise to human patients; in particular, exercise-related factors may be promising pharmacological targets to counteract, or at least to prevent, the progression of NDs.

Gliomas

Gliomas (GLs) are primary brain tumours that arise from glial or precursor cells and represent approximately 25.1% of all CNS tumours and 80.8% of malignant tumours. Although the 5-year survival rate for non-malignant primary brain tumours is 91.7%, for malignant tumours this rate drops to 36% and the worst prognosis is for patients diagnosed with glioblastoma (the most malignant GLs) whose 5-year survival rate is 7.2% (Ostrom et al., 2020).

Researchers are paying more and more attention to the glioma microenvironment and to the crosstalk between glioma and peritumoral cells. Glioma cells secrete an aberrant quantity of the excitatory neurotransmitter glutamate, which leads peritumoral neurons to an excitotoxic death via hyperactivation of NMDAR and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPAR) in peritumoral neurons that in turn can make it easier for glioma to invade nearby CNS parenchyma (Ye and Sontheimer, 1999). Concurrently, the autocrine activation of NMDAR and AMPAR promotes glioma invasion (Lyons et al., 2007; Piao et al., 2009; Nandakumar et al., 2019). Another line of research explores the ability of glioma cells to create contact with neurons forming the neuron-glioma synapses (NGS). Under normal conditions, there are synaptic contacts between neurons and normal oligodendroglial precursor cells (OPCs) (Bergles and Jahr, 2000). Recently, Venkatesh et al. showed that the characteristics of NGS are similar to synapses formed between neurons and normal OPCs (Venkatesh et al., 2019). NGS, whose activity is mediated by AMPA receptors, promotes glioma progression (Venkatesh et al., 2019); indeed, AMPA antagonism reduces the glioma cell proliferation (Venkataramani et al., 2019). Furthermore, the increase in glutamate release stimulates peritumoral neurons to release BDNF and Neuroligin 3 (NLGN3) that in turn stimulate the glioma cells to proliferation and infiltration and sustained NGS formation (Venkatesh et al., 2015, 2017, 2019; Venkataramani et al., 2019).

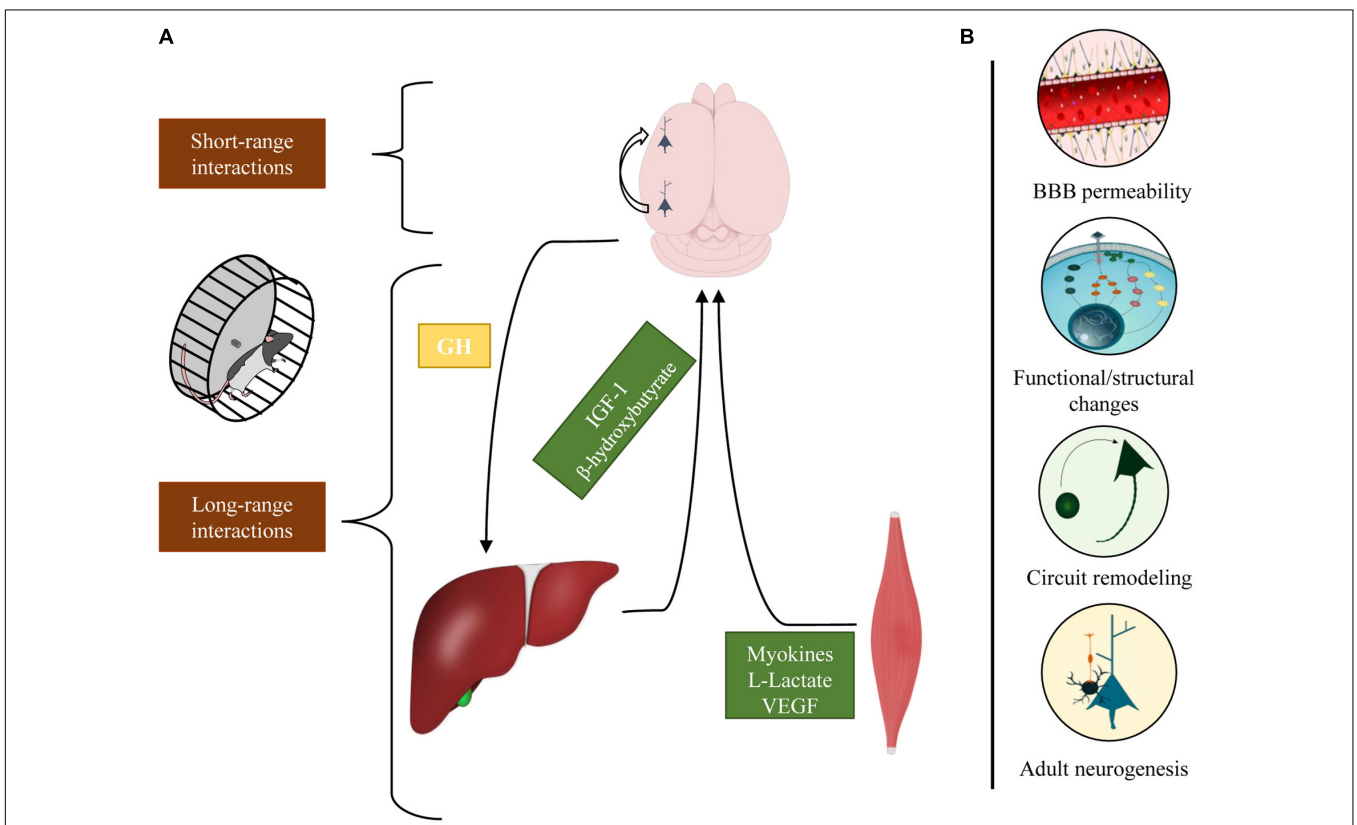

FIGURE 2 | Schematic representation of the different cellular interactions engaged by physical exercise. **(A)** In response to physical exercise, multiple peripheral organs release molecular factors that modulate different neurophysiological processes. The brain, in turn, regulates the inflow of these factors through the somatotropic axis (long-range interactions). Concomitantly, exercise activates intra-cortical neuronal circuits that enhance perceptual and cognitive functions (short-range interactions). **(B)** Physical exercise exerts various effects on the brain modulating BBB permeability, eliciting functional/structural changes, stimulating neurogenesis, and shaping cortical circuits.

Physical exercise might represent a safe, easy, and side-effect-free treatment for GLs. Tantillo et al. (2020) have recently examined the effect of physical exercise in mice injected with tumour cells (GL261) into the primary motor cortex. Compared to the sedentary group, the trained group showed a reduction in glioma cell proliferation and a slowdown in the emergence of motor deficits, while no difference in tumour volume emerged between the two groups (Tantillo et al., 2020). Consistent with this evidence, other studies have shown that physical exercise suppresses tumour growth and can decrease the risk of a number of cancers such as those of the colon, breast and endometrium (Booth et al., 2012). Hence, physical exercise could be used as a protective factor as well as an adjuvant treatment. Indeed, it has been shown that physical exercise, performed during the temozolomide treatment, prolongs the survival of glioma-mice (Lemke et al., 2016). Consistently, physical exercise can also ameliorate the quality of life in human patients (Cordier et al., 2019).

Exercise increases IGF-1 levels both in the periphery (Carro et al., 2001) and the brain (Carro et al., 2000) and increases the BDNF expression (Vaynman and Gomez-Pinilla, 2005) in specific brain areas. These exercise-related factors may be responsible for reducing or slowing down the progression of glioma modifying the glioma microenvironment (Garofalo et al., 2015, 2017). BDNF stimulates the production of Interleukin-15 (IL-15) in the brain of glioma-mice, and IL-15, in turn, stimulates natural kill (NK) cells to produce Interferon-gamma (IFN-γ) that affects the phenotype of myeloid cells, promoting the transition to an anti-tumour state (Garofalo et al., 2017). Aerobic respiration, which occurs in the mitochondria of eukaryotic cells, generates energy and, as a result of this oxidative metabolism, several reactive species are produced [among which reactive oxygen species (ROS) is the most abundant]. But, when the physiological balance between production and elimination of reactive species is broken, oxidative stress is generated. Oxidative stress is related to a wide variety of human diseases, including cancer (Sosa et al., 2013). Physical exercise promotes brain function, increases the resistance against oxidative stress and facilitates recovery from oxidative stress (Radak et al., 2013; Accattato et al., 2017).

Further studies are needed to better understand the molecular mechanisms and the cellular interactions underlying the efficacy of physical exercise as a therapy for GLs. Nevertheless, the obtained results suggest that physical exercise might be a valid and effective strategy, not only in slowing down the progression of glioma but also in improving the life quality of GLs patients.

CONCLUSION

We analyzed the impact that physical exercise exerts on brain activity (**Figure 2**). The work reviewed in this article has shown that physical exercise and brain health are tightly related; indeed, the interplay between brain and muscle activation begins with long-range communications: motoneurons activate myocytes that, in turn, release a large number of factors in the circulation that may reach the brain and regulate the somatotropic axis. On the other hand, in different brain regions, exercise promotes the activation of specific short-range cellular interaction that mediates disparate processes, encompassing from sensory integration to plastic and metabolic changes.

The first consideration, that should emerge from the present work, is that a complete comprehension of the mechanisms that act during exercise on the brain and vice-versa is far from being reached. We marked some boundaries of this tangled network, and we hope they could represent a helpful starting point for future research. As a consequence of that, from a research point of view, the application of multiple experimental approaches but also the commitment of scientists with different formations and perspectives is necessary to develop a field that engages many aspects of general physiology.

Finally, it is important to mention the enormous impact that lifestyle exerts on physiological and pathological brain processes. Effective treatments for most neurodegenerative and neurodevelopmental disorders are still lacking, a fact that entails a huge economic impact on healthcare systems and tremendous consequences on the life quality of families that face such conditions. Given this situation, maintaining an active life, both intellectually and physically, remains one of the few compelling strategies to prevent cognitive decline in the elderly.

AUTHOR CONTRIBUTIONS

GS and AC conceived the work and wrote the manuscript. ID wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Old Stars and New Players in the Brain Tumor Microenvironment

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In recent years, the direct interaction between cancer cells and tumor microenvironment (TME) has emerged as a crucial regulator of tumor growth and a promising therapeutic target. The TME, including the surrounding peritumoral regions, is dynamically modified during tumor progression and in response to therapies. However, the mechanisms regulating the crosstalk between malignant and non-malignant cells are still poorly understood, especially in the case of glioma, an aggressive form of brain tumor. The presence of unique brain-resident cell types, namely neurons and glial cells, and an exceptionally immunosuppressive microenvironment pose additional important challenges to the development of effective treatments targeting the TME. In this review, we provide an overview on the direct and indirect interplay between glioma and neuronal and glial cells, introducing new players and mechanisms that still deserve further investigation. We will focus on the effects of neural activity and glial response in controlling glioma cell behavior and discuss the potential of exploiting these cellular interactions to develop new therapeutic approaches with the aim to preserve proper brain functionality.

Keywords: glioblastoma, glioma, peritumoral tissue, neural activity, glial cells, tumor-associated microglia/macrophages (TAM), reactive astrocyte, OPCs

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INTRODUCTION

Gliomas are the most common primary brain tumors in adults and children. They account for almost 30% of all primary brain tumors and 80% of malignant brain tumors (Weller et al., 2015). Although new therapeutic strategies are continuously under investigation, gliomas remain associated with high morbidity and mortality, especially in the case of glioblastoma multiforme (GBM), the most aggressive form of glioma (Patel et al., 2019; Liang et al., 2020). Hence, treatment of gliomas represents one of the hardest challenges of our times for neuro-oncologists. The standard of care for GBM patients consists in a maximal safe surgical resection followed by cycles of radio- and chemotherapy. However, this therapeutic regimen gives only partial benefits to the patients. Complete glioma eradication is usually prevented by the invasive nature of these tumors and by recurrence due to therapy-resistance (Bahadur et al., 2019). In addition, GBM patients also suffer from devastating neurological deterioration induced by the growing tumor mass as well as the aggressive treatments. In most of the cases, neurodegeneration and other tumor-associated secondary effects contribute to an unfavorable prognosis in glioma patients (Gibson and Monje, 2012; Zetterling et al., 2020).

For a long time, cancer research has mainly focused on understanding the biology of glioma cells and investigating the aberrant pathways that guide tumor onset and progression. The concept that the interaction between glioma cells and the tumor microenvironment (TME) is crucial in driving tumor growth has emerged lately (Hanahan and Coussens, 2012). Understanding microenvironmental determinants that contribute to glioma progression is therefore a major focus of current research, with the aim of discovering new therapeutic targets and strategies (Johung and Monje, 2017).

In order to sustain their growth, glioma cells co-opt brain-resident cells by establishing different communication routes which include secreted molecules, gap junctions, tunneling nanotubes, and extracellular vesicles (Osswald et al., 2015; D'Alessio et al., 2019; Jung et al., 2019; Lane et al., 2019; Matarredona and Pastor, 2019; Gao et al., 2020). Some of these mechanisms have been recently unraveled and exploited to develop new therapeutic strategies. However, the complex cell-cell interactions within the glioma TME are still largely unknown. The TME is also dynamically shaped during disease progression and eventually adapts in response to therapies (Venkatesh et al., 2015; D'Alessio et al., 2019).

Neurons and glial cells (microglia, astrocytes, and oligodendrocytes) not only cooperate to maintain brain homeostasis, but also represent major TME components that substantially contribute to tumor growth. Although the role of microglia has been extensively investigated over the last decade, some important questions still remain open. In contrast, the contribution of oligodendrocytes and, to some extent, astrocytes have been poorly addressed. Important advances have been made in the study of neuronal regulation of glioma development, but some aspects remain controversial. In addition, secondary effects due to glioma growth, like epilepsy and edema, can profoundly affect neuron functionality and lead to cognitive deficits through mechanisms that are not completely known. Therefore, a better understanding of the cellular and molecular dynamics within the TME is urgently needed in order to efficiently counteract glioma progression and improve patients' survival and quality of life.

TUMOR-ASSOCIATED MICROGLIA AND MACROPHAGES: A LEADING PLAYER IN THE TME

Microglia cells are the main immune cell population in the brain (Korin et al., 2017). By continuously scanning the central nervous system (CNS) tissue, they act as "sentinels" that react to any type of pathogens or insult, and are implicated in virtually all CNS pathologies (Hanisch and Kettenmann, 2007). In the glioma field, microglial cells are commonly grouped together with infiltrating monocyte-derived macrophages (MDMs). Microglia and MDMs are collectively named as tumor-associated macrophages (TAMs). Accounting for up to 50% of the tumor mass, TAMs are a central component of the TME, and they play essential roles during tumor progression, recurrence and response to therapy (Gutmann and Kettenmann, 2019). For these reasons, TAM-targeting therapies have recently gained attention in the field as a promising way to treat gliomas (Kowal et al., 2019).

Microglia and MDMs are phenotypically similar and share the expression of markers such as Iba1, CD11b, F4/80, MerTK, and CSF-1R (Greter et al., 2015). Traditionally, their discrimination has been based on the different expression levels of CD45: while microglia are $CD11b^{+}CD45^{low}$-expressing cells, MDMs express high levels of CD45 ($CD11b^{+}CD45^{high}$). Thanks to gene expression profiling studies, more specific markers have been recently identified and are now widely used. These include P2RY12 (Butovsky et al., 2014), Sall1 (Buttgereit et al., 2016), Tmem119 (Bennett et al., 2016), and Hexb (Masuda et al., 2020) that are exclusively expressed by brain-resident microglia cells, while CD49d (Bowman et al., 2016) or Cxcr4 (Werner et al., 2020) are preferentially expressed by peripheral MDMs. However, activated microglial cells acquire some morphological and phenotypical characteristics of MDMs, making the discrimination of these two myeloid cell types problematic (Greter et al., 2015). Identifying the brain-resident vs. peripheral TAMs is important in light of functional differences that have been recently revealed and that possibly arise from a distinct ontogeny. Despite a common origin of microglia and MDMs in the yolk sac, MDMs derive from subsequent wave(s) of progenitors during development and are constantly replenished by bone marrow-derived monocytes in some adult tissues, while in others they maintain themselves locally (Ginhoux and Guilliams, 2016). In contrast, microglia cells originate from unique progenitors and colonize the brain early on during development. They remain in the brain tissue throughout life and self-maintain through a fine balance of proliferation and apoptosis with no need of contribution from the periphery, at least in physiological conditions (Ginhoux et al., 2010; Askew et al., 2017; Tay et al., 2017). In pathological conditions such as brain tumors, the blood brain barrier is compromised and a significant number of MDMs can colonize the brain and participate in disease evolution. Interestingly, when transplanted into a healthy brain, MDMs acquire some microglial-like traits under the influence of the host microenvironment, but they also maintain specific ontogeny-driven features (Bruttger et al., 2015; Bennett et al., 2018). A similar phenomenon has been observed in gliomas, where common and ontogeny-specific programs cooperate to educate microglia and MDMs to support tumor growth (Bowman et al., 2016). For instance, in the TME microglia usually show higher expression of pro-inflammatory genes (Bowman et al., 2016). Conversely, the expression of antigen processing and presentation molecules and of immunosuppressive cytokines is enriched in MDMs and, therefore, they might be more effective in hampering a proper T cell-mediated anti-tumor response (Bowman et al., 2016; Klemm et al., 2020). Differences in the expression profile of brain-resident microglia and peripheral MDMs have been recently confirmed also in humans, where they even vary depending on the brain tumor type. While the TME of less aggressive IDH mutant gliomas is dominated by microglia, in high grade gliomas the contribution of peripheral MDMs is higher. Moreover, TAMs with an immunosuppressive phenotype are particularly abundant in brain metastasis compared to

primary gliomas (Friebel et al., 2020; Guldner et al., 2020; Klemm et al., 2020). Finally, a specific MDMs-related gene signature correlates with poor survival of glioma patients (Bowman et al., 2016; Friebel et al., 2020; Klemm et al., 2020). Different transcriptional responses have also been observed in microglia and MDMs after radiotherapy (Akkari et al., 2020) or anti-CD47 immunomodulatory treatment (Hutter et al., 2019).

Microglia activation in response to brain tumors has been extensively studied in mouse models of GBM (Bowman et al., 2016; Gutmann and Kettenmann, 2019; Friebel et al., 2020; Klemm et al., 2020) and in glioma patients (Müller et al., 2017; Sankowski et al., 2019; Friebel et al., 2020; Klemm et al., 2020). However, it remains unclear whether TAMs initially attempt to control tumor growth before being reprogrammed by tumor cells. Some studies have suggested that microglia and macrophages from healthy individuals may have the ability to delay the proliferation of brain tumor initiating cells *in vitro* (Sarkar et al., 2014), although these data still need to be confirmed. What is certainly known is that shortly after encountering tumor cells, TAMs are "educated" to promote and sustain tumor growth and invasion (Hambardzumyan et al., 2015). Multiple mechanisms inducing TAMs education have been described and these include tumor-released cytokines (Komohara et al., 2008; Zhou et al., 2015; Kloepper et al., 2016; De Boeck et al., 2020), metabolites (Colegio et al., 2014; Shen et al., 2016; Takenaka et al., 2019) and extracellular vesicles (Maas et al., 2020), or cell-cell contact between TAMs and malignant cells (Chia et al., 2018). Tumor cells can also produce chemokines like CCL2, CCL5, CX3CL1, and CSF1 to attract microglia and MDMs (Gutmann and Kettenmann, 2019).

Once activated, TAMs are reprogrammed to produce factors that stimulate glioma cell proliferation (Coniglio et al., 2012; Hammond et al., 2019), invasion (Markovic et al., 2009; Hu et al., 2014), and therapy resistance (Quail et al., 2016). For example, TAMs favor glioma invasion by remodeling the extracellular matrix (ECM). Factors released by both TAMs and tumor cells induce the reciprocal expression of MMP2, MMP9, and MT1-MMP, that degrade the ECM facilitating glioma migration (Wick et al., 2001; Markovic et al., 2009; Hu et al., 2014). Interestingly, MMP2 is released as an inactive molecule and requires the presence of MT1-MMP on the surface of TAMs to be activated (Markovic et al., 2009), indicating that TAMs and glioma cells can coordinate their functions in the TME. In addition, TAMs can indirectly promote glioma progression by favoring angiogenesis (De Palma et al., 2008) and immunosuppression. *In vitro* studies demonstrated that conditioned medium from glioma cells was sufficient to inhibit the anti-cancer properties of TAMs, including phagocytosis and T-cell stimulation (Wu et al., 2010). TAMs isolated from human glioma specimens showed an impaired ability to activate anti-tumor T cells (Hussain et al., 2006), possibly due to an unbalanced expression of T cell stimulatory vs. inhibitory molecules (Klemm et al., 2020). Therefore, upon activation, TAMs acquire a unique multifunctional activation state (Hambardzumyan et al., 2015) which has been recently confirmed and expanded by single cell RNA-sequencing (scRNA-seq) studies. A complex scenario has emerged describing an heterogeneous population of TAMs which comprises multiple activation states and distinct subpopulations characterized by specific expression profiles (Müller et al., 2017; Sankowski et al., 2019; Friebel et al., 2020; Guldner et al., 2020; Klemm et al., 2020; Pombo Antunes et al., 2021). This might be the consequence of an intrinsic heterogeneity and plasticity of CNS myeloid cells in response to different stimuli (Masuda et al., 2020) and/or it might reflect spatial heterogeneity within the TME. For example, TAMs expressing pro-inflammatory and immunostimulatory genes co-exist with anti-inflammatory TAMs populations (Guldner et al., 2020; Klemm et al., 2020). However, pro-inflammatory cytokines genes, like IL1, are more expressed by TAMs at the tumor periphery compared to the tumor core where, instead, anti-inflammatory genes like TGFβ are upregulated (Darmanis et al., 2017). Within the tumor core, a subpopulation of $CD206^{+}CD169^{+}CD209^{+}$ macrophages was found specifically associated with blood vessels (Friebel et al., 2020) and TAMs expressing hypoxic and pro-angiogenic genes HIF1A and VEGF-A have been described in human gliomas and might be enriched in hypoxic regions (Darmanis et al., 2017; Sankowski et al., 2019). However, a more precise functional characterization of those TAM subpopulations is largely missing. In contrast to other neurodegenerative and inflammatory diseases, for which sequential microglia activation states have been described (Keren-Shaul et al., 2017; Mathys et al., 2017; Sousa et al., 2018; Hammond et al., 2019; Masuda et al., 2019), the temporal dynamics of TAMs education in brain tumors are still largely unknown. In addition, the functional differences among distinct TAM subpopulations and their clinical relevance is still poorly defined.

Interestingly, specific mutations in tumor cells can indirectly shape the immune TME and, in particular, TAMs recruitment and activation. NF1 or PTEN deletion in glioma cells increased microglia and macrophages infiltration *in vitro* and in mouse models (Wang et al., 2017; Chen et al., 2019). AKT1 overexpression also enhances physical interaction of microglia with $AKT1^{+}$-tumor cells in a zebrafish model of brain tumors (Chia et al., 2019). In contrast, IDH mutation is associated with diminished production of chemoattractant cytokines and consequent lower numbers of TAMs in mouse models, and with a less immunosuppressive TAM phenotype in humans (Amankulor et al., 2017; Friebel et al., 2020; Klemm et al., 2020). This evidence highlights the ability of microglia and MDMs to respond and adapt to the TME and emphasize the complexity and heterogeneity of TAMs.

TAM Re-education as a Therapeutic Strategy to Cure Gliomas

Given their central role in tumor progression, TAMs represent a promising therapeutic target. Over the last years, multiple approaches have been tested in preclinical and clinical studies with the goal of re-educating TAMs to exploit their anti-tumorigenic potential (Kowal et al., 2019). These strategies include the use of inhibitors of myeloid-specific receptors like CSF-1R (Pyonteck et al., 2013) and MARCO (Georgoudaki et al., 2016), molecules targeting key factors involved in TAM polarization (Kaneda et al., 2016), or agents that boost

TAMs phagocytic activity like anti-CD47 antibodies (Hutter et al., 2019). However, despite promising results in different cancer types (Mantovani et al., 2017), these approaches have failed to significantly improve the overall survival of GBM patients compared to traditional treatments (Butowski et al., 2016). Indeed, after an initial phase of tumor regression, recurrence occurred upon CSF-1R inhibitor treatment in preclinical mouse models of glioma (Quail et al., 2016). Interestingly, therapy-resistance is induced by tumor-derived factors that activate pro-survival pathways in TAMs (Quail et al., 2016), suggesting a continuous and dynamic TAMs-tumor cells crosstalk during tumor evolution in response to therapy. Moreover, a population of Lgals3$^+$-myeloid cells resistant to CSF-1R inhibitors was recently identified in physiological conditions (Zhan et al., 2020) and also observed in mouse models of GBM (Ochocka et al., 2021). However, additional investigations are needed to elucidate the mechanisms driving therapy-resistance and develop approaches that increase the efficiency of TAM re-educating agents. Promising results come from combinatorial strategies where TAM immunomodulatory molecules significantly enhanced the efficacy of other treatments (Quail et al., 2016; Kowal et al., 2019; Akkari et al., 2020). Also, some non-cancer related FDA-approved drugs have demonstrated an unexpected ability to stimulate a TAM anti-tumor activity and might be repurposed as adjunctive treatments: examples are the antifungal Amphotericin B (Sarkar et al., 2014), the antibiotic thiostrepton (Hu et al., 2021), the iron supplement ferumoxytol (Zanganeh et al., 2016), and the vitamin B3 (Sarkar et al., 2020). Interestingly, TAM re-educating strategies were also effective at reducing astrocytes reactivity, oligodendrocytes loss and at ameliorating the neurological deficits associated with chemotherapy (Gibson et al., 2019).

Although the main line of evidence points toward a pro-tumorigenic role of TAMs and prompts the implementation of TAM re-educating therapies, there are some important exceptions that need to be considered. Maximov et al. (2019) described a surprising anti-tumorigenic role of TAMs, and in particular CCR2$^+$ MDMs, in SHH-medulloblastoma, where TAMs deletion significantly accelerated tumor growth. These findings could be explained by the higher phagocytic activity of cerebellar microglia compared to other brain areas and potentially reflect regional-differences (Ayata et al., 2018). But they also suggest caution when designing TAM re-educating strategies for brain tumors.

ANOTHER PROTAGONIST OF THE TME: REACTIVE ASTROCYTES

Astrocytes are the most abundant glial cells in the CNS. One of the main characteristics of these cells is their ability to respond to multiple types of injuries and to participate in different diseases (Buffo et al., 2010). It is therefore not surprising that they can react also to the presence of tumor cells. In a similar way to microglia, astrocytes are efficiently co-opted by tumor cells to sustain their proliferation, survival, migration and therapy resistance (Wasilewski et al., 2017; Brandao et al., 2019). GBM patients with high expression of reactive astrocytes genes show a worse prognosis and tumor cells co-transplanted with astrocytes develop more aggressive tumors in mice (Mega et al., 2020). Moreover, astrocytes can be a cell-of-origin of gliomas, as they give rise to high-grade brain tumors upon transformation (Bachoo et al., 2002; Zhu et al., 2005). Interestingly, different mutations can impose an astrocytic vs. oligodendrocytic fate to tumor cells, with the first usually associated with more aggressive tumors. For example, cells with aberrant EGFR and Ink4A/Arf deletion acquire expression of astrocytes markers (Bachoo et al., 2002), while PDGFB overexpression is sufficient to de-differentiate astrocyte cultures and to form oligodendroglioma or oligoastrocytoma *in vivo* (Dai et al., 2001). These findings are consistent with the differences described in human glioma subtypes, where an astrocytic gene signature is enriched in the classical subtypes characterized by EGFR amplification and Ink/Arf deletion (Verhaak et al., 2010). Also the contribution of different astrocyte subpopulations to the TME can vary among GBM subtypes (John Lin et al., 2017). Interestingly, a gene signature defining astrocytes activation upon co-culture with GBM cells was significantly associated with mesenchymal GBM (Mega et al., 2020), suggesting a prominent role of non-malignant reactive astrocytes in this tumor subtype.

Astrocytes are characterized by broad morphological and functional heterogeneity that may be the consequence of regional and ontogenetic differences (Khakh and Deneen, 2019). Astrocyte heterogeneity could also affect the tumorigenic potential of these cells, although the mechanisms behind are yet to be unraveled. It has been shown that GFAP$^+$ and GLAST$^+$ astrocytes, when transformed, give rise to GBM with different features: GFAP$^+$ tumor cells are intrinsically more proliferative than GLAST-derived tumors that, instead, may rely more on the TME, as suggested by enhanced recruitment of non-malignant glial cells (Irvin et al., 2017). Marker combinations can be used to subdivide astrocytes in discrete subpopulations that co-exist in healthy brains and can give rise to aggressive gliomas. These astrocytes subpopulations and their malignant analogs display different molecular and functional properties (John Lin et al., 2017).

Whether such heterogeneity characterizes also non-malignant astrocytes in TME remains undefined. By comparing gene expression profiles of TME-associated astrocytes in low and high grade gliomas, Katz et al. (2012) identified a subpopulation of astrocytes specifically localized in perivascular regions of aggressive tumors, where also a population of CD44$^+$ stem-like glioma cells reside. These astrocytes express high levels of osteopontin (or SPP1), a CD44 ligand that induce stemness in glioma cells (Pietras et al., 2014). Both osteopontin and CD44 correlate with a poor overall survival in patients, suggesting a role of perivascular-niche astrocytes in supporting glioma stem cells. Astrocytes expressing a phosphorylated form of PDGFRβ and associated with blood vessels favor brain metastases dissemination and can be targeted to impair metastatic growth (Gril et al., 2013). Another subpopulation of astrocytes that displays high levels of the immune checkpoint PD-L1 and activation of the immunomodulatory factor STAT3 has also been observed in the peritumoral area, where it might constitute a

barrier against anti-tumor T lymphocytes (Priego et al., 2018; Henrik Heiland et al., 2019).

Peritumoral regions are implicated in tumor invasion and multiple studies have demonstrated a role of astrocytes in promoting tumor migration in different ways: by expressing the chemoattractant factor GDNF (Shabtay-Orbach et al., 2015), by releasing MMPs (Le et al., 2003) or by inducing MMPs upregulation in tumor cells (Chen W. et al., 2016). Similar to TAMs, astrocytes can cooperate with tumor cells in remodeling the ECM *via* a mechanism involving plasminogen activation. Plasminogen is produced by glioma cells and converted to plasmin by the protease uPA, which is activated by the uPA receptor expressed on astrocytes. Plasmin mediates MMP2 activation and ECM degradation favoring tumor invasion (Le et al., 2003). Nonetheless, in different contexts, such as the initial phase of brain metastasis, astrocyte-derived PA and plasmin can be cytotoxic for tumor cells that enter in a naïve brain parenchyma. To overcome this effect, tumor cells upregulate the PA inhibitors serpins allowing brain infiltration and metastasis dissemination (Valiente et al., 2014). Hence, although in the early phase of metastasis astrocytes seem to counteract brain invasion, later on they are converted by metastatic cells to promote tumor growth, similarly to what they do in primary glioma (Wasilewski et al., 2017). The events triggering the pro-tumorigenic education of astrocytes in primary and metastatic brain tumors are partially unknown. One described mechanism involves the establishment of direct cell-cell contacts to transfer signaling molecules from tumor cells to astrocytes. These molecules induce astrocytes to release cytokines which, in turn, promote tumor growth (Chen Q. et al., 2016). Astrocytomas, but not oligodendrogliomas, exploit similar gap junctions-mediated connections to form functional cellular networks of tumor cells and rapidly exchanged second messengers and propagate calcium signaling (Osswald et al., 2015). Using similar intercellular communication, astrocytes can also protect metastatic tumor cells from apoptosis by sequestering calcium accumulated in tumor cells upon chemotherapy treatment (Lin et al., 2010).

Immunomodulation is another critical aspect of astrocyte reactivity (Linnerbauer and Rothhammer, 2020). Astrocytes can regulate the immune TME by responding and releasing a broad spectrum of cytokines and by communicating with multiple immune cell types (Priego and Valiente, 2019). Astrocytes expressing high levels of immunosuppressive cytokines, such as TGFβ and IL10, have been recently identified in human GBM samples (Henrik Heiland et al., 2019). JAK/STAT and STAT3 inhibition efficiently reverts astrocytes-related immunosuppression and delay tumor growth, indicating a central role of this pathway in controlling astrocytes immunomodulatory properties (Priego et al., 2018; Brandao et al., 2019; Henrik Heiland et al., 2019). Intriguingly, the presence of phosphorylated-STAT3^{+} immunosuppressive astrocytes is promoted by tumor and microglia cells. Reactive astrocytes can also induce the upregulation of immunosuppressive and tumor-promoting molecules in microglia and macrophages, establishing a positive feedback loop between TAMs and astrocytes in the TME (Priego et al., 2018; Henrik Heiland et al., 2019). Hence, the bidirectional crosstalk between astrocytes and microglia is crucial to define the immunosuppressive microenvironment in brain tumors (Matias et al., 2018). Both cell types, once activated, release cytokines that can boost each other's activation. Importantly, distinct types of cytokines differently influence the activation states and, consequently, the secretome of astrocytes and microglia (Liddelow et al., 2017). Therefore, the microglia-astrocytes interconnection could be a promising therapeutic target that certainly deserves further studies.

Astrocytes have a major role in inducing brain tumor resistance to therapy. Radiation, for instance, can stimulate the release of multiple factors by reactive astrocytes. These factors can support glioma stemness and promote tumor recurrence (Berg et al., 2021). Astrocytes collaborate with microglia to clear oncolytic viral particles, leading to severe implications for the therapeutic use of oncolytic viruses in gliomas (Kober et al., 2015). Also, it remains to be addressed whether astrocytes affect immunotherapy responses by virtue of their immunomodulatory properties and expression of immune checkpoint molecules (Priego et al., 2018; Henrik Heiland et al., 2019; Priego and Valiente, 2019).

Altogether, it is clear that astrocytes actively shape the TME of gliomas and metastatic brain tumors, playing mainly a tumor-promoting role. In the recent years some, although limited in number, innovative approaches targeting astrocytes in brain tumors have been tested in preclinical and clinical trials (Wasilewski et al., 2017). The JAK/STAT pathway is one of the best candidates, being at the crossroad of multiple astrocytes-secreted cytokines, like IL10 and IL6, as well as a critical regulator of astrocytes immune response. Legasil®, a nutraceutical product containing a natural polyphenolic flavonoid called Silibinin, inhibits activation of STAT3 in reactive astrocytes and is able to reduce brain metastasis and significantly increase overall survival rate (Priego et al., 2018). Although tested in a small cohort of patients only with metastatic brain tumors, the results are certainly promising. Interfering with intercellular gap junctions is another way to limit tumor-astrocytes communication. Carbenoloxone, a pan-connexin inhibitor, efficiently blocks intercellular calcium waves propagation among astrocytoma cells (Osswald et al., 2015). Beyond its anti-inflammatory function, meclofenamate was found to inhibit also Cx43 gap junctions (Harks et al., 2001) and is now under investigation in a clinical trial for brain metastatic patients. Moreover, astrocytes heterogeneity and astrocytes-specific factors might also be exploited as novel diagnostic strategies. For example, Katz et al. (2012) defined a tumor-associated astrocytes gene signature which efficiently predicts the survival of patients specifically with proneural GBM subtype. However, many biological and molecular aspects of TME-associated astrocytes still need to be untangled in order to fully exploit their therapeutic potential.

THE ROLE OF NEURAL STEM AND PROGENITOR CELLS

Neural stem cells (NSCs) and progenitors of the subventricular zone have been proposed as a cell-of-origin of gliomas

(Alcantara Llaguno et al., 2009; Lee et al., 2018). Notably, differentiation of NSCs toward the neuronal lineage represents a hurdle for the initiation of brain tumors. Immature and mature neurons are resistant to transformation after loss of tumor suppressor genes, a condition that rapidly triggers tumor formation in neural progenitors and glial cells (Alcantara Llaguno et al., 2019).

In addition to being a cell-of-origin of tumors, neural progenitors can contribute to shape the TME. Non-malignant neural progenitor cells are strongly attracted by tumor cells and preferentially accumulate at the tumor border. Differently from the majority of cells composing the TME, their presence delays tumor growth, as demonstrated by enhanced mice survival when GBM cells are co-transplanted with neural precursors (Glass et al., 2005). Mechanistically, neural progenitors trigger cell death by secreting endovanilloids which stimulate the vanilloid receptor TRPV1, specifically expressed by mouse and human GBM cells (Stock et al., 2012). However, glioma cells might have developed a way to counteract the anti-tumorigenic effect of neural progenitors. In a recent study on mouse NCSs, Lawlor et al. (2020) discovered that, when in direct contact, malignant-NSCs induce quiescence in wild-type (wt)-NSCs *via* NOTCH signaling activation. In this way, they rapidly outcompete wt-NSCs in numbers. A crosstalk between tumor cells and neural progenitors remains to be confirmed in humans, but it might be particularly relevant in young patients, where progenitor cells are still present. However, it should be considered that in inflammatory contexts NSCs can interact with microglia/macrophages inducing immunosuppression (Peruzzotti-Jametti et al., 2018). Given their high tropism for tumors and their tumor-suppressive function, neural progenitors could be exploited as an alternative therapeutic strategy to counteract gliomas.

OLIGODENDROCYTES AND OLIGODENDROCYTE PROGENITOR CELLS: AN UNDERESTIMATED PLAYER IN THE TME

Using multiple transgenic mouse models, different groups have demonstrated that oligodendrocyte progenitor cells (OPCs) can be a cell-of-origin of gliomas (Liu et al., 2011; Sugiarto et al., 2011; Galvao et al., 2014). The presence of oligodendrocytes with the typical "fried egg-shape" has been historically the main histopathological criteria to define human oligodendroglioma (Gupta et al., 2005). More recently, comprehensive molecular genetic profiling and scRNA-sequencing studies have confirmed the presence of OPC-like glioma cells not only in oligodendroglioma but also in other glioma entities, like astrocytoma and GBM, although with varying abundance (Venteicher et al., 2017; Neftel et al., 2019). Of note, the proneural GBM subtype is characterized by alteration of *PDGFRA* and high expression of oligodendrocytic markers (Verhaak et al., 2010; Wang et al., 2017).

The function of OPCs and oligodendrocytes within the TME has been neglected for a long time. A recent bioinformatic method that predicts tumor-TME interactions from scRNA-seq datasets revealed a significant infiltration of non-malignant oligodendrocytes in the TME of proneural GBM compared to other GBM subtypes (Caruso et al., 2020). The authors also identified ligand-receptor pairs that suggest crosstalk between malignant and non-malignant oligodendrocytes. For example, infiltrating oligodendrocytes express high levels of PDGFA that, *via* activation of its receptor PDGFRα on glioma cells, could fuel tumor proliferation (Cao, 2013; Caruso et al., 2020). Other evidence, mainly *in vitro*, suggests the existence of a bidirectional communication between oligodendrocytes and tumor cells within the TME. Conditioned media from human glioma cell lines increases the survival of OPCs. In turn, OPC-derived factors like EGF and FGF1, promote stemness and chemotherapy resistance of glioma cells (Hide et al., 2018). In contrast, a negative correlation between Wif-1$^+$ non-malignant oligodendrocytes and the proliferation index of human astrocytoma cells has been reported, suggesting an anti-tumorigenic role of oligodendrocytes *via* Wnt inhibition (Asslaber et al., 2017). Although the function of non-malignant OPCs needs further investigation, the accumulation of these cells at the tumor border of human gliomas suggests that they might play a strategic role within the TME (Asslaber et al., 2017; Hide et al., 2018). In the tumor periphery TAMs are also particularly abundant suggesting a TAMs-OPCs crosstalk. Indeed, it has been shown that macrophage-derived soluble factors sustain OPCs viability (Hide et al., 2018). In addition, Huang and colleagues described a population of glioma-associated OPCs that promotes neovascularization and facilitates tumor growth (Huang et al., 2014). These PDGFRα$^+$ OPCs are recruited at the tumor border through PDGFC released by TME cells, including TAMs. Interestingly, mice with either *PDGFRA* or *PDGFC* deletion display a normalized vascular network and a significant decrease in tumor volume. OPCs could also influence microglia *via* TGFβ, which is essential for proper microglia development and maintenance. Pharmacological and genetic depletion of NG2$^+$ OPCs indirectly causes downregulation of TGFβ signaling in microglia with consequent alteration of microglia homeostasis. Although in the absence of a pathological condition no changes of activation markers were detected in microglia (Liu and Aguzzi, 2020), it would be interesting to investigate the effects of OPCs depletion on microglia in the context of a tumor. IGF-1 is, instead, crucial for OPCs survival and myelination during brain development, when it is mainly produced by CD11c$^+$ microglia cells (Wlodarczyk et al., 2017), a subset of microglia also identified in human gliomas (Friebel et al., 2020). IGF-1, released by TAMs, promotes brain tumor progression and recurrence in response to prolonged administration of CSF-1R inhibitors (Quail et al., 2016; Yao et al., 2020) and might have also some still unexplored effects on OPCs/oligodendrocytes of the TME.

The crosstalk between glioma cells and oligodendrocytes is demonstrated also by the observation that tumor cells use white matter tracts to disseminate into the brain and escape radiotherapy, precluding a complete tumor eradication. Despite being a well-known characteristic of aggressive

gliomas (Cuddapah et al., 2014), the mechanisms behind this phenomenon are unclear. Wang et al. (2019) proposed a NOTCH-SOX2-SOX9 axis driving tropism of glioma cells for white matter tracts. They found that $CD133^+$ bona-fide glioma stem cells are preferentially associated with portions of nerve fibers, where myelin is lost and unconstrained axonal signals stimulate glioma cell survival. Partial demyelination nearby white matter infiltration was also recently reported by Brooks et al. (2021). The authors described, in addition, axonal degeneration, OPCs recruitment and microglia activation: all events usually occurring in demyelinated lesions (Franklin and Ffrench-Constant, 2008). Surprisingly, this white matter injured-like microenvironment is sufficient to stop proliferation of infiltrating tumor cells and to upregulated SOX10 which induces differentiation of tumor cells toward the oligodendrocyte lineage (Brooks et al., 2021). Therefore, in white matter tracts tumor cells may encounter opposite signals stimulating stemness or differentiation. What determines the final outcome is still unclear. More importantly, these reports revealed two intriguing aspects: (i) white matter tracts may play an active role in shaping the fate of tumor cells and not just be a route for tumor cell invasion; (ii) myelinating oligodendrocytes directly interact with tumor cells. Mechanism triggering myelin damage as well as signals regulating OPCs/oligodendrocytes-glioma communication need to be clarified with further investigation.

In addition, it is important to remember that neuronal electrical activity not only promotes tumor growth, as we will describe later (Venkatesh et al., 2015, 2017), but can also stimulate OPCs proliferation and restore myelination in demyelinated lesions (Gibson et al., 2014; Ortiz et al., 2019). Notably, OPCs express high levels of NLGN3 which, together with neuronal neurexin, forms axo-glial contacts that favor OPCs proliferation and myelination (Proctor et al., 2015). Activity-dependent release of NLGN3 by OPCs has been observed, but the molecular mechanism is still unknown and doesn't involve ADAM10 cleavage, as in the case of neuronal-derived NLGN3 (Venkatesh et al., 2017). However, more studies are required to clarify the role of OPC-derived NLGN3 in the TME. OPCs possess also additional characteristics which might be relevant during tumor evolution, like the formation of functional synapses with neurons, the establishment of cellular networks that propagate calcium signaling and the ability to buffer the potassium released after neuronal activity (Menichella et al., 2006; Bergles et al., 2010; Parys et al., 2010; Venkatesh et al., 2019). All these features might allow non-malignant OPCs to functionally integrate into tumor cell networks and tune depolarizing currents in glioma cells, modulating their proliferation (Osswald et al., 2015; Venkatesh et al., 2019). Additional studies will help to understand a possible neuron-OPC-glioma interplay that could drive tumor progression.

The fact that oligodendrocytes possess immunomodulatory properties is a relative recent concept that is still poorly investigated in gliomas, but that might have important implications for disease progression and therapy response. Data from other CNS diseases have recently proven the ability of OPCs to directly interact with immune cells and to modulate inflammation. In a mouse model of preterm white matter injury, OPCs and particularly immature oligodendrocytes are capable of responding to neuroinflammation by upregulating cytokines and the innate immune response receptor TLR3. OPCs/oligodendrocytes stimulated *in vitro* with a natural ligand of TLR3 secrete factors that activate an inflammatory and phagocytic response in microglia (Boccazzi et al., 2021). OPCs can also upregulate MHC-I/II in response to IFNγ and are able to cross-present antigens and stimulate T cells (Falcão et al., 2018; Kirby et al., 2019). In multiple sclerosis plaques, this ability leads to presentation of myelin antigens and a consequent increase of inflammation, OPCs death and impaired remyelination (Kirby et al., 2019). However, in contexts where a T cell response is required to kill tumor cells, the ability of OPCs to activate cytotoxic T cells might be helpful to counteract tumor growth. The immunomodulatory activity of OPCs/oligodendrocytes can also be exerted through recruitment of innate immune cells. However, OPCs can also impair immune cells function and limit inflammation by upregulating factors, like Sema3A (Majed et al., 2006) or FasL (Dowling et al., 1996), inducing immune cells apoptosis. In addition, microglia phagocytosis and clearance can be inhibited by an excess of myelin debris (Shen et al., 2021) which can be a consequence of tumor infiltration along white matter tracts.

Finally, cell adhesion and ECM molecules, as well as MMPs and metalloproteases inhibitors, have been found enriched in OPCs *in vitro* and *in vivo* (Parolisi and Boda, 2018), indicating that these glial cells are an additional and crucial player in tissue remodeling. Moreover, oligodendrocytes release of MMP9 upon IL1β stimulation or after white matter injury, facilitates endothelial tube formation and angiogenesis (Pham et al., 2012), further suggesting a potential implication of oligodendroglia in neovascular remodeling within the TME.

Therefore, although increasing evidence propose OPCs and oligodendrocytes as potential key players in the TME, more work is required to elucidate their roles during disease progression.

THE MULTIFACETED ROLE OF NEURONAL CELLS IN GLIOMA

Recent important findings unravel an active role of neurons within the TME. However, complex relationships between multiple types of neurons make this mechanism difficult to disentangle (Deisseroth et al., 2004; Ge et al., 2007). During normal development, electrical activity influences the formation of central and peripheral neural system (Gafarov, 2018). In particular, CNS maturation is shaped by patterned waves of electrical activity inducing calcium transients (Wong et al., 1995; Corlew et al., 2004). In the adult brain, excitatory neurons modulate proliferation and differentiation of stem cells in the subgranular zone of the dentate gyrus and in the subventricular zone (Deisseroth et al., 2004; Paez-Gonzalez et al., 2014). Moreover, adult neurons regulate Schwann cell proliferation and survival in the PNS (Maurel and Salzer, 2000) and glial precursors proliferation and myelin plasticity in the CNS, conditioning brain structure and function (Scholz et al., 2009; Takeuchi et al., 2010).

Similarly, neurons can also affect glioma proliferation (Johung and Monje, 2017; Tantillo et al., 2020b).

Recent studies have proven that neural activity affects glioma cells behavior in a region-specific manner. Indeed, visual deprivation only affects glioma proliferation when the tumor is located in the visual cortex, but not in the motor cortex (Tantillo et al., 2020b). In addition, the activation of different neuronal populations may have a differential action on glioma growth. In the latest years it has been demonstrated that, while pyramidal cell activity promotes proliferation in both murine and patient-derived high-grade gliomas (Venkatesh et al., 2015, 2017; Tantillo et al., 2020b), the selective stimulation of parvalbumin-positive interneurons elicits the opposite effect in glioma-bearing mice (Tantillo et al., 2020b). Similarly, in other types of cancer such as breast cancer, the stimulation of parasympathetic (cholinergic) afferents reduces tumor growth and the stimulation of sympathetic (noradrenergic) nerves accelerates it (Kamiya et al., 2019). Several studies suggest also that the release of secreted molecules such as neurotransmitters and neurotrophins exert a relevant role in the neural regulation of glioma proliferation. For instance, direct interaction between glioma cell lines and neurons leads to an upregulation of functional GABA A receptor in glioma cells, which strongly correlates with the malignancy of brain tumors (Synowitz et al., 2001). However, since both neurons and glioma cells can induce activity-dependent secretion, unraveling the cellular source of these fundamental factors is technically challenging and require further investigation.

Despite the latest evidence demonstrating a tumor-promoting effect of neurons, an anti-proliferative effect of peritumoral neurons on glioma cells has also been described (Liu et al., 2013). Peritumoral neurons are capable of restraining glioma progression through the pivotal role of PD-L1, whose signaling in brain cells is important for GBM patient survival (Liu et al., 2013). High expression of PD-L1 by peritumoral neurons positively correlates with GBM prognosis, demonstrating the existence of a PD-L1-dependent interaction between peritumoral neurons and tumor cells (Liu et al., 2013). On the contrary, its expression on GBM cells promotes PD-1 receptor activation in TAM and T cells, inhibiting tumor cells phagocytosis and killing (Gordon et al., 2017; Litak et al., 2019).

All these studies depict a complex scenario of bidirectional interactions between neuronal and glioma cells. This entangled crosstalk needs to be completely dissolved in order to achieve a clear understanding of its underlying mechanisms, which may pose the foundation for the development of more effective therapeutic approaches.

The Complex Role of Neurotransmitters and Neurotrophins

It is well-known that glioma cells express functional neurotransmitter receptors, which could interfere with mitogenic pathways. Glutamate is one of the most abundant molecules of the CNS, but its extracellular levels must be maintained low, in order to prevent hyperexcitability and guarantee a normal brain function. Therefore, the scavenger role of astrocytes, through Na^+-dependent glutamate transporters (i.e., EAAT1 or EAAT2), is of paramount relevance to maintain this equilibrium (Bergles and Jahr, 1997; Danbolt, 2001; Robert and Sontheimer, 2014). Similarly, glioma cells express high levels of EEAT1/2. When EEAT1/2 is absent or mislocalized glutamate uptake is compromised; this event causes high levels of glutamate in the extracellular space that leads to excitotoxicity (Ye et al., 1999; Buckingham and Robel, 2013; Robert and Sontheimer, 2014; Campbell et al., 2015).

Tumor cells can also extrude glutamate, predominantly through the cystine-glutamate antiporter (system xc-, SXC) that exchanges intracellular glutamate for extracellular cysteine, normally involved in the generation of antioxidant glutathione (de Groot and Sontheimer, 2011). The biochemical source of glutamate for glioma cells is not entirely known. Some studies suggest that glutamate may be generated from glutamine *via* a glutaminase reaction. Other works, instead, propose that glutamate could be accumulated due to other metabolic impairments, as the decreased conversion in α-ketoglutarate by GLUD2 enzyme (de Groot and Sontheimer, 2011; Franceschi et al., 2018). Glioma cells express both ionotropic and metabotropic glutamate receptors (de Groot and Sontheimer, 2011) and the increased extracellular glutamate levels in proximity of the tumor mass (Behrens et al., 2000) promote survival, growth and migration in part through the activation of AMPA receptors and, consequently, Rho-Akt pathway (Ishiuchi et al., 2007). The high amount of glutamate acts in a paracrine/autocrine manner on tumor cells, facilitating their spread into the brain parenchyma. This event results in excitotoxicity, which damages neurons located in the tumor vicinity. Moreover, gliomas are known to increase the neural excitability of peritumoral neurons (Venkatesh and Monje, 2017), even if the mechanism that links hyperactivity and cell proliferation has still not completely clarified.

In a normal brain, GABA is the most important inhibitory neurotransmitter of the CNS and controls stem cell proliferation in the subventricular zone and hippocampus, limiting the generation of new neuroblasts (Young and Bordey, 2009; Song et al., 2012; Giachino et al., 2014; Blanchart et al., 2017). It has been demonstrated that glioma cells express GABA receptors, whose function is still under debate. Studies on patient-derived glioma cultures show that the expression of functional GABA A receptors correlates more with low-grade gliomas and oligodendrogliomas than GBM (Labrakakis, 1998; Smits et al., 2012). On the contrary, in GBM GABA A receptors are downregulated to reduce GABA inhibition of tumor cell proliferation (Jung et al., 2019). At the same time, other *in vitro* studies on human GBM samples suggest the opposite (D'Urso et al., 2012). In a p16 $Arf^{-/-}$ PDGFB murine glioma model an endogenous ionotropic signaling has been identified within tumor cells, demonstrating that glioma cells not only respond to GABA, but they can also release it in the TME, limiting cell proliferation of both murine and patient-derived GBM cells (Blanchart et al., 2017). It has been proposed that GABA could have a role in tumor progression, as suggested by *in vivo* experiments using GABA B agonist (muscimol) and GABA A antagonist (bicuculline). Intriguingly, the effect of GABA

is more pronounced in glioma cells with stem-like properties, suggesting a possible strategy to maintain a pool of quiescent tumor initiating cells (Blanchart et al., 2017).

Neurotrophins are a family of secreted molecules that influence many aspects of brain physiology. During malignancies, it is not clear whether they exert a pro or anti-tumorigenic effect on glioma cells (Garofalo et al., 2015; Venkatesh et al., 2015). Their controversial role is still under debate and may depend on their multifaceted functions. For instance, it is known that in pediatric tumors, such as neuroblastoma or medulloblastoma, tumor cells express neurotrophin receptors and the associated clinical outcome is favorable (Donovan et al., 1993; Nakagawara et al., 1993; Eberhart et al., 2001). In adult low-grade gliomas TrkA and TrkB are highly expressed, while their presence in GBM is weak, suggesting that those receptors might be involved only in the less aggressive phase of the disease (Wadhwa et al., 2003). Some studies have shown that the activation of the TrkA receptor through NGF treatment has an anti-mitotic and pro-differentiating effect on C6 glioma cells (Rabin et al., 1998; Kimura et al., 2002). Conversely, on human GBM cell lines, NGF seems to improve tumor proliferation *via* Notch signaling (Park et al., 2018). In addition, a phase II clinical trial with administration of NGF to patients with childhood optic gliomas led to a statistically significant improvement in vision respect to placebo-treated patients (Falsini et al., 2016).

Also the role of BDNF in the regulation of glioma growth remains controversial. BDNF is a peptide secreted in an activity-dependent manner (Hong et al., 2008). *In vitro* studies demonstrate that BDNF, through its receptor TrkB, stimulates high grade glioma cells proliferation (Xiong et al., 2013) and that BDNF release increases with neural activity (Venkatesh et al., 2015). At the same time, it has also been shown that BDNF overexpression in the hypothalamus has immune-augmenting properties, provoking an increased anti-tumor immune response and reducing the activity of proteins that would normally confer resistance to chemotherapy (Radin and Patel, 2017). Moreover, glioma-bearing mice reared in an enriched environment (EE) showed enhanced levels of BDNF together with significantly reduced tumor growth, suggesting a pivotal role for this neurotrophin in glioma proliferation (Garofalo et al., 2015). BDNF infusion was also found to reduce TAMs infiltration and activation, and to dampen glioma migration *via* inhibition of RhoA through the truncated TrkB.T1 receptor (Garofalo et al., 2015). These data also emphasize the idea that lifestyles and physical activity can have a direct impact on the brain microenvironment (Garofalo et al., 2015; Tantillo et al., 2020a), counteracting glioma progression.

NEURONAL ALTERATIONS INDUCED BY GLIOMA GROWTH

Emerging evidence suggests that glioma cells exert a strong influence on neurons and neuronal activity. For instance, deficits in neurocognitive functioning frequently occur in glioma patients, heavily affecting their quality of life (Aaronson et al., 2011; Seano et al., 2019). The tumor mass can hamper brain functionality not only by mechanical compression on brain structures but also by releasing excitotoxins which provoke neuronal death and perturb synaptic transmission (van Kessel et al., 2017).

Approximately 30–50% of patients with brain tumors manifest seizure as an initial symptom of disease progression (van Breemen et al., 2007), making tumor-associated epilepsy (TAE) among the most common hallmarks of comorbidity in glioma patients (Radin and Tsirka, 2020). Interestingly, the incidence of developing TAE is higher in low compared to high grade tumors (van Breemen et al., 2007; You G. et al., 2012), but the reasons of such difference are not known. TAE often manifests as focal seizures with secondary generalization and, despite its major clinical and social impact, the pathophysiological causes are poorly understood. In addition, due to the complex heterogeneity of perturbed mechanisms, this kind of epilepsy is often refractory to antiepileptic treatments (You G. et al., 2012; Cowie and Cunningham, 2014). An important aspect that determines the insurgence of seizures is the localization of the tumor mass in the brain. The proximity to the cortical gray matter is a fundamental factor: tumors that are localized closely to the cortex or in the limbic or perilimbic areas are highly epileptogenic, whereas tumors located in the inner parts of the brain are less prone to manifest seizures (Berntsson et al., 2009; Cowie and Cunningham, 2014). The pathogenesis of TAE is likely to be multifactorial and involves the interaction of genetic factors, changes in the peritumoral microenvironment, hypoxia, acidosis, and metabolic impairments that could affect neural morphology and function (You G. et al., 2012; Cowie and Cunningham, 2014; Armstrong et al., 2016). In addition, the balance between excitatory and inhibitory networks certainly plays a pivotal role in the generation of seizures (Nelson and Turrigiano, 1998; You Y. et al., 2012; MacKenzie et al., 2017). Indeed, the high amount of glutamate extruded by glioma cells overactivates both NMDA and AMPA receptors resulting in excitotoxicity, tumor invasion and hyperexcitability of neural tissues (Rzeski et al., 2001; Savaskan et al., 2008; Sontheimer, 2008; Vanhoutte and Hermans, 2008; Marcus et al., 2010; Robert and Sontheimer, 2014). The prolonged activation of NMDA receptors provokes a sustained influx of Ca^{2+} into the cell mediating excitotoxicity (Terunuma et al., 2010), whereas the reduction of neuronal cell density creates room for tumor expansion (Buckingham et al., 2011) and the overactivation of AMPA receptors further boosts neural excitability (Savaskan et al., 2008). In order to adapt to a glutamate-rich environment and avoid the toxic effects of glutamate, glioma cells downregulate AMPA or NMDA receptors in a grade- and spatial-dependent manner. Indeed, expression analyses of different tumor regions showed that their expression is highest at the infiltrating front of the tumors, suggesting a role in mediating tumor invasion (Radin and Tsirka, 2020).

Multiple factors are necessary to establish long-lasting hyperexcitation and concur to support the onset of peritumoral epilepsy (Campbell et al., 2015). For instance, pyramidal peritumoral neurons downregulate KCC2 and upregulate NKCC1, two transporters responsible for exporting and importing chloride, respectively. The consequent abnormal high amount of intracellular chloride causes GABA-mediated

depolarization, reversing chloride potential and leading to an excitatory and detrimental action of GABA receptor in pyramidal neurons (Conti et al., 2011; Di Angelantonio et al., 2014; Pallud et al., 2014; Campbell et al., 2015; Huberfeld and Vecht, 2016; Jung et al., 2019). In addition, peritumoral regions show a significant loss of approximately 35% of GABAergic interneurons, together with a reduction in their firing rates and in synapses with pyramidal neurons (Campbell et al., 2015; Tewari et al., 2018; Yu et al., 2020). Both altered chloride homeostasis and decreased GABAergic inhibition contribute to render the peritumoral tissue more prone to seizures (Buckingham et al., 2011; Venkatesh et al., 2019).

Finally, several studies have shown that the perineuronal nets (PNNs), which surround fast spiking interneurons acting as ionic buffer, neuroprotecting and stabilizing their synaptic activity, resulted degraded by glioma-released proteases. This provokes an increased membrane capacitance and, in turn, reduces the firing rate of the remaining peritumoral inhibitory interneurons within 400 μm from tumor edge. In particular, the pro-seizure effect of PNN degradation seems to be due to the lacking role of electrostatic insulators affecting the physiological properties of fast-spiking interneurons (Buckingham et al., 2011; Tewari et al., 2018; Venkatesh et al., 2019).

Astrocytomas are able to directly interact with the surrounding environment, extending ultra-long membrane protrusions called tumor microtubes (TMs) that represent a route for brain invasion (Osswald et al., 2015). Recently, it has been discovered that glioma cells can create an intricate interconnection with neurons, forming an electrically coupled network. Within these networks neuronal activity evokes non-synaptic activity-dependent potassium currents amplified by tumor mediated gap junctions (Venkataramani et al., 2019, 2021). The presence of these structures correlates with the worst prognosis in human gliomas and confers resistance against all the available therapies (Osswald et al., 2015; Weil et al., 2017). Studies in Drosophila demonstrate that brain tumor cells protrude TMs to enwrap neurons, inhibiting the Wnt pathway through the accumulation of Fz1 receptor or through the secretion of pathway antagonists (such as Impl2), thus causing neurodegeneration (Portela et al., 2019; Jarabo et al., 2021). In addition, glioma cells interact with peritumoral excitatory neurons forming functional bona fide AMPA-mediated synapses (Venkataramani et al., 2019), through which pro-mitotic peptides as NLGN3 can stimulate glioma progression (Venkatesh et al., 2019). Surprisingly, even tumor cells of non-brain origin, like metastatic breast cancer cells, establish synaptic contacts with neurons (Zeng et al., 2019). This suggests that tumor-neuron synapses are an efficient way that tumor cells exploit in order to obtain glutamate and sustain their proliferation.

NEURONAL ALTERATIONS INDUCED BY GLIAL CELLS IN THE TME

In addition to neuronal alterations directly induced by glioma cells, glial cells response can also profoundly affect brain functionality. Being coopted by tumor cells, glial cells can no longer support brain homeostasis. In addition, activated glial cells contribute to establish a TME milieu rich of cytokines, metabolites and signaling molecules that have potential detrimental effects on neuronal activity. The inflammatory status of gliomas is the result of complex interactions of different TME players. Astrocytes, TAMs and oligodendrocytes all possess immunomodulatory properties, including the production of a broad spectrum of cytokines. Beside their prominent role in stimulating or suppressing immune and glial cells activity, the cytokines present in the TME can also affect neuron survival and activity. Although there are some examples of chemokines with neuroprotective roles (Trettel et al., 2020), an excessive accumulation of pro- and anti-inflammatory cytokines has usually neurotoxic effects. Cytokines like IL1α/β, TNFα, and TGFβ, which are highly enriched within the TME, have been shown to induce neurons loss, impair neuronal morphogenesis and are associated with many neurodegenerative diseases (Glass et al., 2010; Nakashima et al., 2018).

Inflammation is also a consequence of aggressive chemo- and radiotherapies that can exacerbate a neurotoxic microglia-astrocytes crosstalk with long-term consequences on neurons integrity, myelin plasticity as well as neurogenesis (Monje et al., 2003; Gibson et al., 2019). It is well-known that anti-cancer treatments, beyond their unquestionable advantage of delaying tumor progression, are accompanied by multiple side effects that in most of the cases are short-term and temporary. However, what is less known are the long-term consequences of these therapies, especially on brain functions, with patients experiencing deficits in memory, attention, information process, and even mental health (Gibson and Monje, 2021). In addition, neurotoxicity associated with cerebral edema has been reported after immunomodulatory treatments, although the underlying mechanisms are still undefined (Gust et al., 2017; Spain et al., 2017).

Cerebral edema is a common complication of brain tumors and is generally associated with increased intracranial pressure, reduced blood flow and hypoxia, all events that lead to neuronal deficits (Roth et al., 2013). These phenomena can arise as consequence of the presence of a tumor mass, that physically compresses and remodels the brain parenchyma. For example, by migrating along blood vessels, tumor cells displace astrocytic endfeet from endothelial cells causing loss of endothelial tight junctions and breaches in the BBB (Watkins et al., 2014). Similar effects have also been described in some multiple sclerosis active plaques, where OPCs, due to a defective migration, accumulate along the blood vessels disrupting the BBB. Mechanistically, an excessive Wnt signaling in OPCs triggers perivascular accumulation and release of Wif-1 which inhibits Wnt ligand essential for endothelial cells tight junction, compromising the integrity of the endothelial barrier (Niu et al., 2019). OPCs expressing Wif-1 were also found in gliomas (Asslaber et al., 2017), suggesting that their influence on BBB homeostasis might not be limited to multiple sclerosis but might be extended to brain tumors. Disruption of the neurovascular coupling during disease progression impairs the hemodynamic response after seizures, causing hypoxia and exacerbating the seizure-related brain damages (Montgomery et al., 2020). Glial and immune

cells in the TME can also actively contribute to cerebral edema. Indeed, BBB and vessels permeability are altered by cytokines and factors, like VEGF, released by tumor cells, astrocytes and TAMs (Calabrese et al., 2007; Priego et al., 2018; Sankowski et al., 2019; Klemm et al., 2020). Beside its pro-angiogenic functions, VEGF can induce expression of AQ4, a water-channel that is mainly present on astrocytes composing the BBB and that controls its permeability (Lan et al., 2017). Anti-angiogenic treatments that normalized tumor vasculature have demonstrated efficacy also in reducing brain edema in GBM patients (Batchelor et al., 2007). Corticosteroids are the drug of choice to treat glioma-induced cerebral edema for their ability to downregulate VEGF, modulate the expression of channels and tight junctions' components and, therefore, limiting BBB permeability. However, among other side effects, corticosteroids, and in particular dexamethasone, show immunosuppression and might have additional and unknown effects on cells of the TME that deserve further scrutiny (Cenciarini et al., 2019).

As discussed earlier, PNNs degradation alters neuronal activity concurring to TAE. The contribution of glial cells to PNNs remodeling has been widely demonstrated in physiological conditions and in other diseases (Crapser et al., 2020; Nguyen et al., 2020), but it remains speculative in glioma. Indeed, TAMs, astrocytes and OPCs are important sources of MMPs and can assist tumor cells in ECM remodeling, possibly including PNNs degradation. Exposure to an EE, achieved by continuous physical, sensory and social stimulation, is also known to shape ECM and PNNs, to induce plasticity and neurogenesis and to modulate microglia and astrocytes activation (Baroncelli et al., 2010; Rodríguez et al., 2013; Kempermann, 2019; Yates, 2020). Interestingly, an EE is sufficient to significantly prolong the survival of glioma-bearing mice through re-education of TAMs toward a less immunosuppressive state and through IL15-mediated recruitment of anti-tumor NK cells, which also contribute to TAMs re-polarization (Garofalo et al., 2015, 2017).

Neuron hyperexcitability and acute seizures could be attenuated by microglia *via* P2RY12 (Eyo et al., 2014) which is, however, downregulated in glioma (Sankowski et al., 2019; Friebel et al., 2020; Guldner et al., 2020; Klemm et al., 2020). In a zebrafish brain tumor model, this mechanism was hijacked by tumor cells that transiently increased intracellular Ca^{2+} levels and ATP release, in order to coopt $P2RY12^{+}$ microglia and establish direct microglia-to-tumor contacts that stimulate tumor proliferation (Chia et al., 2019). It remains to be investigated whether tumor cells can exploit also the synapse pruning ability of microglia and astrocytes to form and remodel glioma synapses, possibly at the expense of neurons (Paolicelli et al., 2011; Chung et al., 2013). Of note, specific astrocyte subpopulations have been shown to support synapse formation and their emergence in gliomas correlates with increased hyperexcitability and seizures (John Lin et al., 2017).

In the healthy brain, astrocytes are responsible for removing the excess of extracellular glutamate *via* EAAT1/2 transporters and converting glutamate into glutamine, which is released and re-used by neurons (Mahmoud et al., 2019). However, in gliomas, peritumoral reactive astrocytes show altered electrophysiological properties as well as decreased glutamate uptake and glutamine production, that lead to GABAergic disinhibition and neuronal hyperexcitability (Campbell et al., 2020). Therefore, reactive astrocytes might propagate the glutamate excitotoxicity and exacerbate, instead of preventing, neuronal death and glioma-induced epilepsy.

CONCLUSION AND FUTURE PERSPECTIVES

In physiological conditions a balance among glial cells, immune cells and neurons is necessary and established with the final goal of sustaining neuronal activity and brain homeostasis. In case of malignancy, tumor cells represent an additional player introduced in the game, forcing the equilibrium at its advantage. Indeed, tumor cells coopt neurons and glial cells to create a microenvironment that influences their growth (**Figure 1**). In this context, new interactions are established between tumor and brain resident cells. Interestingly, some of these interactions vary depending on the type of glioma, suggesting a preferred mode of communication that might be intrinsically related to the tumor cell-of-origin or be dictated by specific mutations (Gao et al., 2020).

Tumor cells exploit and even strengthen pre-existing interactions within the TME. Astrocytes, TAMs and OPCs/oligodendrocytes actively communicate and modulate each other's activation in physiological conditions, as well as during tumor progression. Yet, neuronal activity is affected by direct interaction with tumor cells and also indirectly by changes in the TME. Although some of the cellular and molecular mechanisms behind these complex networks have been recently uncovered, many aspects still need to be further investigated. For example, it still remains unclear to what extent cell-cell interactions dynamically change during disease progression and in response to treatments. In addition, cell types like OPCs, that have been so far neglected in gliomas, definitely deserve more attention.

Novel therapeutic strategies that target the TME have recently emerged, showing promising results especially when used in combination with other treatments (Kowal et al., 2019). This suggests that acting on several fronts is a successful strategy to impair cellular interplays in the TME and tumor-TME crosstalk. Nevertheless, there is still a great deal to be done. Indeed, in glioma patients, clinical trials of TME-targeting therapies, and especially immunomodulatory therapies, didn't meet the expectations. Part of the failure is certainly due to the intrinsic therapeutic resistance of gliomas and the particularly immunosuppressed brain microenvironment (Sampson et al., 2020). However, it would be also important to understand the contribution of cells in the TME that are not directly targeted by these treatments.

Interestingly, cells in the TME can undergo senescence due to aging (Fane and Weeraratna, 2019) or upon chemo/radiotherapy (Wang et al., 2020). Cellular senescence is a complex cellular state which determines not only cell intrinsic effects, like cycle arrest and DNA damage response, but also non-cell-autonomous mechanisms that influence the surrounding microenvironment.

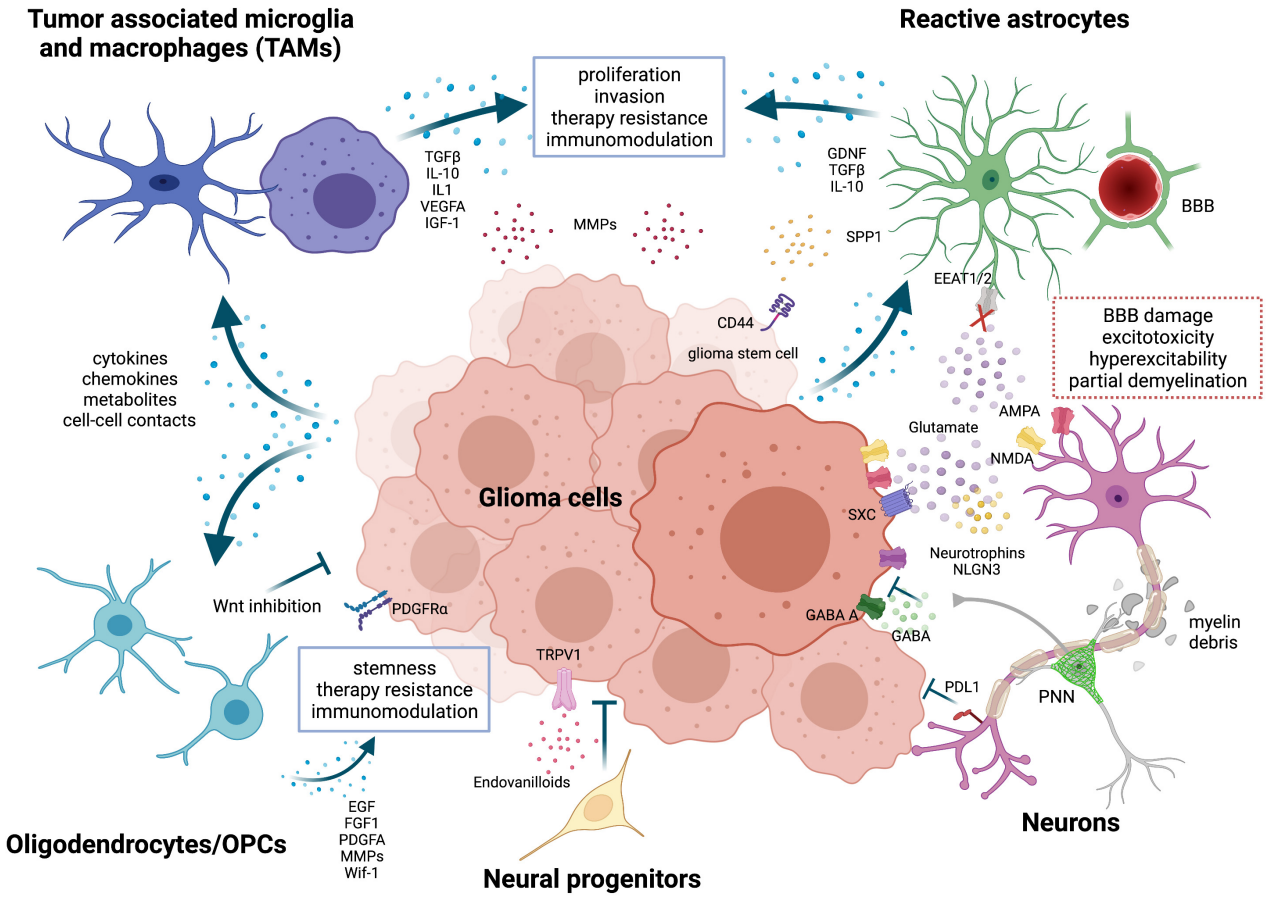

FIGURE 1 | Neurons and glial cells in the glioma microenvironment. Tumor-associated microglia and macrophages (TAMs) are reprogrammed by tumor cells through the release of cytokines, chemokines, metabolites or through cell-cell contacts. Activated TAM produce factors that stimulate glioma proliferation, invasion, angiogenesis, and therapy resistance. Metalloproteinases (MMPs) are released by both tumor cells and TAMs to facilitate glioma cells migration. TME-associated astrocytes are efficiently coopted by tumor cells. They promote tumor migration by expressing chemoattractant factors like GDNF and support stemness *via* SPP1-CD44 axis. Both TAMs and astrocytes also play a role in immunomodulation, expressing high levels of immunosuppressive cytokines, such as TGFβ and IL-10. Oligodendrocytes and oligodendrocyte progenitor cells (OPCs) release factors like EGF, FGF1 and PDGFA that favor proliferation, stemness and chemotherapy resistance of glioma cells. But, at the same time, OPCs can also impair tumor growth through Wnt inhibition. Neural progenitor cells delay tumor growth and induce tumor cell death *via* endovanilloids stimulation of TRPV1. Neurons have a multifaceted role in glioma. Peritumoral neurons were found to counteract glioma progression through programmed death-ligand 1 (PD-L1) protein, but recent evidence has shown that the activation of peritumoral excitatory neurons was able to increase glioma proliferation. Selective activation of peritumoral parvalbumin interneurons elicited, instead, the opposite effect. In addition, tumor cells extrude glutamate outside predominantly through the overexpression of the cystine-glutamate antiporter (system xc-, SXC); this event is not harmonized by peritumoral astrocytes, which show a compromised glutamate uptake *via* the Na^+-dependent glutamate transporters (i.e., EAAT1 or EAAT2). High amount of glutamate provokes an over-activation of both NMDA and AMPA receptors on neuronal cells, resulting in excitotoxicity and hyperexcitability of neural tissues and eventually promotes survival, growth and migration of glioma cells. Glutamatergic neurons can enhance glioma progression also by releasing NLGN3 and neurotrophins and by establishing functional bona fide AMPA-mediated synapses with tumor cells. Invading glioma cells, together with activated glial cells in the tumor microenvironment, cause also secondary alterations like perineural nets (PNNs) remodeling, partial demyelination and blood-brain-barrier (BBB) leakage. Image created with BioRender.com.

The senescence-associated secretory phenotype (SASP) involves the secretion of pro-inflammatory factors, including cytokines and MMPs, that promote various aspects of tumor development like tumor cell proliferation (Kuilman et al., 2008) and immunosuppression (Ruhland et al., 2016). Although numerous studies have reported that non-malignant cells can acquire a senescent phenotype and promote tumor progression in multiple cancers, little is known about senescent non-malignant cells in the brain TME (Wang et al., 2020). Chemotherapy triggers in microglia and astrocytes a pro-inflammatory secretome (Gibson et al., 2019), which is similarly induced by physiological aging (Li et al., 2015; Clarke et al., 2018) or neurodegenerative diseases (Bhat et al., 2012; Liddelow et al., 2017). Progenitor cells and OPCs also show a senescence phenotype in pathological conditions (Nicaise et al., 2019; Zhang et al., 2019) and secondary detrimental effects of senescent glial cells have been reported also on neurons (Limbad et al., 2020). Senolytic therapies have been efficiently used to eliminate senescent cells in multiple diseases, including cancers (Sieben et al., 2018). JAK2/STAT3 inhibitors, beside their immunomodulatory role on

tumor-associated astrocytes (Priego et al., 2018), have been used to reprogram SASP and delay prostate cancer growth (Toso et al., 2014). Moreover, CAR T cells, genetically modified T cells which usually recognize tumor cells-specific antigens, have also been recently engineered to target senescent cells, with promising results in mouse models (Amor et al., 2020). Interestingly, the senescence-specific antigen used to target senescent cells was uPAR, which is also highly upregulated by tumor-associated astrocytes (Le et al., 2003). Therefore, senolytic treatments could be considered as an alternative strategy to target the TME and might be used in combination with more classic anticancer therapies. However, further investigations are certainly necessary to unravel the mechanisms inducing senescence in brain TME cells and in order to identify the factors produced by senescent cells to sustain tumor progression.

We should also not forget secondary alterations, that can be due to direct detrimental effects of tumor cells or to indirect glial cells dysregulation in the TME. In both cases such alterations profoundly affect brain functionality and, consequently, patients' quality of life; therefore, these events should be considered in the choice of the therapeutic intervention and in the development of alternative treatments.

Finally, some technical considerations should be made. We have here reviewed the most important recent studies that have contributed to add pieces to the complex jigsaw of the brain TME, employing different models (e.g., human, mouse, Drosophila, zebrafish) and approaches (e.g., *in vitro*, *in vivo*). Certainly, every model has its own advantages and disadvantages that must be taken into account in the interpretation of the obtained results; remarkably, using different approaches could help to tackle the issue from different points of view, producing a more complete and reliable scenario of the cellular and molecular players in the TME. Since the immune system exerts a critical role in the TME (Lei et al., 2020; Sampson et al., 2020) and, as reported in this review and in many studies, all the TME cells possess immunomodulatory functions, it would be desirable to choose models that do not exclude this important player of the TME (i.e., immunocompetent animal models). In order to address some of the current gaps of knowledge, innovative experimental approaches have been more recently developed. Patients-derived organoids or organotypic cultures are, for example, 3D *in vitro* models that reproduce the TME interactions and could be very useful for testing novel drugs or screening the patient-specific response and subsequently choose the proper therapeutic intervention (Pamies et al., 2020). However, due to their complexity, at the moment these types of models are of difficult adaptation to high-throughput screening. New technologies are also currently aiming at overcoming other limitations, such as the integration of microfluidics or 3D bioprinting of tissue structures to circumvent the lack of a complete vasculature system and the infiltration of peripheral immune components (Li et al., 2020; Pamies et al., 2020).

Dissecting the contribution of different cells of the TME and understanding their interactions is of paramount importance to shed light on the mechanisms that glioma cells exploit to support their growth and invasion. A profound knowledge of the TME is a puzzling but relevant issue, because it will highlight the molecules that could be targeted for the development of novel therapeutic strategies aimed at counteracting brain tumors.

AUTHOR CONTRIBUTIONS

EP, ET, and EV: conceptualization. EP, MS, and EV: review and editing. EP, MS, EM, ET, and EV: contribution to draft preparation. MS and EM: figure making. All authors contributed to the draft preparation, have read and agreed to the published version of the manuscript.

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GLOSSARY

ADAM10	A Disintegrin And Metalloprotease domain 10
AKT	Serine/Threonine Kinase
AKT1	Serine/Threonine Kinase 1
AMPA	Alpha-amino-3-hydroxy-5-Methyl-4-isoxazolePropionic Acid
AMPAR	Alpha-amino-3-hydroxy-5-Methyl-4-isoxazolePropionic Acid Receptor
AQ4	Aquaporin-4 ATP Adenosine Triphosphate BBB BloodŰBrain Barrier
BDNF	Brain-Derived Neurotrophic Factor
CAR T	Chimeric Antigen Receptor
T CCL2	C-c motif Chemokine Ligand 2
CCL5	C-c motif Chemokine Ligand 5
CCR2	C-c motif Chemokine Receptor 2
CD11b	Cluster of Differentiation molecule 11b
CD11c	Cluster of Differentiation molecule 11c
CD133	Cluster of Differentiation molecule 133
CD169	Cluster of Differentiation molecule 169
CD206	Cluster of Differentiation molecule 206
CD209	Cluster of Differentiation molecule 209
CD44	Cluster of Differentiation molecule 44
CD45	Cluster of Differentiation molecule 45
CD47	Cluster of Differentiation molecule 47
CD49d	Cluster of Differentiation molecule 49d
CNS	Central Nervous System
CSF-1R	Colony Stimulating Factor 1 Receptor
CSF1	Colony Stimulating Factor 1
CX3CL1	C-X3-c motif Chemokine Ligand 1
Cx43	Connexin 43
Cxcr4	C-x-c motif chemokine receptor 4
EAAT1	Excitatory Amino Acid Transporter 1
EAAT2	Excitatory Amino Acid Transporter 2
ECM	Extracellular Matrix
EE	Enriched Environment
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FasL	Fas Ligand
FGF1	Fibroblast Growth Factor 1
Fz1	Frizzled class receptor 1
GABA	Gamma-Aminobutyric Acid
GABA A	Gamma-Aminobutyric Acid type A
GABA B	Gamma-Aminobutyric Acid type B
GBM	Glioblastoma Multiforme
GDNF	Glial cell line-Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GLAST	Glutamate-ASpartate Transporter
GLUD2	Glutamate Dehydrogenase 2
Hexb	Hexosaminidase subunit beta
HIF1A	Hypoxia Inducible Factor 1 subunit Alpha
Iba1	Ionized calcium-binding adapter molecule 1
IDH	Isocitrate Dehydrogenase
IFNγ	InterFeroN Gamma

IGF-1	Insuline-like Growth Factor 1
IL1	Interleukin 1
IL6	Interleukin 6
IL10	Interleukin 10
IL15	Interleukin 15
IL1α	Interleukin 1 alpha
IL1β	Interleukin 1 beta
Impl2	Imaginal morphogenesis protein-late 2
Ink	Inhibitors of cyclin-dependent kinase
Ink4A/Arf or p16	Inhibitors of cyclin-dependent kinase 4a/Alternate reading frame
JAK/STAT	Janus Kinase/Signal Transducers and Activators of Transcription
KCC2	Potassium Chloride Cotransporter 2
Lgals3	Galectin 3
MARCO	Macrophage Receptor with Collagenous structure
MDMs	Monocyte-Derived Macrophage
MerTK	Mer Tyrosine Kinase
MHC-I	Major Histocompatibility Complex class I
MHC-II	Major Histocompatibility Complex class II
MMP2	Matrix MetalloPeptidase 2
MMP9	Matrix MetalloPeptidase 9
MT1-MMP	Membrane Type 1ŰMatrix MetalloProteinase
NF1	NeuroFibromatosis type 1
NG2	Neural/Glial antigen 2
NGF	Nerve Growth Factor
NK	Natural Killer
NKCC1	Na-K-Cl Cotransporter 1
NLGN3	Neuroligin 3
NMDA	N-Methyl-D-Aspartic acid or N-Methyl-D-Aspartate
NMDAR	N-Methyl-D-Aspartic acid or N-Methyl-D-Aspartate Receptor
NSC	Neural Stem Cell
OPC	Oligodendrocyte Progenitor Cell
P2RY12	Purinergic Receptor P2Y12
PA	Plasminogen Activator
PD-1	Programmed cell Death protein 1
PD-L1	Programmed Death-Ligand 1
PDGFA	Platelet-Derived Growth Factor subunit A
PDGFB	Platelet-Derived Growth Factor subunit B
PDGFC	Platelet-Derived Growth Factor C
PDGFRA/PDGFRα	Platelet-Derived Growth Factor Receptor Alpha
PDGFRβ	Platelet-Derived Growth Factor Receptor beta
PI3K	Phosphoinositide 3-Kinase
PNNs	Perineuronal Nets
PTEN	Phosphatase and Tensin homologue
RhoA	Ras homolog family member A
Sall1	Spalt like transcription factor 1
SASP	Senescence-Associated Secretory Phenotype
Sema3A	Semaphorin 3A
SHH	Sonic HedgeHog signaling molecule
SOX10	Sry-box transcription factor 10
SOX2	Sry-box transcription factor 2
SOX9	Sry-box transcription factor 9

SPP1	Secreted Phosphoprotein 1
STAT3	Signal Transducer and Activator of Transcription 3
SXC	System XC- cystine/glutamate antiporter
TAE	Tumor-Associated Epilepsy
TAMs	Tumor-Associated Microglia/Macrophages
TGFβ	Transforming Growth Factor Beta
TLR3	Toll-Like Receptor 3
TME	Tumor Microenvironment
Tmem119	Transmembrane protein 119
TMs	Tumor Microtubes
TNFα	Tumor Necrosis Factor Alpha
TrkA	Tropomyosin receptor kinase A
TrkB	Tropomyosin receptor kinase B
TrkB.T1	Truncated isoform of tropomyosin receptor kinase B
TRPV1	Transient Receptor Potential Vanilloid 1
uPA	urokinase-type Plasminogen Activator
uPAR	urokinase-type Plasminogen Activator Receptor
VEGF	Vascular Endothelial Growth Factor
VEGF-A	Vascular Endothelial Growth Factor A
Wif-1	Wnt inhibitory factor 1
Wnt	Wingless/integrated

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REVIEW
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Psychological Stress as a Risk Factor for Accelerated Cellular Aging and Cognitive Decline: The Involvement of Microglia-Neuron Crosstalk

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The relationship between the central nervous system (CNS) and microglia is lifelong. Microglia originate in the embryonic yolk sac during development and populate the CNS before the blood-brain barrier forms. In the CNS, they constitute a self-renewing population. Although they represent up to 10% of all brain cells, we are only beginning to understand how much brain homeostasis relies on their physiological functions. Often compared to a double-edged sword, microglia hold the potential to exert neuroprotective roles that can also exacerbate neurodegeneration once compromised. Microglia can promote synaptic growth in addition to eliminating synapses that are less active. Synaptic loss, which is considered one of the best pathological correlates of cognitive decline, is a distinctive feature of major depressive disorder (MDD) and cognitive aging. Long-term psychological stress accelerates cellular aging and predisposes to various diseases, including MDD, and cognitive decline. Among the underlying mechanisms, stress-induced neuroinflammation alters microglial interactions with the surrounding parenchymal cells and exacerbates oxidative burden and cellular damage, hence inducing changes in microglia and neurons typical of cognitive aging. Focusing on microglial interactions with neurons and their synapses, this review discusses the disrupted communication between these cells, notably involving fractalkine signaling and the triggering receptor expressed on myeloid cells (TREM). Overall, chronic stress emerges as a key player in cellular aging by altering the microglial sensome, notably via fractalkine signaling deficiency. To study cellular aging, novel positron emission tomography radiotracers for TREM and the purinergic family of receptors show interest for human study.

Keywords: microglia, neuron, synapse, cognitive aging, oxidative stress, chronic psychological stress, major depressive disorder

INTRODUCTION

Since their discovery, microglia have been described as the resident macrophages of the central nervous system (CNS). They migrate from the yolk sac to the brain around embryonic day 9.5 in mice (Ginhoux et al., 2010) and can be observed for the first time in the developing human brain around the 4.5–5th week of gestation (Andjelkovic et al., 1998; Monier et al., 2006; Verney et al., 2010). Following their migration and colonization, microglia remain distributed unevenly between the gray and white matters and across individual brain regions (Lawson et al., 1990; Mittelbronn et al., 2001). Indeed, Lawson et al. (1990) described the distribution of microglia in adult mice and found that these immune cells ranged from 5 to 12% of all brain cells. In humans, microglial distribution, which was discovered to be greater in the white matter, varied from 0.3 to 16.9% of all brain cells depending on the region (Mittelbronn et al., 2001). Microglial numbers are then maintained through local self-renewal in physiological conditions, a phenomenon conserved across species (Lawson et al., 1992; Askew et al., 2017; Füger et al., 2017; Réu et al., 2017; Tay et al., 2017).

Microglia are categorized most notably by their morphological state (e.g., surveillant, bushy or hyper-ramified, ameboid, dystrophic) and molecular signature (Martinez and Gordon, 2014; Ransohoff, 2016; Stratoulias et al., 2019). While previously termed as "quiescent" or "resting," surveillant microglia are quite dynamic; their ramified processes retract and extend, constantly surveying the parenchyma for environmental cues (Davalos et al., 2005; Nimmerjahn et al., 2005). This allows them to interact with numerous cellular elements including astrocytic and neuronal cell bodies, synapses, but also with the basement membrane of the brain vasculature (Tremblay et al., 2010; Bisht et al., 2016; Stogsdill and Eroglu, 2017; Joost et al., 2019; Matejuk and Ransohoff, 2020; Vainchtein and Molofsky, 2020). Microglia contacting neuronal cell bodies are known as "satellite" microglia (Wogram et al., 2016; Stratoulias et al., 2019), a position recently associated with the regulation of neuronal activity (Cserép et al., 2020). Another interesting aspect of microglia is their ability to quickly alter morphologically to appropriately respond to the functional needs of the brain. Once microglia detect CNS insults through local stimuli, they undergo drastic morphological transformations. Their morphology can range from ameboid to hyper-ramified states (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009), tightly following changes in their transcriptome and proteome (Reynolds et al., 2008; Dungrawala et al., 2010; Zhurinsky et al., 2010; Marguerat and Bähler, 2012; Rangaraju et al., 2018; Bell et al., 2019; Miedema et al., 2020; Rayaprolu et al., 2020). This microglial remodeling is observed in conditions of psychological stress, where among various brain regions that include the prefrontal cortex (PFC) and hippocampus, microglia were found to dynamically transform their morphology, gene and protein expression, as well as function (Bordeleau et al., 2020; Picard et al., 2021). These microglial changes to stress become further exacerbated with aging (Tay et al., 2017). Psychological stress is well-known to accelerate cellular aging (Yegorov et al., 2020), hence potentially predisposing to various neuropsychiatric disorders and neurodegenerative diseases across the lifespan (Musazzi and Marrocco, 2016; Justice, 2018; Desmarais et al., 2020; Smith and Pollak, 2020).

Microglia entertain a particular relationship with neurons. Developmental studies taught us that microglia notably influence neurogenesis by controlling the maturation, density and migration of neuronal progenitors during the first weeks of life (Paolicelli and Gross, 2011; Bordeleau et al., 2019). Acting specifically on neurons, microglia can influence their axonal growth (Pont-Lezica et al., 2014) and ability to form synapses by inducing the genesis of filopodia at dendritic elements (Miyamoto et al., 2016). Microglia are key players in synaptic plasticity, both structural and functional, which allows them to modify the neuronal circuitry via synaptic pruning, synaptic stripping, the secretion of neurotrophic factors and regulation of synaptic activity (Ji et al., 2013a,b; Sipe et al., 2016; Zhou et al., 2019), phenomena confirmed in the olfactory bulb, hippocampus, as well as cerebral cortex in mice (Tremblay and Majewska, 2011; Kettenmann et al., 2013; Morris et al., 2013; Schafer et al., 2013; Milior et al., 2016; Miyamoto et al., 2016). Microglia can modulate learning and memory, including auditory-cued fear conditioning and novel object recognition. This was for instance shown in mice depleted of microglia, which displayed altered performance in these tasks (Parkhurst et al., 2013; Torres et al., 2016). During early postnatal development, microglia are also able to shape the visual cortex of mice in an experience-dependent manner (Tremblay et al., 2010). By sensing synaptic activity, microglia remove weaker signaling synapses in the visual thalamus (Schafer et al., 2013). This is an important concept, as the best correlate for cognitive decline is synaptic loss (Scheff et al., 2006; Jackson et al., 2019; Colom-Cadena et al., 2020). Reduced synaptic density in the PFC is observed upon chronic psychological stress (Csabai et al., 2018), although some studies also show region-specific synaptic growth, for instance in the amygdala, in association with an emotional memory component (Picard et al., 2021). These various roles of microglia in the healthy brain were additionally shown to differ between sexes (Bordeleau et al., 2019).

In this review, we describe recent discoveries on the bidirectional communication between microglia, neurons, and their synapses, as well as discuss how their interactions are altered upon psychological stress and in major depressive disorder (MDD), leading to accelerated brain and cognitive aging. Studies on these emerging mechanisms in humans by positron emission tomography (PET) imaging are also briefly mentioned because of their potential to investigate MDD. Previous investigations using PET imaging have shown decreased metabolites in the limbic lobe and basal ganglia of MDD patients (Su et al., 2014). Further investigation tracking microglia-neuron receptor/ligand communication could offer monitoring opportunity for disease progression and treatment response in MDD patients.

MICROGLIA-NEURON COMMUNICATION AND INTERACTION IN HEALTH, STRESS, AND AGING

The communication between microglia and neurons is bidirectional (Tremblay, 2011; Eyo and Wu, 2013; Szepesi et al., 2018), essential for homeostatic function and implicated in neurodegenerative disorders (Sheridan and Murphy, 2013). It is achieved via the complex microglial sensome constituting a myriad of various key receptors that constantly receive signals from surrounding neurons (Hickman et al., 2013) as well as their synapses (Roumier et al., 2004, 2008; Bessis et al., 2007; Béchade et al., 2013; Cantaut-Belarif et al., 2017; Tay et al., 2017; Comer et al., 2020). Several essential molecular mechanisms such as fractalkine signaling, the classical complement pathway, purinergic signaling, and triggering receptor expressed on myeloid cells 2 (TREM2) are discussed below. Diverse types of signaling can influence each other, acting as on and off systems (Biber et al., 2007).

Fractalkine Signaling

A main mediator of this communication is signaling between the neuronal chemokine fractalkine (CX3CL1) and its unique receptor (CX3CR1) expressed on the surface of microglia (Harrison et al., 1998; Ransohoff and Perry, 2009; Paolicelli et al., 2014; Lauro et al., 2019). During development, neurons expressing CX3CL1 (Meucci et al., 1998) modulate microglial pruning of synapses and brain functional connectivity, thus participating to proper brain maturation and social behavior in mice (Zhan et al., 2014; Arnoux and Audinat, 2015; Gunner et al., 2019). In adult mice, loss of fractalkine signaling causes widespread deficits in glutamate release at hippocampal synapses, which are associated with defects of adult hippocampal neurogenesis as well as learning and memory (Maggi et al., 2009; Rogers et al., 2011; Paolicelli et al., 2014; Zhan et al., 2014; Basilico et al., 2019), while psychological stress is associated with lower CX3CL1-CX3CR1 signaling between neurons and microglia (Han et al., 2019b). Throughout life, fractalkine signaling plays a part in the stress response, as CX3CR1 knockout in mice delays or prevents the response to chronic stress (Hulshof et al., 2003; Wohleb et al., 2013; Milior et al., 2016; Rimmerman et al., 2017; Winkler et al., 2017). In aging, fractalkine signaling is important for the regulation of adult neurogenesis in the hippocampus, but not the olfactory bulb (Gemma et al., 2010; Reshef et al., 2017; Bolós et al., 2018). However, hippocampal fractalkine signaling decreases steadily over the course of aging in mice, where its consequences still remain unknown (Mecca et al., 2018). Moreover, young adult (2-month old) mice with knockout for CX3CR1 were demonstrated to have a microglial transcriptome resembling that of aged mice based on its expression of inflammatory genes, suggesting a protective role of fractalkine signaling in aging (Gyoneva et al., 2019).

Classical Complement Pathway

Complement signaling is an essential part of the innate immune system bearing the role of opsonization, the secretion of molecules enhancing phagocytosis, contributing to overall pathogen removal (Veerhuis et al., 2011). Growing evidence places the complement protein 3 (C3) as essential for proper brain development (Lee et al., 2019), considering that C3 guides microglial synaptic pruning (Schafer et al., 2012; Luchena et al., 2018). In aging rhesus monkeys, the expression of C1q protein, an upstream complex of C3, is increased within synaptic elements of the PFC, signaling to microglia which synapses need to be pruned (Datta et al., 2020). Evidence for an implication of the complement pathway in stress-related disorders shows increased C3 expression in the PFC of mouse models of stress-induced depressive-like disorder (Crider et al., 2018). Distress paradigm in mice has been associated with hyper-ramified microglia and a loss of dendritic spines, paralleled by an increased expression of C1q component in the medial PFC and hippocampus (Smith et al., 2019). These observations are in agreement with an initially beneficial immune response, as shown by other mouse studies, where chronic stress was found to initially increase microglial survival through colony stimulating factor 1 (CSF1) signaling, which additionally promoted neuronal remodeling via the complement pathway (Kreisel et al., 2014; Wohleb et al., 2018; Horchar and Wohleb, 2019). Prolonged stress reduced microglial numbers, hence preventing effective neurogenesis, but this deficit was rescued by stimulating CSF1 signaling (Kreisel et al., 2014).

Purinergic Signaling

Purinergic receptors, many of which are highly expressed by microglia, can be separated into two families, i.e., sensors of adenosine (P1) counting A1, A2A and A3 or nucleotides (P2), which include the receptors P2Y12, P2Y6, P2Y4, P2X4, P2X7, and pannexin 1 (Burnstock, 2013; Illes et al., 2020; Sanchez-Arias et al., 2021). During development and injury, P2Y12 is a key adenosine triphosphate (ATP)-sensitive receptor responsible for determining microglial process motility, an important aspect involved in phagocytosis of both cellular debris or synaptic elements, notably in the mouse visual system (Haynes et al., 2006; Eyo et al., 2014; Sipe et al., 2016). This process was also characterized at the microglial filopodia level, showing that these structures perform a nanoscale surveillance of the mouse brain in homeostasis (Bernier et al., 2019). As microglia sense ATP to put themselves in motion, they also communicate with neurons via the secretion of adenosine binding to adenosine A1 receptor (A1R), which is able to act as negative feedback to suppress neuronal activity by limiting synaptic transmission (Badimon et al., 2020). During prolonged stress exposure, P2X7 receptor is up-regulated in hippocampal and PFC microglia, initiating an inflammatory response via the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome (Franklin et al., 2018; Dias et al., 2021). Assembly of the NLRP3 inflammasome via the P2X7 receptor was shown in the rat hippocampus to take place after 3 weeks of chronic stress (Yue et al., 2017). The implication of purinergic receptors in aging is still elusive, but cognitive decline-related disease conditions involving inflammation, such as Alzheimer's disease (AD), show a consistent up-regulation of P2X7 in microglia (Woods et al., 2016; Francistiová et al., 2020; Pietrowski et al., 2021).

Triggering Receptor Expressed on Myeloid Cells 2 Signaling

TREM2 is a cell surface receptor for putative ligands that include lipids, DNA and pro-inflammatory proteins such as apolipoprotein E and amyloid (β) oligomers, which activate downstream signaling pathways for cell survival, phagocytosis, metabolic fitness and cell motility (Ulland et al., 2017; Konishi and Kiyama, 2018). While TREM2 is a major pathology-induced immune signaling pathway (Deczkowska et al., 2020), it is also required for synaptic pruning during normal development (Filipello et al., 2018). Cell survival-regulating Wnt/β-catenin pathways that are up-regulated after TREM2 binding, notably by one of its ligands Transmembrane Protein 59 (TMEM59), bear great importance for microglia. Knockout of the receptor impairs microglial response in AD, leading to an exacerbated sensibility to stress-induced cell death (Zheng et al., 2017; Liu et al., 2020; McQuade et al., 2020), with reports highlighting the emergence of susceptibility only upon TREM2 haploinsufficiency (Sayed et al., 2018). Later in life, TREM2 sustains a key signaling pathway for microglia, allowing their clearance of myelin debris (Kober and Brett, 2017). This role in myelin recycling is also key in AD, as mutant microglia for TREM2 overly produce autophagic vesicles via a deficit in the mammalian target for rapamycin (mTOR) pathway, leading to AD pathologies in the 5XFAD mouse model (Ulland et al., 2017). Furthermore, aged TREM2 knockout mouse microglia show reduction of phagocytosis and sudden increase in cellular oxidative stress (Linnartz-Gerlach et al., 2019). Overall, TREM2 seems to be major signaling pathway throughout life and shows a great pathological risk when altered in aging.

Tyro3, Axl, and Mer Receptors

Tyro3, Axl, and Mer (TAM) receptors are part of the cellular tyrosine kinase (TK) signaling pathways of the brain (Lemke, 2013). Microglial TAM receptors comprised of Axl and MerTK were shown to be required for their functions during normal physiological conditions as well as injury, specifically their process motility and phagocytosis of apoptotic newborn neurons (Fourgeaud et al., 2016). This is important as TAM receptors can promote hippocampal neurogenesis by preventing the secretion of inflammatory cytokines, as shown *in vitro* using a triple (Tyro3, Axl, MerTK) knockout model for these receptors with cultured microglia (Ji et al., 2013c). During aging, the Axl pathway is implicated in amyloid β clearance, hence preventing cellular toxicity in mice (Zhang et al., 2018) and with amyloid plaque-associated microglia expressing Axl and MerTK (Savage et al., 2015). However, the role of microglial TAM receptors during stress and aging remains elusive.

MICROGLIAL METABOLIC AND MORPHOLOGICAL CHANGES IN STRESS AND AGING

Immune dysregulation crucially impacts an individual's ability to cope with internal and external challenges, such as trauma, infection, but also psychological stress, altering future response to stress outcomes in life and promoting cellular aging (Godbout et al., 2005; Buchanan et al., 2010; O'Donovan et al., 2012; Ritzel et al., 2019). A combination of several mechanisms including cellular senescence, deficits in energetic metabolism, release of inflammatory mediators, dysregulation of protein degradation pathways, activation of DNA repair mechanisms and gut dysbiosis, were suggested to be involved in cellular aging (Franceschi and Campisi, 2014; Franceschi et al., 2018). Upon exposure to immune challenges, stress or pathological aging, microglia are presumed to undergo a *priming* process (Püntener et al., 2012; Frank et al., 2018; Keane et al., 2021) leading to an elevated basal inflammatory activity [i.e., production of tumor necrosis factor alpha (TNFα), interleukin (IL)-1 beta (IL-1β), IL-6] (Sierra et al., 2007; Frank et al., 2010; Marschallinger et al., 2020), further increasing the overall inflammation levels in the brain (Franceschi and Campisi, 2014). Recent evidence showed that microglial priming can be explained by the trained immunity hypothesis, in which a challenge trains microglia to a next insult by promoting their secretion of intracellular metabolites and changing their epigenetic program (Haley et al., 2019; Neher and Cunningham, 2019). These findings are in line with increased gene expression within the interferon (IFN)-pathways observed in rodent models of aging (Grabert et al., 2016; Raj et al., 2017), and result in an inflammatory environment for microglia and neurons (**Figure 1**; Carnevale et al., 2007).

Microglial populations become more heterogeneous with aging as compared to the adult life stage (Olah et al., 2020; Delage et al., 2021). Concurrently, they display similarities with the early developmental period, a stage when microglia actively participate in the assembly and refinement of neural circuits (Schafer et al., 2012; Hammond et al., 2019). Parallel to an increase in the diversity of aging-related molecular signatures, aged microglia are morphologically altered when observed using light microscopy and tend to adopt more of an amoeboid-shaped phenotype. Alternatively, increased density of rod-shaped microglia (Bachstetter et al., 2017) or dystrophic microglia with reduced, gnarly processes have been described (Streit et al., 2004; Damani et al., 2011). These alterations may be of direct relevance to the functional irregularities reported in aged microglia. In this line of idea, transcriptomic investigation in human microglia reveals lower expression of CX3CR1 in aging (Galatro et al., 2017). Similar investigation in stressed microglia showed clustering of microglial population for genes related to inflammation and cytokine secretion (Lehmann et al., 2018), with the main ones illustrated in **Figure 1**. Furthermore, the overall microglial response to stimuli seems to be poorly managed with aging, suffering delays as well as prolonged duration, with potential detrimental consequences for the integrity of the aged CNS (Damani et al., 2011; Hefendehl et al., 2014; Jiang et al., 2020). Long-term sustenance of non-homeostatic microglial states in combination with morphological changes and loss of motility interfering with their optimal area surveillance (Damani et al., 2011; Hefendehl et al., 2014) may be contributing to an excessive accumulation of debris. Underlying mechanisms of the excessive debris accumulation may include increased lysosomal pH and compromised debris clearance notably through phagocytosis. This might be caused by their inability

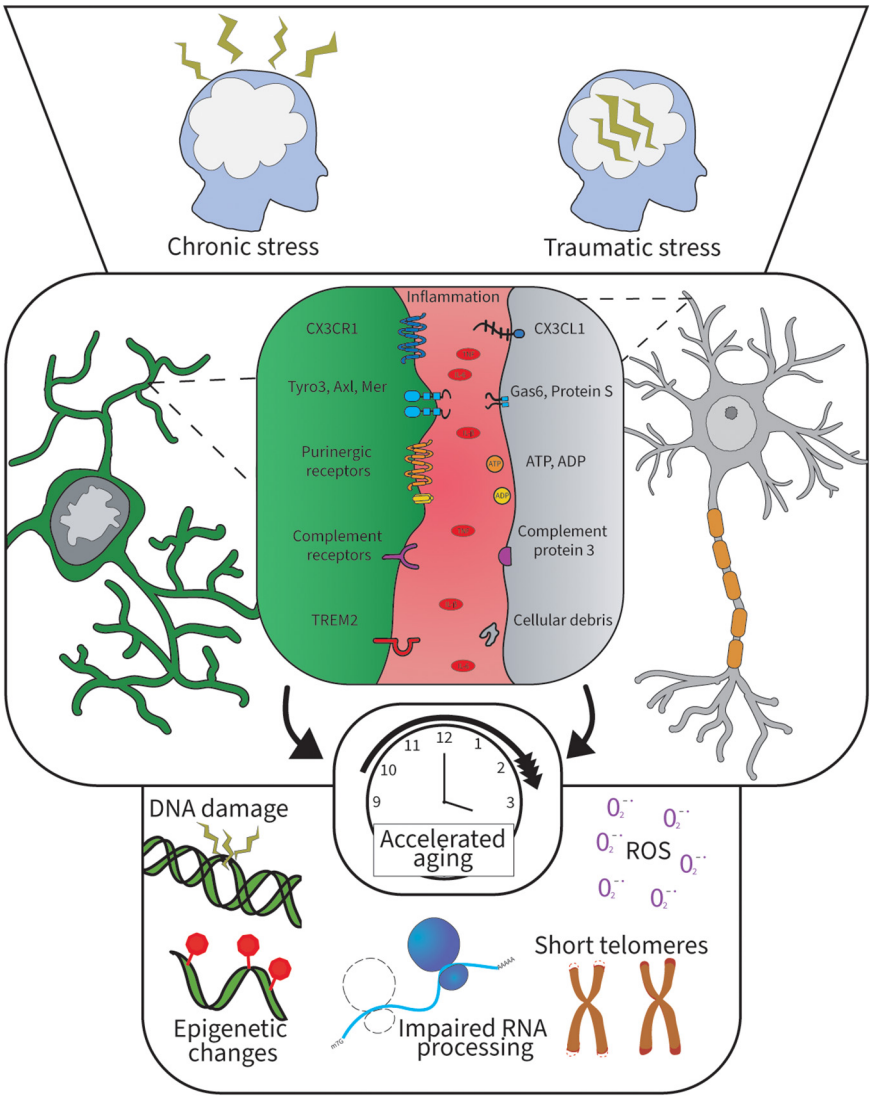

FIGURE 1 | Psychological stress and inflammation alter microglia-neuron communication and lead to cellular aging. Psychological stress induces brain inflammation and oxidative stress resulting in cellular aging when prolonged. This hostile brain environment disrupts the microglia-neuron brain communication and leads to cognitive decline. The top panel illustrates two major types of stress lived by humans. The middle panel shows the different microglia-neuron signaling pathways in the brain and the key inflammatory factors identified in chronic stress. The bottom panel illustrates the different cellular aging processes happening when cells undergo cellular aging, which is often associated with inflammation and aging.

to protect and maintain the physiological function of neurons, synapses, myelin, etc. via trophic factors (Solé-Domènech et al., 2018; Pluvinage et al., 2019). Of note, the concept of senescence, a state when a cell enters division cycle arrest but preserves its metabolic activity and possesses a modified senescence-associated secretory phenotype (Coppé et al., 2010), has recently been a trending topic in the microglial field. Current evidence demonstrated that microglia can preserve their proliferative capacity even in aging as acutely extracted microglial cells from aged murine brains seem to lack up-regulation of senescence-associated markers, e.g., beta-galactosidase, as compared to long-term cultured microglia (Stojiljkovic et al., 2019; Hu et al., 2021). Previously, dystrophic morphological traits have been suggested for this phenotype (Streit et al., 2004). It is yet to be established whether senescent microglia are an independent microglial subtype of aged microglia or a state within the course of microglial aging.

Aged microglia possess elevated levels of mature lysosomal structures, undegradable lipofuscin granules and lipid bodies that are also larger in size (Sierra et al., 2007; Safaiyan et al., 2016; Marschallinger et al., 2020). Moreover, as was shown in a mouse model, aged microglia display reduced expression of the essential autophagy gene, *Atg7* (Berglund et al., 2020), suggesting a limited intracellular maintenance. Metabolic failure occurs alongside impairments in the mitochondrial electron transport chain (ETC) during aging, identified by an altered/abnormal state

of mitochondrial cristae ultrastructure, a marker of oxidative stress (Minhas et al., 2021). Upon exposure to challenges, microglia can undergo a switch from the efficient homeostatic oxidative metabolism (OXPHOS) to a faster ATP production via aerobic glycolysis, to sustain their elevated activity and increased coping strategies (Lauro and Limatola, 2020). In aging, this mechanism may become maladaptive, leading to the formation and accumulation of glycogen (a glucose storage), accompanied by a decreased mitochondrial respiration via the prostaglandin E2 (PGE2) and its receptor (EP2) pathway, as recently shown in mice (Minhas et al., 2021). Microglial proteomic analysis further suggested that this aging-related metabolic shift may relate to their utilization of alternate substrates, such as fatty acid, thus contributing to increased cholesterol trafficking with aging, which may result in neuronal dysfunction and cognitive decline (Flowers et al., 2017). Higher levels of neuronal cholesterol are considered detrimental, as myelinogenesis is slowed in aging, coincident with cytotoxic accumulation of cholesterol and cognitive decline (Kadish et al., 2009). On top of that, increase of intracellular iron, possibly related to an accumulation of lipofuscin, which is able to bind metals (Höhn et al., 2010), may be a contributing factor to aging-related oxidative imbalance and OXPHOS failure. As such, iron overload is considered one of the hallmarks of aged or senescent microglia (Angelova and Brown, 2019).

Several intracellular microglial properties were shown to be affected by aging (Cho et al., 2019). In agreement with observations made in other cell types, aged microglia show several signs of cellular aging such as epigenetic changes via the hypomethylation of IL-β associated with elevated Il-β transcript in microglia (Cho et al., 2015), which are described in **Figure 1**, along with an impaired DNA repair capability, altered transcription machinery and diminished ability of chromatin remodeling, as revealed by proteomic analysis of aged primary mouse microglia (**Figure 1**; Raj et al., 2014; Flowers et al., 2017; Costa et al., 2021). This inadequate microglial remodeling may underlie their impaired ability to dynamically change states according to the needs of the CNS during aging (Flowers et al., 2017). It is important to highlight that the telomere length is chemically affected by oxidative stress, notably via hydrogen peroxidase (H_2O_2), a reactive substrate that can cause prominent DNA damage (Oikawa and Kawanishi, 1999). Together, these cellular changes should be prevented or rescued to promote healthy aging (Depp et al., 2007; Wolkowitz et al., 2011), especially among key brain regions that are impacted by stress and aging.

BRAIN HIPPOCAMPUS AND PREFRONTAL CORTEX: KEY REGIONS IMPACTED BY STRESS AND AGING

The Hippocampus

Hippocampal involvement in memory and learning has been widely studied across mouse, rat, non-human primate and human (Jarrard, 1993; Banta Lavenex and Lavenex, 2009; Voss et al., 2017; Zalucki et al., 2018). While still debated in human (Rodríguez-Iglesias et al., 2019), neurons in animal models are continuously produced throughout the lifespan among the olfactory bulb (Breton-Provencher et al., 2009) and hippocampus (Toda et al., 2019), while microglia also maintain their population by self-renewal (Tay et al., 2017). Several studies indicate that microglia may participate in the hippocampal-dependent learning and memory processes (Jarrard, 1993; Banta Lavenex and Lavenex, 2009; Parkhurst et al., 2013; Torres et al., 2016; Voss et al., 2017; Zalucki et al., 2018). For instance, microglial ablation via the CX3CR1-diphtheria toxin receptor reduces performance in object recognition test in rats (De Luca et al., 2020). The hippocampal CA1 makes projection to other brain regions also affected by stress, including the PFC (Rosene and Van Hoesen, 1977). This association seems to place the hippocampus as the gateway network particularly activated during learning tasks, as shown using magnetic resonance imaging (MRI) in human (Doyère et al., 1993; McEwen et al., 2016). It was also shown that stress resulting from sleep deprivation decreases hippocampal neurogenesis and induces depressive behavior (Murata et al., 2018; Han et al., 2019a). This contrasts with the ability of newborn neurons to contribute to stress resilience by inhibiting the hippocampal dentate gyrus mature granule cells activity as measured using electrophysiology (Anacker et al., 2018). The hippocampal functions are highly sensitive to accelerated aging, showing reduced cholinergic inputs and diminished neuronal activity rhythm in a mouse model of AD pathology (Rubio et al., 2012). Microglia regulate hippocampal neurogenesis in development (Pérez-Rodríguez et al., 2021) and throughout life (Reshef et al., 2017; Szepesi et al., 2018; Augusto-Oliveira et al., 2019; Bordeleau et al., 2019; Tan et al., 2020), in both health and neurodegeneration (De Lucia et al., 2016), via an IL-4 driven brain-derived neurotrophic factor (BDNF)-dependent mechanism (Zhang et al., 2021). Microglial influence on cognition could result from their roles in neurogenesis and at synapses. Indeed, these roles discussed in the section on fractalkine signaling can be performed notably via the secretion of inflammatory mediators and neurotrophic factors (Parkhurst et al., 2013; Sierra et al., 2014). Furthermore, microglia contribute to the control of synaptic activity and plasticity, constituting a quad-partite synapse alongside astrocytes, notably in the hippocampus (Bennett, 2007; Tremblay et al., 2011, 2014; Béchade et al., 2013; Schafer et al., 2013; Sierra and Tremblay, 2014).

The Prefrontal Cortex

The PFC is a key area affected by stress, with major impact on the behavior across species (Arnsten and Goldman-Rakic, 1998; Cerqueira et al., 2007; Holmes and Wellman, 2009; Myers-Schulz and Koenigs, 2012; Arnsten et al., 2015; McKlveen et al., 2015; Page et al., 2019). The stress response involves complex changes in neuron-microglia-astrocyte relationships, notably denoted by increased microglial phagocytosis of dendritic spines, as well as reduced astrocytic coverage areas in the PFC of rats exposed to chronic stress (Woodburn et al., 2021). This is in line with data showing an altered microglial morphology in rats after chronic stress, with females showing a greater proportion of primed

microglia compared to males (Bollinger et al., 2016). Studies performed in aging are nevertheless lacking. Furthermore, PFC-dependent functions such as spatial working memory are affected by chronic stress in aged humans with MDD (Beats et al., 1996; Nebes et al., 2000) and in aged rats, and correlate with increase in staining intensity of ionized calcium binding adapter molecule 1 (Hinwood et al., 2012), a microglia/infiltrating macrophage marker (Imai et al., 1996). In a rat model of working memory deficiency, performance during the task was improved by administering minocycline (Hinwood et al., 2012), which normalizes microglial inflammatory and phagocytic functions (Mattei et al., 2014, 2017), potentially rescuing the homeostatic microglia-neuron crosstalk (Miao et al., 2018). As shown in the hippocampus during aging and in the PFC upon chronic stress, time is of the essence as studies show deleterious effects over time (Hanamsagar and Bilbo, 2017; Angelova and Brown, 2019), notably due to chronic inflammation acting in a negative feedback manner (Comer et al., 2020).

MICROGLIAL INVOLVEMENT IN STRESS AND COGNITIVE DECLINE ASSESSED IN HUMAN

In human, the cognitive abilities of aged or chronically stressed subjects can be assessed on multiple levels. One of them is cumulative knowledge (crystallized intelligence) including general knowledge with vocabulary and historical knowledge, the other being the cognitive processing (fluid intelligence) of information in order to complete a task (Paterniti et al., 2002; Wilson et al., 2013; Murman, 2015). Extensive work from Harada et al. (2013) highlighted deficits in processing speed when performing tasks, attention, memory and language with aging. These deficits were highly correlated with gray and white matter volume reductions revealed by MRI (Harada et al., 2013). A reduced executive control in aging can also be detected using the Wisconsin Card Sorting Task as an impairment often associated with function and volume diminution of the PFC and other frontal areas (Ballesteros et al., 2013). Patients with MDD experience deficits in attention, learning, as well as in a short-term and working memory (Lam et al., 2014). This is accompanied by evidence showing changes in white matter microstructures in adults with MDD including the cingulum and the hippocampus (van Velzen et al., 2020). There is then a clear link between cognitive deficit, stress and anatomical changes in the brain (Lam et al., 2014; van Velzen et al., 2020), but one might ask how this process occurs at the cellular level. Microglial activity can be measured with PET using the coupling of a radiotracer, most commonly [^{11}C]PK11195 (Best et al., 2019) or [^{11}C]PBR28 (Nettis et al., 2020), and its receptor Translocator Protein 18 kDa (TSPO) (**Figure 2**). TSPO has diverse cellular functions, and increases in its expression can signify impaired mitochondrial metabolism, increased oxidative stress, phagocytosis, and inflammation (Shoshan-Barmatz et al., 2019), particularly in glial cells (Pannell et al., 2020). Using this tracer, it was shown that elevated microglial PET activity parallels a decline in cognition with aging evaluated by cognitive processing (Parbo et al., 2017). Hence, the aging population is likely to suffer cognitive symptoms which can be triggered and/or escalated by chronic psychological stress and other environmental factors (Gonçalves de Andrade et al., 2021; Rizzo and Paolisso, 2021).

Measuring TSPO to infer glial activity is informative in various neurological diseases including AD, but also mild cognitive impairment (Hannestad et al., 2013; Holmes et al., 2018; Best et al., 2019; Kreisl et al., 2020; Meyer et al., 2020), and cognitive aging (Gulyás et al., 2011; Suridjan et al., 2014). Most studies have reported increased TSPO expression in patients with MDD compared to healthy controls, and higher levels were measured the longer MDD remained untreated (Meyer et al., 2020). PET studies on aging have reported mixed results (Repalli, 2014), with one using [^{18}F]-FEPPA showing no increase in TSPO levels across the lifespan in healthy individuals (Suridjan et al., 2014), while another using [^{11}C]vinpocetine found that TSPO expression increased linearly with age (Gulyás et al., 2011). A study by Ottoy et al. (2019) found that reduced ^{18}F-fluorodeoxyglucose (^{18}FDG) PET uptake, indicative of brain hypometabolism, was strongly associated with short-term cognitive decline and predicted a short-term transition to AD in mildly cognitively-impaired patients. Inconsistencies between studies may be in part due to limitations with TSPO radiotracers such as low signal-to-noise ratio or partial blood-brain barrier permeability (Kreisl et al., 2020). Moreover, limitations exist with the TSPO target itself, as it is not microglia-specific, being also expressed by astrocytes, endothelial cells and other myeloid cell types which can also be at play upon stress and in aging (Janssen et al., 2018; Carrier et al., 2020). Certain alternatives to TSPO, like the TREM and purinergic family of receptors, which are summarized in **Figure 2**, have gained interest in recent years to assess microglial function and their interactions with neurons in human (Janssen et al., 2018; Narayanaswami et al., 2018). Other than measuring mitochondrial activity, mitochondrial dysfunction can be imaged with ^{62}Cu-ATSM [Cu-diacetyl-bis(N^4-methylthiosemicarbzone)], which tags excessive electron buildup caused by leakage from malfunctioning ETC, suggesting an accumulation of reactive oxygen species (ROS) (Ikawa et al., 2020). ROS might be of particular interest as their cerebral levels are known to increase with stress and aging (Seo et al., 2012; Narayanaswami et al., 2018), limiting microglial ability to interact properly with neurons (Qin et al., 2002; Hur et al., 2010; Spencer et al., 2016; Munoz et al., 2017). Other investigations have developed and tested radiotracers targeting the myeloid cell marker CSF1R, able to track microglia in health and disease (Horti et al., 2019). However, there is more work required to expand the PET imaging radiotracer panel to understand human microglia and neuron crosstalk.

DISCUSSION

Chronic stress was proposed to accelerate the process of cellular aging and promote sensitization or *priming* of microglia toward external stressors (Franklin et al., 2018; Rentscher et al., 2019). This over-sensitization by psychological stress

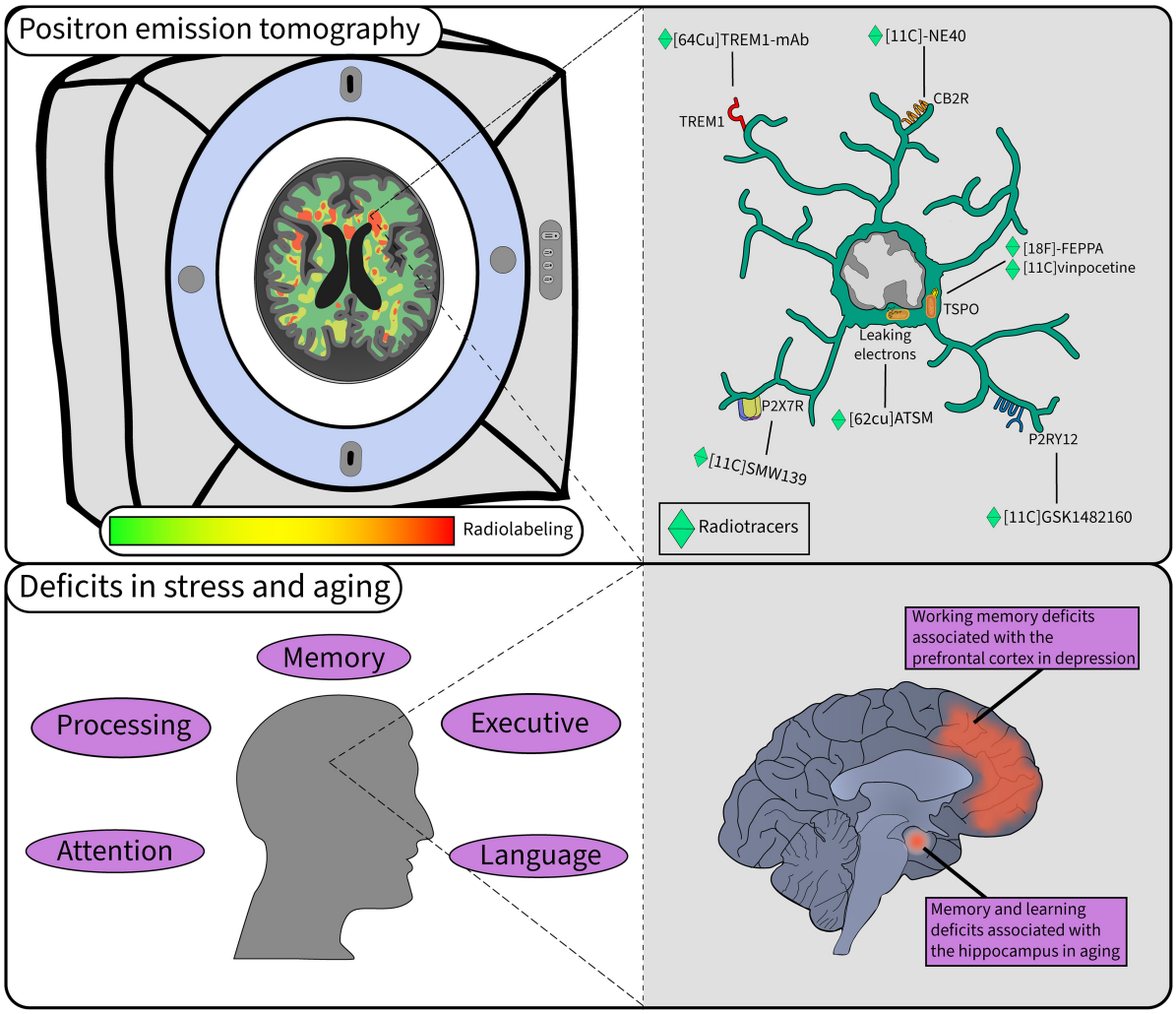

FIGURE 2 | Microglial study using PET in stress and aging. PET is a widely used technique when investigating microglial activity in pathology. In the top left, microglial labeling of radiotracers is quantified to provide insight into their function in the human brain in a non-invasive manner. In the top right a myriad of tracers provide different information on the function of microglia in the brain. The bottom left panel highlights some of the cognitive deficits observed in aging that can be accelerated with chronic stress. The bottom right panel illustrates the key regions and deficits associated with stress and aging.

is related to immunosenescence, i.e., the accumulation of oxidative stress causing deficit in responses revolving around the immune system (Gingrich, 2005; Deleidi et al., 2015) and preventing appropriate communication of microglia with the local environment, reported as hostile (Cornejo and von Bernhardi, 2016). The microglial sensome was identified as a key defective player during cognitive aging in the hippocampus and PFC (Kerr et al., 1991; Miller and O'Callaghan, 2005; Bauer, 2008; Bloss et al., 2010; Tay et al., 2018, 2019; Zannas et al., 2018). **Figure 1** shows how psychological stress can alter the communication between microglia and neurons via inflammation leading to cellular aging of both neurons and microglia through multiple processes. In particular, aged microglia show morphological signs of dystrophy preventing their homeostatic function and often associated with neurodegenerative disease (Angelova and Brown, 2019; Savage et al., 2019; Swanson et al., 2020; Shahidehpour et al., 2021). Overall, microglia in pathological aging recruit different pathways compared to younger ages, with a predominant presence of inflammatory ones including TREM2, TAM and complement signaling (Flowers et al., 2017; Tay et al., 2017; Ulland et al., 2017; Pan et al., 2020; Keane et al., 2021). Microglial TREM2 has been particularly linked to aging, with 24 month old TREM2 knockout mice showing fewer and less phagocytic microglia based on a lower expression of CD68 in brain samples (Linnartz-Gerlach et al., 2019). Future studies are needed to investigate this change in TREM2 expression in humans experiencing chronic stress along the aging trajectory by PET imaging combined with a psychological cognitive assessment. Furthermore, the dynamics of purinergic receptors also require further investigation to determine if A1R is

possibly affected in stress or aging, particularly if a disbalance in P2RY12/A1R may be at play.

There are also genetic predispositions known to accelerate aging by altering the microglial responses to stress. Genetic ablation of the protease cathepsin B (CatB) reduces oxidative stress in mice, thus improving cognitive performance with aging (Ni et al., 2019). Other types of stressors such as obesity-induced oxidative stress can accelerate the adverse effects of aging (Tarantini et al., 2018), resulting in increase of oxidative radicals in microglia which in turn contribute to the accelerating aging process (Raj et al., 2015). It is important to note that even if the role of microglia in oxidative stress is substantial, neurons in culture without glial cells can also show oxidative stress, meaning that glial cells are not solely responsible for brain cellular stress (Sompol et al., 2008; Hui et al., 2016). Indeed, neuronal inflammation is a major contributor to the Werner syndrome mouse model of premature aging, a neurological disorder associated with neuronal oxidative stress and microglial morphological changes (Hui et al., 2018). The overall outcome of impaired microglial aging may lead to impairments of synaptic plasticity, neurogenesis, accompanied by a loss of functions of other glial cells such as oligodendrocytes (Baror et al., 2019) and pathological neovascularization (Jiang et al., 2020). Oligodendrocytes are responsible for the CNS myelination and alteration of these cells contributes to memory deficits in aged mice (Wang et al., 2020). As these processes may play a role in aging-associated cognitive decline, targeting microglia presents an innovative direction of the therapeutic research. Potential therapies that could limit or rescue microglial aging, normalizing their key roles in learning and memory and the adaptation to environmental challenges, most notably include aerobic exercise and stress management in humans, also shown using wheel-running and outdoor living in mice (Tosato et al., 2007; Niraula et al., 2017; Yegorov et al., 2020). Dietary interventions like caloric restriction, in order to limit cellular cytotoxicity and promote anti-inflammatory activities, have also shown a great potential to promote healthy aging (Cheng et al., 2010). For instance, by inhibiting the mTOR pathway (López-Lluch and Navas, 2016) and thus potentially normalizing TREM2 signaling (Zhou et al., 2018), diet could lead to more efficient microglia when looking at the phagocytic ability and response ability to stress. Dietary intervention using ketogenic food made from soybean oil and cocoa butter is also beneficial in mice subjected to chronic stress (Guan et al., 2020; Morris et al., 2020). Similar dietary intervention is also demonstrating high potential to reducing mild cognitive impairment encountered in aging (Fortier et al., 2019, 2021). While we have presented preventive options, other curative therapies have shown interest. For instance, targeting survival signaling by blocking CSF1R was found to reduce Alzheimer's-like pathology in mice (Olmos-Alonso et al., 2016). The monitoring of stress and aging in patients using PET imaging and psychological testing for cognitive decline seems like an interesting strategy to promote healthy aging, even more when paired with diet and healthy living.

CONCLUSION

This work aimed to focus on potential therapeutic targets and highlight preventive therapies that may be beneficial to limit the development of MDD and accelerated aging, as they are often less covered in the literature because of their long-term reward on the patient health. Using the communication pathways altered in stress and aging summarized in **Figure 1**, we can thus orient the development of new radiotracers and investigate pre-existing ones as shown in **Figure 2**, which would allow to monitor microglia-neuron communication in MDD. Some major regions of the brain are altered in stress and aging such as the hippocampus and the PFC, although other regions are also targeted like the insular lobe. Finally, this work proposes the long-term goal of preventing and identifying novel biomarkers for MDD and accelerated cognitive aging using translational research.

AUTHOR CONTRIBUTIONS

MC was the principal manager of the review, wrote the discussion, section on human brain regions, and microglial-neuron communication, took care of the overall revision and formatting of the manuscript, and created the figures included in the manuscript. EŠ oversaw writing of the microglial metabolic changes in stress and aging section. CM oversaw redaction of the PET imaging section. M-KS-P was responsible for writing the introduction. M-ÈT oversaw the conceptual framework of the review while contributing significantly to the organization, design, and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Tight Junctions of the Neurovascular Unit

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The homeostatic balance of the brain and retina is maintained by the presence of the blood-brain and inner blood-retinal barrier (BBB/iBRB, respectively) which are highly specialized barriers. Endothelial cells forming the lining of these blood vessels are interconnected by the presence of tight junctions which form the BBB and iBRB. These tight junctions, formed of numerous interacting proteins, enable the entry of molecules into neural tissues while restricting the entry of harmful material such as anaphylatoxins, bacteria and viruses. If the tight junction complex becomes dysregulated due to changes in expression levels of one or more of the components, this can have detrimental effects leading to brain and retinal pathology.

Keywords: tight junction, neurovasculature, endothelial cells, blood brain barrier, inner blood-retinal barrier

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INTRODUCTION

Vascular heterogeneity is essential for the diverse functions and roles arising across the vascular tree; and is of particularly great importance in the brain and retina. The microvasculature of the brain and the retina differs vastly to other vascular beds due to the presence of the blood-brain (BBB) or inner blood-retinal barrier (iBRB) which are formed from endothelial cells that interconnect via highly specialized and enriched tight junctions that act as selective barriers (Abbott et al., 2006; Hudson and Campbell, 2019). The tight junctions help to regulate the entry of molecules and ions, from the blood into the tissue while restricting entry of potential harmful blood-borne components, including immune cells and pathogens (Abbott et al., 2006). In addition to the endothelial cells, the BBB and iBRB requires the presence of astrocytes, pericytes, microglia, Müller cells, and the basement membrane to help facilitate the barrier properties that are intrinsic within the brain and retina. The microenvironment needs to be stringently controlled to maintain homeostatic conditions, as dysfunction of junctional components can lead to numerous brain and retinal pathologies (as shown in **Table 1**).

COMPOSITION OF THE NEUROVASCULAR UNIT

The neurovascular unit (NVU) is comprised of numerous interacting cells that facilitate the formation, maintenance and functionality of both the BBB/iBRB. The presence of each cell type; astrocytes, pericytes, microglia and Müller cells, with innervation from neurones, are all required to maintain brain and retinal homeostasis. The endothelial cells that form the blood vessel lumen are surrounded by pericytes which are then ensheathed by astrocytic end-feet that forms a continuous layer with the basal lamina (**Figure 1**).

Astrocytes and Müller cells are the most common glial cells in the brain and retina, respectively. Both cells types have essential roles in maintaining tissue homeostasis (Abbott et al., 2006;

TABLE 1 | Contribution of tight junction components to disease pathology.

Tight junction component	Disease pathology
Claudin-5	• EAE/MS: claudin-5 loss and remodeling during leukocyte transmigration • RPE atrophy observed in response to claudin-5 downregulation (animal model of dry AMD) • Decreased claudin-5 levels detected in post-mortem brains of individuals diagnosed with Schizophrenia • Decreased claudin-5 levels found in Epilepsy • Stroke • Cold-induced model of traumatic brain injury (TBI) found decreased claudin-5 levels reduced edema and accelerated recovery • Repetitive mild TBI found decreased claudin-5 levels in association with deposition of hyperphosphorylated tau leading to BBB dysfunction • Alzheimer's disease found increased amyloid-β clearance into blood when claudin-5 and occluding down-regulated. • Claudin-5 knockdown exacerbates social defeat model of depression • Claudin-5 mislocalization and increased expression in oxygen induced retinopathy (OIR) model
Claudin-1	• Increased expression in stroke • Decreased expression in Glioblastoma Multiforme • Expression of claudin-1 reduces vascular leakage in model of EAE
Claudin-3	• Decreased expression observed in EAE and Glioblastoma Multiforme
Occludin	• Lower occludin levels observed in Multiple Sclerosis • VEGF mediated phosphorylation of occludin in Diabetic Retinopathy leads to dysfunctional iBRB • Alzheimer's disease found increased amyloid-β clearance into blood when claudin-5 and occludin down-regulated
Zonula Occludens (ZO-1)	• In Multiple Sclerosis lesions ZO-1 expression reduced leading to junctional instability
LSR	• Downregulated in EAE/middle cerebral artery occlusion leading to junctional instability
JAM-A	• Loss of JAM-A leads to increased neutrophil transmigration • Increased JAM-A expression correlates with increased monocyte migration in HIV-infected individuals
JAM-C	• Down-regulation of JAM-C inhibits wet AMD patient macrophage adhesion to endothelial cells

Reichenbach and Bringmann, 2020). They are involved in (1) regulating ion and water transport due to influencing the expression and locality of influx and efflux transporters such as aquaporin-4, (2) microvascular permeability mediated by calcium signaling to the endothelium, (3) cell-cell communication via junctional components, (4) development and maintenance of the BBB/iBRB as loss of astrocytic end-feet overage leads to an increased BBB permeability (Segarra et al., 2018), and (5) release and uptake of neurotrophic factors such as glutamate and vascular endothelial growth factor (VEGF). Dysfunction of astrocyte or Müller cell behavior can contribute to neuroinflammation due to pro-inflammatory cytokine release or tissue edema due to water retention leading to tissue swelling (Abbott et al., 2006).

The presence of pericytes in the NVU aids in microvessel stability and regulation of blood flow due to pericyte contractility and relaxation (Peppiatt et al., 2006; Hamilton et al., 2010; Lendahl et al., 2019). In contrast to other vascular tissue beds, the ratio of pericytes to endothelial cells with the CNS and retina is significantly higher, with 1 pericyte:1 endothelial cell in the retina (Frank et al., 1990). Pericyte populations vary along the vasculature- differing in their morphology and alpha smooth muscle actin expression depending on their location. Pericytes and the endothelium are usually separated physically by the basement membrane although the two cell types can directly interact at peg-socket contact sites (Lendahl et al., 2019). Platelet-derived growth factor (PDGF) signaling recruits pericytes to the BBB and iBRB and if signaling becomes dysfunctional pericyte numbers are greatly reduced leading to increased barrier permeability and dysfunction, although this may become dispensable in adult mice (Armulik et al., 2010; Daneman et al., 2010b; Park et al., 2017). Pericytes also release factors, such as angiopoietin, that influence barrier properties by inducing tight junction protein expression (Hori et al., 2004). Loss or dysfunction of pericytes has been linked to various neurodegenerative conditions including, Alzheimer's Disease, Amyotrophic lateral sclerosis (Lendahl et al., 2019) and Diabetic Retinopathy (Enge et al., 2002).

Monocyte-derived microglia are CNS-resident macrophages which become activated in response to any changes detected within their microenvironment, such as injury or inflammation. They continually undertake immune surveillance in the tissue they reside accounting for between 10 and 15% of the cell population (Perry et al., 2010; Ronaldson and Davis, 2020). Depending on the signaling pathway initiated microglia can become either pro-inflammatory (M1) or anti-inflammatory (M2) which can influence barrier properties by either upregulation or downregulation of tight junction components in both the brain and retina. M1 microglia have been implicated in BBB dysfunction due to the release and secretion of cytokines and chemokines such as interleukin (IL)- 1β, IL-12 and tumor necrosis factor (TNF)α, and CCL2 which can increase leukocyte extravasation. M2 microglia are believed to play a more protective role by controlling inflammation and resolving injury due to the release of cytokines, including IL-10 and transforming growth factor (TGF)-β (Ronaldson and Davis, 2020).

In addition to microglia, the presence of perivascular macrophages aids in maintaining tissue health. Perivascular macrophages act as antigen-presenting cells phagocytosing potential harmful material to present to leukocytes and subsequently can regulate leukocyte transmigration due to releasing anti-inflammatory cytokines. The presence of perivascular macrophages at the BBB and iBRB can enhance barrier tightness (Lapenna et al., 2018). As observed with the other cell types found within the NVU changes in perivascular macrophage behavior and number can be a causative role in neurodegenerative disease pathogenesis.

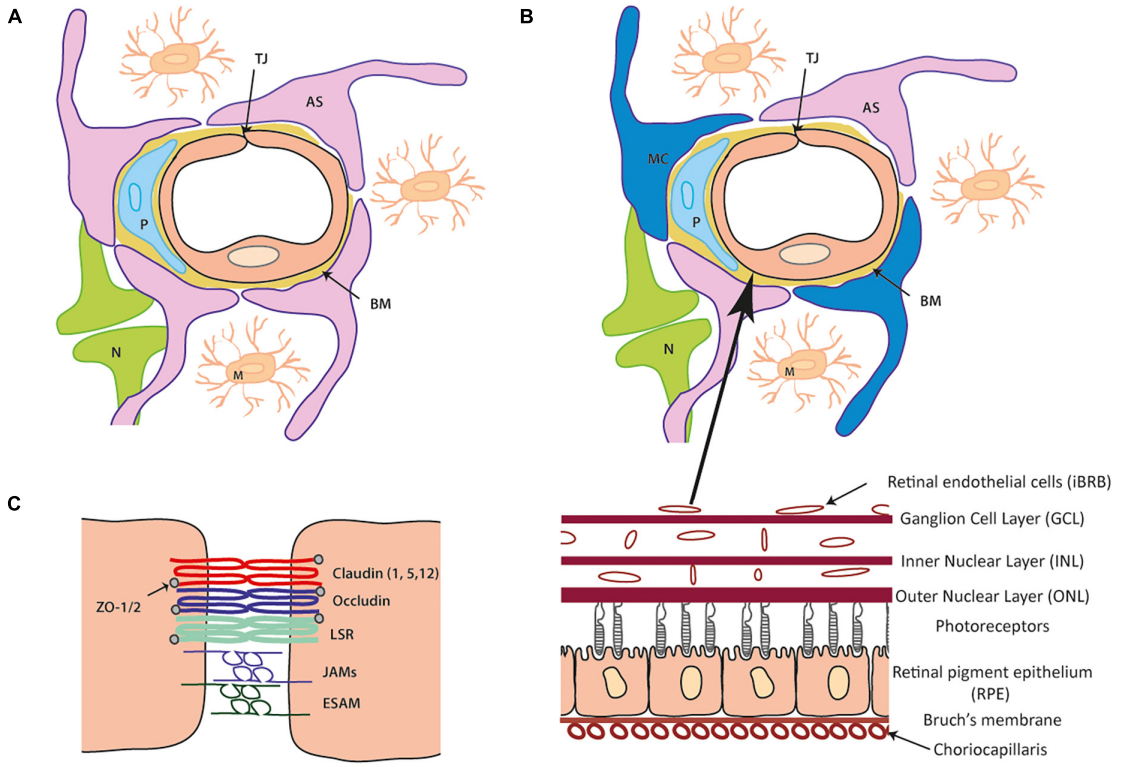

FIGURE 1 | Cellular and tight junction (TJ) protein composition of the blood- brain barrier (BBB) and inner blood-retinal barrier (iBRB). **(A)** Schematic of the blood brain barrier (BBB) neurovascular unit (NVU). A single endothelial cell (EC) forms the lumen of the blood vessels surrounded by a pericyte (P) and the basement membrane (BM) containing laminins, nidogens, collagen IV and heparin sulfate proteoglycans. Astrocytes (AS) end-feet ensheath the cell complex with neurone (N) and microglia (M) present in the microenvironment. **(B)** The iBRB is similar in composition to the BBB (as seen in **A**) although pericytes (P) are at a ratio of 1:1 with endothelial cells (EC) and Muller cell (MC) processes wrap around the blood vessels along with the astrocytes (AS). The iBRB is found in the retina from the ganglion cell layer (GCL) to the outer nuclear layer (ONL). **(C)** Schematic of tight junction proteins expressed that join the same endothelial cell or adjacent endothelial cells to one another. Claudin-5 is expressed most abundantly with contribution from claudin 1 and 12 (other family members shown to be expressed in other NVU cells). The TAMPs (occludin, tricelllin) and LSR along with JAM family members (**A–C** and ESAM) constitute the additional transmembrane proteins. Zonula occludens (ZO) 1 and 2 are expressed cytoplasmic which can form a structural link to the actin cytoskeleton and associate with actin binding proteins.

Basement membrane proteins are essential in supporting role for the cells found within the NVU which are derived from astrocytes, pericytes and the endothelium. In the brain there are two basement membranes- the endothelial and parenchymal basement membrane which under healthy conditions are indistinguishable from one another, keeping a separation between the endothelium and neurones/glial cells. Laminin, collagen IV, nidogen and heparin sulfate proteoglycans (HSPGs) are proteins that form the basement membrane and other additional proteins, such as fibronectin, are also present although their expression is dependent on the developmental or physiologically state (Thomsen et al., 2017). Agrin and perlecan are the most abundant HSPGs which integrate within the collagen IV and laminin network assisting in integrity of the basement membrane as well as having the capability to bind growth factors. The interaction of the basement membrane and the NVU cells is mediated by integrin or dystroglycan receptors that maintain the cells in their correct location. For example, collagen IV of the basement membrane interacts with endothelial β1 integrins. The expression of proteins found within the basement membrane network varies along the vascular beds. Laminin 411 and 511 are expressed in the endothelial basement membrane with low or patchy post-capillary venule expression of laminin 511 being preferential sites for leukocyte transmigration (Wu et al., 2009; Hallmann et al., 2020). Studies investigating neurodegenerative conditions, in conjunction with the use of transgenic animal models, have shown the important role that basement membrane proteins have in a functional and intact BBB (Thomsen et al., 2017). Many transgenic mice that lack the expression of a key basement membrane component are embryonic lethal, such as agrin or perlecan KO (Sarrazin et al., 2011) and collagen IV (Poschl et al., 2004), or die within a few weeks of birth, such as *Lama2*−/− mice (Miyagoe et al., 1997). Altered tight junction expression, resulting in a compromised BBB, can arise due to the loss of basement membrane components as seen in mice lacking astrocytic laminin (Yao et al., 2014).

TIGHT JUNCTIONS

Tight junctions have been described to have "gate" (paracellular permeability) and "fence" (apical/basolateral polarity barrier)

functions which are key to maintaining low endothelial permeability whilst providing a high transendothelial electrical resistance (Otani and Furuse, 2020). Individual cells can regulate the "tightness" of the junction depending on the cells physiological and pathological demands (Tsukita et al., 2001). The tight junction complex is formed from numerous interacting proteins and include the tight-junction-associated MARVEL proteins, claudin family members and junctional adhesion molecules (JAMs). These link to the actin cytoskeleton by a cytoplasmic plaque consisting of adaptor, scaffold and signaling proteins (Zihni et al., 2016). Tight junction complexes not only confer structural integrity but also play a role in numerous signaling pathways influencing their assembly, function and polarity as well as a role in gene expression (Zihni et al., 2016).

CLAUDIN PROTEIN FAMILY

The claudin protein family are integral transcellular components of tight junctions and considered to be the main structural components of intramembrane strands (Furuse et al., 1998; Tsukita et al., 2001). Claudins are a family of 27 proteins which form the primary junctional seal through homophilic or heterophilic interactions (Mineta et al., 2011). Claudins have numerous functions helping to establish barrier properties, restricting permeability to solutes and forming charge specific pores which permit ion diffusion (Zihni et al., 2016). It is believed that the functionality of claudin proteins is specified by the extracellular loop; the tightness and ion selectivity involves the first loop whilst the second loop is important for the two opposing membranes to interact and adhere (Krause et al., 2008). Ion selectivity of each molecule across the barrier is thought to be regulated by a specific claudin protein.

Claudin expression is tissue-specific, with many cells expressing more than one family member which can be altered in response to developmental stage. Junctional "tightness" and ion selectivity arises in response to the combination and ratio of claudin members (Liebner et al., 2000; Tsukita et al., 2001). Expression of Claudins-1, -3, -5, and -12 have been reported in the brain and retinal microvasculature. However, for both vascular beds claudin-5 appears to be the most highly enriched and may indeed be the only claudin expressed at high levels (Daneman et al., 2010a; Luo et al., 2011; Vanlandewijck et al., 2018).

CLAUDIN-5

Claudin-5 is expressed specifically on endothelial cells (Morita et al., 1999b), although during embryonic development it has been shown to be transiently expressed in the retinal pigment epithelium (Kojima et al., 2002). Due to its high enrichment at the BBB, the importance of claudin-5 in maintaining BBB function and integrity has been shown as claudin-5 null mice show a size-selective increase (for small molecules up to 800 Da) in BBB permeability and are embryonic lethal, dying within a few hours of birth (Nitta et al., 2003).

Alterations in claudin-5 expression have been implicated in a number of neurological conditions including schizophrenia, depression, epilepsy and traumatic brain injury (Doherty et al., 2016; Menard et al., 2017; Greene et al., 2018, 2020; Farrell et al., 2019). In addition, claudin-5 remodeling occurs at sites of leukocyte transmigration in both physiological and pathological conditions such as Multiple Sclerosis (Paul et al., 2013; Winger et al., 2014; Castro Dias et al., 2021) and mislocalization of claudin-5 occurs in a mouse model of oxygen induced retinopathy (Luo et al., 2011). Recent work has found the inner retinal blood vessels to be highly dynamic with claudin-5 expression regulated in a circadian-manner and claudin-5 changes being a key mediator in initiating dry age-related macular degeneration like pathology (Hudson et al., 2019). Transient modulation of claudin-5 expression using RNA interference has been shown to be beneficial in animal models of traumatic brain injury, Alzheimer's disease and choroidal neovascularization (Campbell et al., 2009, 2012; Keaney et al., 2015). This technique enabled either the removal of neurotoxic material from brain to blood or the enhanced penetration and efficacy of small molecule therapeutics into the brain or retina. Claudin-5 expression can be modulated by a number of factors including glucocorticoids, hypoxia, hormones and VEGF-A (Koto et al., 2007; Argaw et al., 2009; Burek et al., 2010; Hudson et al., 2014).

CLAUDIN-1

Claudin-1 is ubiquitously expressed in most tissues of the body (Furuse et al., 1998), with a key role in skin barrier formation found as claudin-1 knockout mice die of dehydration due to the loss of the junctional barrier function to water and macromolecules (Furuse et al., 2002). The requirement of claudin-1 in tight junctions of the BBB is highly debated and may vary among different species. In response to pathological conditions claudin-1 expression can be altered leading to BBB disruption. Claudin-1 upregulation has been found in conditions such as stroke (Sladojevic et al., 2019), where it appears to impair interactions with other tight junction components due to its incorporation. In human glioblastoma multiforme claudin-1 was found to be downregulated in tumor vessels (Liebner et al., 2000). In contrast, several groups have shown that claudin-1 mRNA is not detected in brain endothelial cells (Pfeiffer et al., 2011; Vanlandewijck et al., 2018). It was found that in an animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), endothelial specific inducible ectopic BBB expression of claudin-1 reduced BBB permeability and ameliorated clinical disease signs (Pfeiffer et al., 2011).

CLAUDIN-3

The role of claudin-3 in BBB integrity was first shown in studies investigating EAE and glioblastoma multiforme where loss of expression lead to a loss of BBB function (Wolburg et al., 2003). Maturation and stabilization of barrier properties

occurred in response to β-catenin induced claudin-3 expression (Liebner et al., 2008). However recent work utilizing claudin-3 deficient mice and transcriptomic analysis found claudin-3 was not expressed in the BBB endothelium (Vanlandewijck et al., 2018; Castro Dias et al., 2019b). It has been suggested that the detection of claudin-3 at the BBB may arise due to issues with antibody specificity and cross-reactivity.

CLAUDIN-12

Claudin-12 is an atypical claudin family member which is unable to interact with the cytoskeleton due to the inability to bind to accessory adaptor proteins as it lacks a PDZ binding motif. Claudin-12 is expressed in numerous organs, and has been described to be present in the BBB (Nitta et al., 2003) and in the retina (Luo et al., 2011), although its role in the BBB tight junction complex was not fully elucidated. Recent work has found that brain claudin-12 expression is predominantly found in neurons, astrocytes and smooth muscle cells rather than the endothelium (Vanlandewijck et al., 2018; Castro Dias et al., 2019a). In addition, loss of claudin-12 did not impact BBB integrity under physiological or pathological inflammatory conditions such as EAE. Mice lacking claudin-12 do show some behavioral deficits including decreased locomotion and decreased anxiety, along with minor ear and retina phenotypes such as slight changes in hearing sensitivity and a reduction in axial length in the eye (Castro Dias et al., 2019a).

OTHER CLAUDIN FAMILY MEMBERS

Additional claudin family members have been suggested to be expressed at the BBB, although their cellular expression and importance in barrier integrity has not been fully characterized. This is also true for claudin expression in the retina with some family members being expressed in a developmental manner (Luo et al., 2011). Claudin-4 is integral in maintaining astrocytic tight junctions and claudin-4 degradation influences EAE development (Horng et al., 2017). Recent studies has suggested claudin-4 to be a novel BBB tight junction component (Berndt et al., 2019), however, single cell RNA sequencing data could not detect claudin-4 expression in any brain cell types (He et al., 2018; Vanlandewijck et al., 2018). Expression of claudin-11 has been detected in a co-culture primary BBB model of endothelial cells, glial cell and pericytes (Bocsik et al., 2016) as well as in microdissected mouse and human cortical capillaries (Berndt et al., 2019). However, claudin-11 expression may appear to be more specific for oligodendrocytes localizing within the myelin rather than the tight junctions (Bronstein et al., 1996; Morita et al., 1999a; Vanlandewijck et al., 2018). Claudin-20 and -25 have also been implicated as BBB tight junction components (Berndt et al., 2019) although single cell RNA sequencing data detected claudin-20 at very low levels within capillary and arterial endothelial cells and astrocytes and claudin-25 expressed highest in oligodendrocytes (Vanlandewijck et al., 2018).

TIGHT-JUNCTION-ASSOCIATED MARVEL PROTEINS

The tight-junction-associated marvel proteins (TAMP) family of proteins include occludin, tricellulin (MARVEL D2) and MARVEL D3. Occludin was the first integral membrane protein identified to localize to tight junctions (Furuse et al., 1993) and its high expression at the BBB endothelium correlates with low endothelial permeability (Hirase et al., 1997). In contrast to claudin-5 null mice, mice lacking occludin are viable and do not have a deficient BBB due to the presence of morphologically intact tight junctions (Saitou et al., 2000; Tsukita et al., 2001). This suggests that occludin may play more of a regulatory, rather than structural, role in paracellular permeability, which can be compensated for by other tight junction proteins. The phosphorylation status of occludin is important for barriergenesis aiding in formation (Sakakibara et al., 1997), permeability (Antonetti et al., 1999; Harhaj et al., 2006) and tight junction trafficking (Murakami et al., 2009). Occludin domains exhibit distinct functions and regulatory features (Cummins, 2012). The C-terminus of occludin associates with the actin cytoskeleton via accessory proteins, such as zonula occluden (ZO)-1 (Furuse et al., 1994) and is important for paracellular permeability along with essential signaling properties. The phosphorylation status of occludin is important in disease pathology- in response to diabetes an increase in VEGF mediated phosphorylation of occludin leads to a loss of iBRB integrity and subsequent vision loss (Antonetti et al., 1998; Goncalves et al., 2021).

Tricellulin (MARVEL D2) is another transmembrane protein that is normally localized to tricellular junctions in the brain and retina (Ikenouchi et al., 2005; Iwamoto et al., 2014). However, tricellulin relocates to bicellular junctions in the absence of occludin (Ikenouchi et al., 2008). Therefore, tricellulin may have a compensatory role in the absence of occludin in the bicellular tight junction formation. Several studies have found tricellulin to be specifically enriched in brain endothelial cells (Daneman et al., 2010a; Vanlandewijck et al., 2018; Castro Dias et al., 2021). Similar to mice lacking occludin, tricellulin-deficient mice are viable although they develop hearing loss (Kitajiri et al., 2014; Kamitani et al., 2015). Under inflammatory conditions within the brain endothelium tricellulin expression is reduced leading to increased leukocyte transmigration in response to destabilization of both bi- and tricellular junctions (Castro Dias et al., 2021).

MARVEL D3 is a transmembrane protein which lacks the C-terminus found in occludin and tricellulin (Steed et al., 2009; Raleigh et al., 2010). The role of MARVEL D3 at the BBB and iBRB is still unknown although it has been found to be down-regulated in response to oxygen-glucose deprivation (Tornabene et al., 2019).

LIPOLYSIS-STIMULATED LIPOPROTEIN RECEPTOR (LSR/ANGULIN-1)

LSR recruits tricellulin to tricellular tight junctions (Masuda et al., 2011) and has been found to be specifically expressed in the BBB

and iBRB (Daneman et al., 2010a; Iwamoto et al., 2014; Sohet et al., 2015). Mice deficient for *LSR* are embryonic lethal (Mesli et al., 2004) and show impaired barriergenesis as the BBB fails to seal and is leaky to small molecules (Sohet et al., 2015). As found for tricellulin, expression of LSR was found to be down-regulated in response to inflammation, such as EAE, and middle cerebral artery occlusion which led to destabilization of the bi- and tri-cellular junctions (Sohet et al., 2015; Castro Dias et al., 2021).

ZONULA OCCLUDENS

Zonula occludens (ZO) proteins are cytoplasmic plaque proteins that form a structural link to the actin cytoskeleton and can bind to actin binding proteins including α-catenin and cortactin (Pachter et al., 2003). ZO-1 was the first tight junction protein to be discovered in both epithelial and endothelial cells, although only the ZO-1 α^- form is expressed in endothelial cells (Stevenson et al., 1986; Balda and Anderson, 1993). ZO-2 and ZO-3, have similar sequence homology to ZO-1, also localizing to tight junctions (Zihni et al., 2016), although ZO-3 is not expressed in BBB tight junctions (Inoko et al., 2003). Cells deficient for ZO-1/2 fail to form tight junctions showing the importance of ZO proteins for tight junction assembly (Umeda et al., 2006) while ZO-1 knockout mice are embryonic lethal which is believed to be due to ZO-1 importance in endothelial tissue organization (Katsuno et al., 2008). ZO proteins have specific domains that allow for various protein-protein interactions; PDZ domains enable ZO-1 to interact with ZO-2, ZO-3, and claudin family C-terminus and occludin interacts via guanylate cyclase domain (Itoh et al., 1999).

In addition to tight junction complex formation, ZO-1 and ZO-2 have a role in gene transcription regulating transcription factors as well as cell proliferation via its ability to bind ZO-1-associated nucleic acid binding (ZONAB) (Balda and Matter, 2009). Accumulation of ZONAB in the nucleus occurs when cell density is low, but if cell density is high ZONAB interacts with ZO-1 at cellular junctions (Balda and Matter, 2000; Balda et al., 2003). ZO-1 has also been found to mediate a role in endothelial cell-cell tension, cell migration and angiogenesis (Tornavaca et al., 2015). Like claudin-5 and occludin, ZO-1 expression is reduced in certain neurological diseases leading to barrier instability.

JUNCTIONAL ADHESION MOLECULES

JAMs are single span members of the immunoglobulin superfamily (Martìn-Padura et al., 1998) that are important for tight junction assembly and integrity (Ebnet, 2017). There are three family members JAM-A, -B, and -C which can all interact with PAR-3, a core component of the cellular polarity regulating machinery which localizes to tight junctions (Ebnet, 2017). All three JAMs have the capacity to interact with ZO-1, while JAM-A can also regulate the localization of ZO-1 within the junction complex. JAM-A is the predominant isoform in the brain and retinal endothelium regulating permeability changes (Aurrand-Lions et al., 2001; Tomi and Hosoya, 2004). Furthermore, JAM-A and JAM-C have been implicated in leukocyte trafficking as well as junction integrity (Woodfin et al., 2007, 2011; Williams et al., 2015; Hou et al., 2021). JAM-C has been shown to play a specific role in regulating microvascular permeability during inflammation by targeting the adherens junction protein vascular endothelial cadherin which can regulate claudin-5 expression via a β-catenin and FoxO1 dependent pathway (Taddei et al., 2008).

Endothelial selective cell adhesion molecule (ESAM) has a similar structure to JAM proteins. ESAM localization is supported by its interaction with ZO-1 in the brain capillaries (Nasdala et al., 2002) and it plays a role in endothelial cell-cell interaction during vascular development and neutrophil extravasation during early stages of inflammation (Wegmann et al., 2004).

CONCLUSION

Tight junctions found in the BBB and iBRB are complex and dynamic in nature, comprising numerous interacting proteins that aid in the gate and fence function. The contribution of other cell types found in the NVU, astrocytes, pericytes, and microglia/macrophages as well as the presence of the basement membrane are essential in ensuring the highly specialized barrier properties. All components are integral in maintaining a homeostatic balance and the integrity of the brain and retina in both healthy and disease states. Of particular importance in maintaining BBB and iBRB integrity is claudin-5, the most highly enriched tight junction component which when dysregulated has been linked to a number of neurodegenerative pathologies. In recent years the involvement of claudin–1, –3, and –12 in BBB integrity and function has come into dispute as these claudin family members are found to be expressed at extremely low levels in the brain endothelium.

AUTHOR CONTRIBUTIONS

NH and MC wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Altering Cell-Cell Interaction in Prenatal Alcohol Exposure Models: Insight on Cell-Adhesion Molecules During Brain Development

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Alcohol exposure during pregnancy disrupts the development of the brain and produces long lasting behavioral and cognitive impairments collectively known as Fetal Alcohol Spectrum Disorders (FASDs). FASDs are characterized by alterations in learning, working memory, social behavior and executive function. A large body of literature using preclinical prenatal alcohol exposure models reports alcohol-induced changes in architecture and activity in specific brain regions affecting cognition. While multiple putative mechanisms of alcohol's long-lasting effects on morphology and behavior have been investigated, an area that has received less attention is the effect of alcohol on cell adhesion molecules (CAMs). The embryo/fetal development represents a crucial period for Central Nervous System (CNS) development during which the cell-cell interaction plays an important role. CAMs play a critical role in neuronal migration and differentiation, synaptic organization and function which may be disrupted by alcohol. In this review, we summarize the physiological structure and role of CAMs involved in brain development, review the current literature on prenatal alcohol exposure effects on CAM function in different experimental models and pinpoint areas needed for future study to better understand how CAMs may mediate the morphological, sensory and behavioral outcomes in FASDs.

Keywords: prenatal alcohol exposure (PAE), fetal alcohol spectrum disorder (FASD), cell adhesion molecule (CAM), central nervous system development, cell interaction

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INTRODUCTION

Maternal alcohol consumption during pregnancy is well recognized as an important public health concern. In the United States, 1 in 9 pregnant women drink alcohol (May et al., 2009; Tan et al., 2015; Fontaine et al., 2016; Popova et al., 2017), while almost 16% of European women consume alcohol during pregnancy (Mårdby et al., 2017). Approximately 20 years ago, the term Fetal Alcohol Spectrum Disorders (FASDs) was introduced to recognize the broad range of effects induced by maternal alcohol exposure (Koren et al., 2003; Sokol et al., 2003; Chudley et al., 2005; Cook et al., 2016). FASDs are characterized by impairments in working memory, response inhibition, and behavioral flexibility (Streissguth et al., 1991; Mattson et al., 1999; Green et al., 2009; Marquardt et al., 2020).

Fetal Alcohol Spectrum Disorder Symptoms

The symptoms of FASDs are classified into three categories including craniofacial malformations, sensory and cognitive abnormalities, and brain structure anomalies. Both clinical and preclinical studies have demonstrated that prenatal alcohol exposure (PAE) can induce craniofacial anomalies including a flat nasal bridge, an upturned and short nose, thin upper lip, a smooth philtrum, and micrognathia (Moore et al., 2002, 2007; Wattendorf and Muenke, 2005). Moreover, FASD infants commonly exhibit low body weight, short height and smaller head size (Murawski et al., 2015).

Abnormalities in sensory processing including taste, smell and tactile sensitivity are reported after PAE (Franklin et al., 2008; Bower et al., 2013). In addition, vision and auditory processes can be affected, including symptoms such as microphthalmia with reduced palpebral fissure length, convergent strabismus and low visual acuity (Strömland and Pinazo-Duran, 2002; Strömland et al., 2015) and hearing loss (Tesche et al., 2014; Yoshida et al., 2018). While both changes in morphology and sensory systems are commonly seen when level of exposure is high, if has become increasingly clear that impairments in executive functioning, memory and attention can be present even without the hallmark physical changes seen in Fetal Alcohol Syndrome (FAS) (Mattson et al., 1997, 2011; Bertrand et al., 2005).

In the last decade, studies have reported fine and gross motor deficits, poorer manual coordination and balance problems in children with FASD (Doney et al., 2014; Taggart et al., 2017). Moreover, neuroimaging studies have revealed several changes in brain structure including hypoplasia of the corpus callosum and cerebellum (Chen et al., 2012; Colby et al., 2012; Yang et al., 2012; Boronat et al., 2017), and reduction in overall cortical volume in those with FASD (Rajaprakash et al., 2014).

In addition to commonly reported alterations in brain morphology in PAE, studies have also described correlations between alcohol consumption during pregnancy and anomalies in other organs including heart, kidney, liver, and endocrine system (Caputo et al., 2016). Congenital heart defects, structural anomalies of the heart and great vessels have also been observed in children with FASD (Yang et al., 2015). The correlation between PAE, kidney, and liver is still not well understood, as studies performed have generally reported non-specific anomalies with FASD such as kidney hypoplasia and hydronephrosis and liver hyperbilirubinemia (Hofer and Burd, 2009).

Alcohol Consumption During Pregnancy

The global prevalence of FASDs is ∼1% of the general population (Popova et al., 2016; Lange et al., 2017), with rates in the US of ∼2–5% (May et al., 2009, 2013, 2014, 2015), and an estimated prevalence in Europe of ∼2% (Lange et al., 2017). The highest numbers of FASD cases reflect the high alcohol consumption, in fact the 7.3% of pregnancies are alcohol-exposed (Green et al., 2016), considering that the 45% of pregnancies are unplanned and/or unrecognized during the first days (Finer and Zolna, 2016; Wozniak et al., 2019).

The amount, pattern and the timing of alcohol consumption during the pregnancy are critical factors in determining the impact of PAE on development. Data from animal models demonstrate a clear correlation between drinking pattern and the effects of PAE on brain size and volume. Bonthius and West conducted a study comparing different drinking patterns in which three groups of newborn rats were exposed to alcohol during the early postnatal period (PND 4–10). The first group was exposed to 4.5 g/kg/day for 4 h per day, while the second received the same dose, but for 8 h per day. The last group received a higher dose of alcohol (6.6 g/kg/day) for 24 h. Interestingly, the results showed that a lower daily dose was more dangerous than a higher consumed in 24 h, as brain weights from the first experimental group were significantly lower than the third group (Bonthius and West, 1990). Human studies support these conclusions as maternal binge drinking during the month prior to pregnancy recognition has been found to correlate with neurobehavioral deficits in attention, memory and in cognitive flexibility in children at age 7.5 (Streissguth et al., 1990).

Recent neuroimaging studies have also shown that children and adolescents exposed to heavy alcohol consumption *in utero* (more than seven drinks/week) have anomalies in cortical thickness and reductions in brain volume (Donald et al., 2015; Robertson et al., 2016; Hendrickson et al., 2018; Treit and Beaulieu, 2018; Zhou et al., 2018). A recent population study utilizing functional MRI data found that children (9–10 years old) exposed to heavy alcohol doses (around 90 drinks consumed during pregnancy) had lower volume and surface area in parietal and temporal lobes compared with children exposed to lighter alcohol doses (around 40 drinks consumed during pregnancy). Strikingly, even the light prenatal alcohol exposure still induced abnormalities in size of brain areas and psychological and behavioral problems (Lees et al., 2020).

Timing of consumption or exposure during pregnancy also plays a role in the symptomology seen in offspring. Alcohol exposure during the first trimester in human pregnancy (gestational day GD 1–10 in rodents) affects the gastrulation and neurulation stages and is associated with characteristic craniofacial dismorphology of FAS, specifically a wider interorbital distance, shorter midface and cranium width. In addition, alterations in cortical volume and alterations in white matter tracts have been seen (Sulik and Johnston, 1983; Lipinski et al., 2012; Parnell et al., 2013; Cao et al., 2014; Petrelli et al., 2018). PAE during the second trimester (GD 11–21 in rodents) leads to midfacial dysmorphologies, smaller skull volume and circumference, and reduction in frontal, parietal and occipital areas (Anthony et al., 2010; Coleman et al., 2012; Kajimoto et al., 2013; Shen et al., 2013). In addition, significant reduction in olfactory bulb and hippocampus volumes has been reported in rodent model (Akers et al., 2011; Petrelli et al., 2018).

The effects observed after alcohol exposure during the third trimester (postnatal day PND 1–10 in rodents) include impairments of the developing visual system, reduction in total brain volume and in total neurons and interneurons number (Dursun et al., 2011, 2013; Coleman et al., 2012). Additionally, neurodevelopmental disorders including cognitive impairments

in learning and memory have been reported following third-trimester equivalent exposure (Wilson et al., 2016).

In summary, PAE during gestation alters many crucial and important developmental processes, including neurogenesis, neuronal differentiation and migration (Miller, 1992; Goodlett and Horn, 2001; Guerri et al., 2001, 2009; Olney et al., 2002; Guizzetti et al., 2014). While there is currently a consensus that no amount of alcohol during pregnancy is "safe," the mechanisms of these alterations seen across development are still not fully understood.

Cell-Adhesion Molecules: A Possible Target of Alcohol During Development

The Central Nervous System (CNS) is a complex network of interconnected neurons whose efficient structure and functionality requires the establishment of cell-cell interactions both for efficient migration and synaptogenesis (Goodlett and Horn, 2001). These cell-cell interactions are a selective process that requires the presence and function of a class of cell adhesion molecules (CAMs). CAMs may be a critical target of alcohol during development considering the crucial role played in the brain development and in the formation of functional synaptic connections (Washbourne et al., 2004; Li et al., 2017). Astrocytes are now recognized as an essential component of synapses and their interactions with neurons have been shown to be mediated by CAMs. Evidence also suggest that alcohol exposure during development affects neuron-glia interactions (Guerri and Renau Piqueras, 1997; Ullian et al., 2001; Fields and Stevens-Graham, 2002; Yang et al., 2003; Guizzetti et al., 2008; Wilhelm and Guizzetti, 2016; Tan and Eroglu, 2021).

In this review we provide an overview of structures and roles of different types of CAMs involved in CNS development. In addition, we will discuss experimental evidence that PAE impacts CAMs and how these affects may be involved in the sensory, morphological and behavioral features of FASD previously described. The principal aim of the review is to provide a better understanding of how alcohol affects CAMs following PAE, and to identify gaps in the existing literature related to these molecular targets that will further our understanding of how alcohol alters the developing brain.

ANIMAL AND *IN VITRO* EXPERIMENTAL MODELS USED FOR FETAL ALCOHOL SPECTRUM DISORDERS

Studies in animal models of PAE have been an essential tool as the characterization of molecular mechanisms and behavioral alterations associated with FASD are difficult to carry out given the complexity in controlling and measuring variables such as maternal health and nutrition, volume and timing of alcohol exposure during the pregnancy. In order to avoid those limitations, numerous animal and *in vitro* models have been developed.

Rodents are the most widely models used in this field and have demonstrated a high utility in characterizing the PAE effects in the brain and in the complex behaviors. However, the long-lasting developmental, neurobiological and neurobehavioral changes in offspring induced by PAE vary largely depending on the specifics of the model studied (Jones and Smith, 1973; Jones, 1975; Sokol et al., 2003; Chudley et al., 2005; Bird et al., 2015; Marquardt et al., 2020; Licheri et al., 2021). Rodent models have a short gestational period and a large number of offspring (Almeida et al., 2020). In addition, they are useful for investigating the molecular mechanism altered by PAE in relation to exposure time, pattern of exposure and dosage (Almeida et al., 2020). Despite data showing the several similarities in brain architectures and functions between rodent and human brains, rodent models all have limitations. Rodent pregnancy is shorter compared to human pregnancy, and the third trimester equivalent to human gestational period occurs postnatally. Considering the overall size differences across species, in the translational studies it is critical to consider and acknowledge differences in the processes of alcohol administration, distribution, metabolism and elimination.

Several *in vitro* studies have been useful for identifying the molecular mechanisms affected by prenatal alcohol exposure (Lussier et al., 2017). Most of the studies were performed using neural progenitor cells and *ex vivo* primary cell culture (Balaraman et al., 2012; Veazey et al., 2013; Tunc-Ozcan et al., 2016). The *in vitro* models show numerous advantages including the low cost, large number of experimental groups, controlled environments. At the same time with this model is impossible to perform study on alcohol metabolism and on tissue. Recently human cerebral organoids have been developed using induced pluripotent cells grown in Matrigel, a scaffold resembling the extracellular matrix (Lancaster et al., 2013). A newly developed approach, cerebral organoids, which are similar to fetal brains in the aspects of development and structure have also been utilized to examine the role of alcohol on development. For example, recent studies were performed using this model for studying the alcohol effects, in particular the neural pathology phenotypes and signaling pathways (Arzua et al., 2020), including alterations in CAMs (Zhu et al., 2017).

A more recently adopted model of PAE on neural development is the zebrafish (Danio rerio). Although in this model embryonic development is outside of the body, and the probability of alcohol penetration can vary in relation to the concentrations administered (Ali et al., 2011; Meyers, 2018), the short development period and a large amount of offspring have made the zebrafish an important tool in the study of the effects of alcohol on development (Facciol and Gerlai, 2020). Importantly, it has shown strong utility and has yielded important results regarding the effects of PAE on CAMs.

ROLE OF CELL-ADHESION MOLECULES IN CENTRAL NERVOUS SYSTEM DEVELOPMENT

During the last 50 years various families of CAMs have been described (Rutishauser et al., 1976) and their role in development of brain structures explored (Franco and Müller, 2013;

de Agustín-Durán et al., 2021). CAMs play a pivotal role in neural migration, axon growth, and synaptogenesis (Hirano and Takeichi, 2012; Hippenmeyer, 2013; Mandai et al., 2015; Martinez-Garay, 2020). Typically, CAMs are transmembrane proteins with an extracellular domain mediating the interaction with the extracellular matrix (ECM) or other CAMs. The intracellular domain is responsible for the binding to cytoskeleton proteins and for interaction with signal transduction (Chotia and Jones, 1997; Leshchyns'ka and Sytnyk, 2016; **Figure 1**). Some CAMs are anchored to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor, called GPI-anchored CAMs. Despite GPI-CAMs do not show the typical intracellular domain, they interact with signal transducer and synapse regulators (Tan et al., 2017). Furthermore, CAMs are classified by whether they are calcium-dependent or calcium-independent; most CAMs are traditionally divided into four groups: the immunoglobulin superfamily (IgSF), the cadherins, the integrins and the selectins (Shapiro et al., 2007). We will briefly review the structure and function of three major classes of CAMs, Neural Cell adhesion Molecules, Cadherins and Integrins, and then discuss evidence that these classes of CAMs are a target of alcohol during development.

NEURAL CELL ADHESION MOLECULE: STRUCTURE AND FUNCTION

Neural cell adhesion molecule (NCAM) is a membrane-bound cell recognition molecule belonging to immunoglobulin (Ig) superfamily (Sytnyk et al., 2017). Its cytoplasmic domain is composed of 110 amino acids, while the extracellular region has five Ig-like and two fibronectin 3 (FN3) domains (**Table 1**; Grumet, 1997; Kiselyov et al., 2005; Shapiro et al., 2007; Homrich et al., 2015). The Ig-like domains are responsible for homophilic binding (Shapiro et al., 2007), while Ig1 and Ig2 only or all five domains are involved in *trans*-homophilic binding. The NCAM *cis*-homophilic binding is mediated by Ig1 and Ig2, and Ig1 and Ig3 domains (Frei et al., 1992; Ranheim et al., 1996; Atkins et al., 2004). The homophilic interaction is involved in the cell adhesion between the same molecules on membranes of adjacent cells; furthermore, NCAMs can also interact heterophilically with other cell adhesion molecules and proteins of the extracellular matrix (Leshchyns'ka and Sytnyk, 2016).

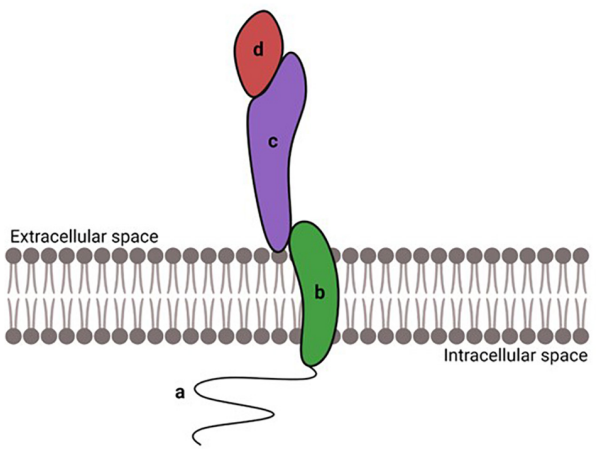


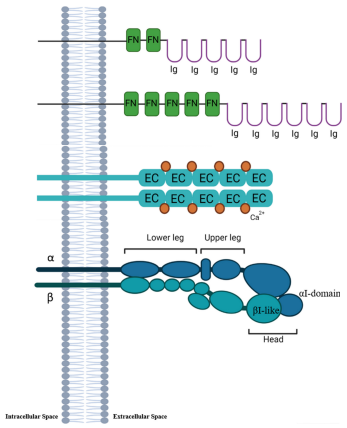
FIGURE 1 | Schematic structure of CAMs. CAMs have an intracellular domain **(a)**, a transmembrane domain **(b)**, and an extracellular domain **(c)** responsible for the binding to ligands **(d)**. Created with BioRender.com.

Three main isoforms have been identified; two are transmembrane forms NCAM-140 and NCAM-180, while the third, NCAM-120 is anchored to the cell membrane through glycosylphosphatidylinositol (GPI) linkage (Cox et al., 2009). Additionally, there is the polysialylated form of the neural cell adhesion molecule called PSA-NCAM; it is well documented that during CNS development, the PSA-NCAM is involved in precursor migration and in neuronal differentiation (Hu et al., 1996; Petridis et al., 2004; Bonfanti, 2006; Rutishauser, 2008; Schuster et al., 2020), and also in the regulation of synaptic plasticity in adult brain (Seki and Arai, 1993; Muller et al., 2000; Saini et al., 2020).

NCAM-140 and NCAM-180 are highly expressed during fetal and postnatal development, respectively (Chuong and Edelman, 1984; Gennarini et al., 1986; Oltmann-Norden et al., 2008), in fact NCAM-140 is localized to growth cones and axon of developing neurons (Sytnyk et al., 2006; Cox et al., 2009). While, NCAM-180 can be found in the postsynaptic membrane of mature neurons (Sytnyk et al., 2006). NCAM-120 is the predominant CAM expressed in glia (Noble et al., 1985), and shows high levels during adult life (Gennarini et al., 1986; Brennaman and Maness, 2008). NCAMs are involved in cell migration and neurite outgrowth through FN3 domains (Schachner, 1997; Crossin and Krushel, 2000; Rønn et al., 2000; Sytnyk et al., 2017; Huang et al., 2020). It has also been shown that NCAMs regulate the synaptic development and plasticity (Muller et al., 2000; Stoenica et al., 2006; Sytnyk et al., 2017; Cameron and McAllister, 2018; Duncan et al., 2021).

Another CAM belonging to immunoglobulin (Ig) superfamily is L1. Similar to NCAM, L1 has an extracellular region composed of six Ig ad five FN3 domains, followed by a transmembrane and an intracellular region containing a specific motif mediating the binding to cytoskeleton (**Table 1**; Bennett and Baines, 2001; Bian, 2012). The Ig-1 domain mediates the homophilic interactions (Jacob et al., 2002). L1 can interact with other Ig superfamily members through homo- or heterophilic interactions (Jacob et al., 2002; Maness and Schachner, 2007), and ECM molecules, extracellular signal-regulated kinases (Erk), cytoplasmic and traffic proteins (Maness and Schachner, 2007; Bian, 2012). L1 plays an important role during extrinsic signaling transduction regulating cell migration, differentiation, and axon growth through interaction between ECM molecules (Maness and Schachner, 2007). Moreover, the cell-adhesion interactions mediated by NCAM and L1 are calcium-independent process (Bian, 2012). Additionally, some studies have shown the involvement of NCAM and L1 in the axon-fasciculation during early postnatal period (Fischer et al., 1986;

TABLE 1 | Structure and role of CAMs during central nervous system development.

Family	Structure	Role
Ig-superfamily (NCAMs, L1)		• Cell migration • Cell differentiation • Neurite growth • Axon fasciculation • Synaptic plasticity
Cadherins (N-cadherins, E-cadherins)		• Structural integrity of neural tube and cortical structure • Cell migration and synapse formation
Integrins		• Cell adhesion • Cell migration • Synaptogenesis • Synaptic plasticity

NCAM, neural cell adhesion molecule; N-cadherin, neural cadherin; E-cadherin, epithelial cadherin. FN, fibronectin domain; Ig, immunoglobulin domain; EC, extracellular cadherin repeat. Created with BioRender.com.

Kamiguchi and Lemmon, 1998; Crossin and Krushel, 2000; Barry et al., 2010; Frei and Stoeckli, 2017).

Neural Cell Adhesion Molecule and Prenatal Alcohol Exposure

Exposure to alcohol during development has been shown to interfere with cell proliferation (Miller, 1996), migration (Miller, 1993), differentiation (Valles et al., 1996) synaptogenesis (Lancaster et al., 1989), gliogenesis (Lancaster et al., 1982), and apoptosis (Liesi, 1997). Common to most of these intrinsic processes, including gene expression and cell-cell interaction, is the involvement of CAMs. Specifically, experiments using *ex vivo* and in *in vivo* models have demonstrated that alcohol exposure during gestational periods affects NCAMs. Almost 30 years ago, it was reported that alcohol exposure (10 mg/50 microliters/day) to chick embryos at embryonic day 1–3 (E1-3) induced a significant increase of PSA-NCAM expression measured via Western blot in cerebral hemispheres between E8 and E10; while no significant change was observed in cerebellum from E10 to E20 (Kentroti and Vernadakis, 1996). Moreover, in cortical cultures NCAMs were found to have altered neuronal growth patterns after alcohol exposure. Neuroblast-enriched cultures obtained from 3-day-old whole chick embryos 3 h post-plating were treated with 50 mM ethanol from 0 to 4 DIV, and then fixed at 3, 6 or 9 DIV. To characterize the possible effects after alcohol exposure, cells were double-stained for NCAM and neurofilament, and the data collected showed changes in growth patterns of developing neurons and an intense NCAM staining. Interestingly, the altered NCAM expression in cerebral hemispheres corresponds temporally with the shift in neuronal phenotype from cholinergic to catecholaminergic and GABAergic (Kentroti et al., 1995; Kentroti and Vernadakis, 1996). Taken together, these data suggest the effects of alcohol on neuronal growth patterns and on NCAM expression might influence the establishment of neurotransmitter phenotype.

The alterations of NCAM expression have been confirmed by a study in rat offspring from dams exposed to alcohol liquid diet [5% (wt/vol) alcohol resulting in 14.3 ± 0.8 g of ethanol/kg/day] before mating, during gestation and lactation. The NCAM expression was measured at different postnatal days using Western blot and it was found that from postnatal day 5 to 7 (PND5-7) the PSA-NCAM showed higher levels in cerebral cortex in alcohol-exposed litters. It was also found that alcohol groups had a delay in the decrease of protein expression between PND7-30 while in parallel, the levels of NCAM-140 and NCAM-180 were significantly reduced (Miñana et al., 2000). In addition, alterations in NCAM isoforms have been observed in primary cultures of cortical neurons obtained from 16-day-old rat fetuses and treated with alcohol (400 mg/dl) for 48 h. This study also found that alcohol exposure significantly up-regulated the expression of NCAM-120 and 140, but not significant modulation of NCAM 180 (Miller and Luo, 2002).

These reported alterations in NCAM expression may change cell-cell interactions that affect neural migration, glial development and synaptogenesis. Even given differences in model, dose and timing of exposure, the findings have shown a considerable and consistent modulation of NCAM after PAE in the cortex. Given the role of multiple cortical subregions in mediating cognitive function, these alterations might mediate impairments in learning and behavioral flexibility induced by gestational alcohol exposure. To our knowledge, there are no studies directly examining the involvement of NCAM in the cognitive deficits induced by PAE in rodent models. However, there is evidence that negative modulation of NCAM function can induce learning and memory deficits (Doyle et al., 1992; Cremer et al., 1994; Sandi et al., 1995). In contrast to these cortical effects, PAE models using a alcohol liquid diet (5%(w/v) ethanol) between gestational days 10 and 21 was shown to alter the distributions of mossy fibers in dorsal hippocampus from rat offspring; but these effects were not mediated by NCAMs (Sakata-Haga et al., 2003).

Effects of Prenatal Alcohol Exposure on Specific Neural Cell Adhesion Molecule Subtypes

In addition to more global effects of PAE on NCAMs and brain development, several studies have examined the effects of alcohol on specific NCAMs. In order to characterize the effects of alcohol on both L1 and NCAM-140, mouse fibroblasts were transfected with human L1, and treated with 0.3–50 mM of alcohol for 30 min. The aggregation assay reported a significant inhibition of cell-cell adhesion mediated by L1 at 1 mM; in addition, the half-maximal inhibition was observed at 7 mM concentration, while the concentration of 50 mM completely blocked cell-cell adhesion (Ramanathan et al., 1996). Considering that 7 mM is the concentration reached in the blood after 1 or 2 drinks, these data underlying the harmful effects of maternal alcohol exposure induced by light drinking. In the same study, the authors investigated if the alcohol was able to affect the cell-cell adhesion processes also in cerebellar granule cells obtained from PND8 rats, and clinically concentrations of alcohol (5, 10, 25, and 50 mM) were added for 90 min. Here, as well, the authors observed that alcohol inhibited the L1 cell-adhesion, while had no effect on NCAM-140 (Ramanathan et al., 1996). Intriguingly, the same experiments performed in mouse fibroblasts transfected with human NCAM reported that the alcohol exposure did not affect the cell-cell adhesion mediated by NCAM (Ramanathan et al., 1996).

Similarly, Bearer and colleagues demonstrated the inhibitory effect of alcohol at concentration 3–5 mM on L1-mediated neurite outgrowth of cerebellar granule cells obtained from PND6 rats within 12 h (Bearer et al., 1999). PAE effects on L1 in cerebellum has also been investigated *in vivo*. In one study, rat pups on PND6 received 4.5, 5.25 and 6 g/kg of alcohol divided into 2 doses 2 h apart, then sacrificed. Analysis found that the percent of L1 in lipid rafts was significantly increase after alcohol exposure (6 g/kg/day) (Littner et al., 2013). The inhibitory effect mediated by alcohol on L1 adhesion, and the resultant disruption of L1 cell-cell adhesion could justify the accumulation of L1 in the lipid rafts (Tang et al., 2011).

The molecular mechanism behind this inhibitory effect mediated by alcohol on L1-adhesion has also been examined. Cerebellar granule neurons and dorsal root ganglion neurons were exposed to 100 mM of alcohol. Utilizing confocal microscopy the authors reported that alcohol exposure did not affect L1 distribution to the growth cone, while an immunoblot study revealed that the effect of alcohol on L1 is on its activation of $pp60^{src}$ (Yeaney et al., 2009). Interestingly, a similar study performed using cerebellar slices from PND7 rats reported that the treatment with 20 or 100 mM of alcohol for 4, 24 h and 10 days did not alter L1 expression (Fitzgerald et al., 2011).

Importantly, the alcohol-binding site in the extracellular domain of L1 has been identified utilizing photolabeling. This work found that alcohol interacts with this site localized at the interface between Ig1 and Ig4 domains (Arevalo et al., 2008; Dou et al., 2011). Furthermore, the alcohol-inhibition of L1 adhesion can be abolished by decreasing the phosphorylation of serine 1248 (S1248), an Erk2 substrate located to the L1 cytoplasmic domain (Dou et al., 2013). Recently, a study identified three highly conserved sites on L1 cytoplasmic domain involved in L1 sensitivity to alcohol; in fact, Dou and colleagues found that the phosphorylation of L1 cytoplasmic domain at S1152, S1176, S1181, and S1248 promotes L1 coupling with ankyrin-G and spectrin-actin cytoskeleton facilitating L1 sensitivity to alcohol (Dou et al., 2018). In order to determine if there is a correlation between this molecular pathway and susceptibility to FASD, the authors studied the genes involved in phosphorylation of L1 cytoplasmic domain. They found that polymorphisms in genes encoding ankyrin-G and p90rsk, a kinase that phosphorylates S1152 are associated with facial anomalies observed in children exposed to heavy maternal alcohol consumption (Dou et al., 2018).

Taken together, these studies clearly demonstrate that alcohol exposure in particular during the third trimester of gestation inhibits the cell adhesion process mediated by L1 without changes in the protein expression, but through L1-ankyrin G association. Intriguingly, the inhibitory effect is brain region-specific given that the L1 association with lipid rafts is only observable in cerebellum between PND 8–28 (Nakai and Kamiguchi, 2002).

The relation between L1 and FASD is further supported by studies reporting that neuroprotective peptides are able to block alcohol-inhibition of L1 adhesion in C57BL6J mouse embryos. Embryos at gestational day 8 were exposed to alcohol (100 mM), or in combination with the peptides NAPVSIPQ (NAP) and SALLRSIPA (SAL). The incubation with alcohol for 20 h induced neural tube defects, while co-incubation with neuropeptides rescued these alterations (Chen et al., 2005). A more recent study performed combining immunoprecipitation, Western blotting and immunofluorescence in fibroblasts transfected with human L1 demonstrated the protective effect of NAP. In fact, NAP is able to stimulate the phosphorylation of the tyrosine-1229 at the ankyrin binding motif of the L1 cytoplasmic domain, blocking L1-ankyrin-G and spectrin-actin cytoskeleton association through the activation of EphB2, a kinase that phosphorylates L1-Y1229 (Dou et al., 2020). It is already established that the interaction of EphB2 and L1 plays an important role during brain development contributing to the signaling during hippocampal development (Robichaux et al., 2014).

Overall, studies performed in the last 20 years have characterized not only the inhibitory effect of alcohol on L1 adhesion process, but also the molecular mechanism involved and the possible rescue processes. These findings might be useful for future pharmacological approaches. Furthermore, the study performed by Dou and colleagues, where they identified the association between polymorphisms in genes encoding ankyrin-G and FASD facial anomalies, suggests new studies in order to understand if genetic regulation can alter FASD susceptibility (Dou et al., 2018).

As previously mentioned, zebrafish have more recently become utilized as a model of PAE. A study examining the immersion of zebrafish embryos into 1% alcohol solution (vol/vol%) at 24 h post-fertilization for 2 h found that this approach reduced the NCAM expression in different brain regions (Mahabir et al., 2018; **Table 2**). Interestingly, these data could explain the lower serotonin and dopamine levels observed

in zebrafish embryos after alcohol exposure (Buskea and Gerlaia, 2011), considering previous findings reporting the reduction of serotonin transporter protein levels in different brain regions of adult NCAM(-) (/) mice (Aonurm-Helm et al., 2015). Moreover, it has been shown the involvement of NCAM in the trafficking of the neurotransmitter receptor dopamine 2 (Xiao et al., 2009). Taken together these findings demonstrated that different prenatal alcohol exposure models affect significantly the NCAMs in brain region-dependent manner, confirming the role of this class of CAM as like alcohol target during CNS development.

CADHERINS

The cadherins represent a large family of proteins expressed in simple and complex organisms, many of which participate in Ca^{2+}-dependent cell-cell adhesion process. There are more than 100 family members divided in four subgroups including classical cadherins, protocadherins, desmosomal and unconventional cadherins, which have a similar extracellular Ca^{2+}-binding region known as extracellular cadherin repeats (ECs) (**Table 1**; Nollet et al., 2000; Angst et al., 2001; Shapiro et al., 2007; Bian, 2012). Structurally, the classical cadherins are single-pass transmembrane proteins, with a cytoplasmic actin-binding site, while the extracellular site is composed of five EC domains (EC1-5) (Shibata-Seki et al., 2020; **Table 1**). Cadherins mediate homophilic or heterophilic interaction through a dimer of EC1-5 (Patel et al., 2006; Brasch et al., 2018). Classical cadherins are further divided into type I and II based on sequence comparison. The first group includes neural cadherins (N-cadherins) and epithelial cadherins (E-cadherins), which have a conserved histidine-alanine-valine (HAV) amino acid sequence in the distal EC (EC1), which is important for homophilic adhesion (Takeichi, 1995; Halbleib and Nelson, 2006). Moreover, type I class mediates a strong cell adhesion (Paulson et al., 2014). In contrast, type II classical cadherins do not have a HAV motif, and consequently are associated with less strong cell-adhesion (Thiery et al., 2012). Furthermore, the catenins connect N-cadherin to the actin cytoskeleton mediating the cadherin-mediated cell-adhesion (Meng and Takeichi, 2009; Takeichi, 2014).

N-cadherin plays an important role in maintaining the structural integrity of the neural tube and cortical structure during development (Radice et al., 1997; Kadowaki et al., 2007; Punovuori et al., 2021). The complexity of the CNS depends on key role played by N-cadherin, which controls cell migration, synapse formation and maintenance of progenitor pool (Togashi et al., 2002; Bekirov et al., 2008; Rieger et al., 2009; Camand et al., 2012; de Agustín-Durán et al., 2021). For this reason N-cadherin levels are tightly regulated, and upregulation and downregulation can lead to significant alterations during CNS development. The overexpression of N-cadherin inhibits the differentiation of neural progenitors, while downregulation induces a premature differentiation (Barami et al., 1994: Rousso et al., 2012).

Additionally, E-cadherins are required for cell movement during gastrulation (Babb and Marrs, 2004; Kane et al., 2005; Solnica-Krezel, 2006; Morita and Heisenberg, 2013; Song et al., 2013), and for developmental signaling pathways, including including non-canonical Wnt (Ulrich et al., 2005), heterotrimeric G-protein (Lin et al., 2009) and Pou5f1/Oct4 signaling pathways (Song et al., 2013).

Numerous studies have demonstrated the pivotal role played by Type I and Type II cadherins in the formation of specific synaptic connections, in fact they are localized in both pre- and post-synaptic terminals (Bekirov et al., 2002; Arikkath and Reichardt, 2008; Williams et al., 2011; Basu et al., 2015). A recent study described the important role of cadherins in developing CNS including the organization into layers and the formation of neuronal circuits (Polanco et al., 2021).

Prenatal Alcohol Exposure Affects Cadherin Expression and Interactions

During CNS development, cadherin 8 is expressed in frontal and motor cortices (Dye et al., 2011), and prenatal alcohol exposure is able to alter the protein level of this CAM. Cadherin 8 is a classical type II cadherin (Kido et al., 1998), and during the perinatal and postnatal period it shows a restricted expression patterns in specific brain regions including cortex, hippocampus and striatum (Medina et al., 2004; Lefkovics et al., 2012). To evaluate the effect of prenatal alcohol exposure on cadherin 8, El Shawa and colleague exposed mouse dams to alcohol (25% v/v) for the entire gestational period (GD 0.5-GD 19.5). Pups were sacrificed at postnatal day 0, and *in situ* RNA hybridization showed a significant increase of cadherin 8 expression in cortex (El Shawa et al., 2013). The upregulation of N-cadherin induced by alcohol exposure was further supported in a study where dams mice were exposed to alcohol 10% solution for 17 days before and up to day 10 of gestation (Coll et al., 2011). Western blotting data showed a significant increase of E-and N-cadherins levels in E10 embryos coming from dams exposed to alcohol (Coll et al., 2011).

Disruptions in cadherins have also been shown in zebrafish models. Zebrafish embryos exposed to 100 mM (0.6% vol/vol) alcohol from to 2 to 8 h post-fertilization (hpf), and it was shown that following 4.5 of exposure alcohol affected yolk cell microtubule and E-cadherin distribution (Sarmah et al., 2013). Alcohol effects on cell adhesion process were still observed at 8 hpf (mid-gastrulation) and microarray analysis showed a reduction in gene expression of protocadherin-18a (Pcdh-18a), suggesting that alcohol exposure continued to affect cell-cell communication in treated embryos (Sarmah et al., 2013).

Approximately 30 years ago, the class of protocadherin was identified and described similar to cadherins, but with the difference that they show six or seven ECs (Sano et al., 1993). Despite this structural difference, similar to cadherins, the protocadherin expression is regulated during CNS development, and they play an important role during the specification of neuronal identity (Pancho et al., 2020). Recently, a study performed using chick embryos as experimental model reported that alcohol exposure (2% alcohol) once every 1.5 days for 11 days upregulated the N-cadherin and cadherin 6B expression, and downregulated cadherin 7 in dorsal neural tube (Zhang et al., 2017). These alterations in the protein expression may inhibit neural crest cells migration leading to the craniofacial defects (Zhang et al., 2017; **Table 2**).

Despite the different experimental models used, the data collected clearly demonstrated that alcohol exposure modulates cadherin expression during early development. Overall, the data suggest that abnormal regulation of cadherin expression could explain the abnormalities observed in the neural tube following developmental alcohol exposure.

INTEGRINS

Integrins are a class of CAMs that are major mediators of cell-cell adhesion and cell-ECM interactions (Barczyk et al., 2010; Ringer et al., 2017). Integrins are heterodimeric transmembrane proteins consisting of α and β subunits, and electron microscopy studies show that this class of CAMs has a globular head and two leg regions (one formed by α subunits and the other by β subunits; each region is subdivided in lower and upper leg) into the plasma membrane (**Table 1**; Srichai and Zent, 2010). Each subunit has an extracellular domain, a transmembrane domain, and a cytoplasmic tail (Srichai and Zent, 2010). The α subunits are responsible for ligand binding, while both α and β subunits mediate the cell signal transduction (Srichai and Zent, 2010; Pan et al., 2016). The integrin α subunits can be further divided according to the presence of an I-domain, a crucial motif for ligand binding. The extracellular domains of integrin α subunits have a "metal-ion-dependent adhesive site" (MIDAS) that is able to bind divalent metal cations, while the transmembrane domains through 5 common amino acid sequences "GFFKR" regulate integrin affinity by mediating an alpha-beta subunit cytoplasmic tail interaction (Pan et al., 2016).

The structure of integrin β subunits is well described as an I-like domain similar to I-domain characterized in α subunits. This I-domain is a highly conserved region composed of 240 residues, and it contains two additional sections that either are responsible for ligand binding (Huang et al., 2000; Xiong et al., 2002). Moreover, they show a large extracellular domain, a single-spanning transmembrane domain and a cytoplasmic tail (Pan et al., 2016).

Importantly, integrins are expressed in brain areas heavily involved in learning, memory and cognition including the hippocampus, cerebellum, thalamus and cortex (Pinkstaff et al., 1999; Clegg et al., 2003). There is strong evidence that these proteins mediate the adhesion and migration of neuronal cells during the developing of CNS (Galileo et al., 1992; Zhang and Galileo, 1998; Anton et al., 1999; Clegg et al., 2003). Interestingly, it has been reported that the neuronal migration during CNS development is modulated also by the interaction with integrins and the L1 (Silletti et al., 2000; Thelen et al., 2002).

Several integrin α and β subunits are highly expressed in growth cones and synapses (Wu and Reddy, 2012; Park and Goda, 2016), and it is well established their role in building and maintain synaptic structure during the phases of the development (Benson et al., 2000). *In vitro* studies have also shown that the integrins are able to stabilize long term potentiation (LTP) after induction (Stäubli et al., 1998; Babayan et al., 2012; Kerrisk et al., 2014), therefore, integrins may be another CAM target of alcohol considering their role during brain development.

Integrins and Prenatal Alcohol Exposure Effects

Similarly to its CAM family members, there is some evidence that integrins are affected by alcohol exposure during development. Pharmacological concentrations of alcohol (1, 10, and 100 mM) added to cultures of neural progenitor cells for 3 days modulate the mRNA levels of genes involved in the cell adhesion pathways. In particular, the expression of α integrin 5 and β integrin 3 was significantly increased, while the β integrin 2 was downregulated

TABLE 2 | Prenatal alcohol exposure modulates CAMs expression in *ex vivo* and *in vivo* experimental models.

CAM	Experimental model	Doses/treatment	Time of exposure	Effect
PSA-NCAM	Chick embryos	10 mg/50μl/day	E1-5	Increase of protein expression
PSA-NCAM	Rats	5% wt/vol	Before mating, during gestation and lactation	Increase of protein expression
NCAM-140, NCAM-180	Rats	5% wt/vol	Before mating, during gestation and lactation	Reduction of protein expression
NCAM-140, NCAM-180	Cultures of cortical neurons (16 day old fetuses)	400 mg/dl	48 h	Reduction of protein expression
L1	Cultures of cerebellar granule cells (PND8 rats)	5, 10, 25, 50 mM	90 min	Inhibition L1 cell-adhesion
L1	Cultures of cerebellar granule cells (PND6 rats)	3–5 mM	12 h	Inhibition L1- mediated neurite outgrowth
L1	Rats	6 g/kg/day	PND6	Increase in lipid rafts
NCAMs	Zebrafish	1% (vol/vol)	1 day after post-fertilization for 2 h	Reduction of protein expression
Cadherin-8	Mice	25% vol/vol	Gestation	Increase of protein expression
N-cadherin	Chick embryos	2% vol/vol	3 days	Increase of protein expression
N-cadherin	Mice	10% (w/vol)	17 days previous and up to day 10 of gestation	Increase of protein expression
α-integrins and β-integrin 3	Neural progenitor cells	1, 10, 100 mM	3 days	Increase of protein expression
β-integrin 2	Neural progenitor cells	1, 10, 100 mM	3 days	Decrease of protein expression

(Vangipuram et al., 2008). Considering that integrins play a pivotal role in cell proliferation and migration during brain development (Schmid and Anton, 2003), another experimental investigation focused its attention on the integrin protein levels in fetal cortices. The dams were exposed to alcohol (from 6 to 17 gestational days) using a liquid diet with increasing concentrations of alcohol (v/v), precisely during GD 6 and 7 the concentration was 2.2%, then increased to 4.5% during GD 8, 9 and 10, and 6.7% during GD 11 to 19. The offspring cortices analyzed at gestational day 18 showed a significant increase of β integrin 1, while a reduction for α integrin 3 (Rout and Dhossche, 2010; **Table 2**). These results could explain PAE's effects on thickness of cortical areas measured in mouse offspring (Abbott et al., 2016), confirming the involvement of integrins in the formation of cell layers in cortex.

While there is some evidence that integrins may be altered by PAE, less work has focused on this class of CAMs than either the NCAMs or cadherins. Considering the important role by this class of cell adhesion molecules plays not only during CNS development but also in learning and memory processes, further investigation is needed in order to evaluate the possible involvement of integrins in the cognitive deficits observed in FASDs.

AREAS OF FUTURE FOCUS

In the last 50 years, the impact of alcohol consumption during pregnancy has been extensively investigated, but the molecular mechanisms underlining abnormalities observed in PAE offspring are still not understood. Given the evidence reviewed, it can be well established that alcohol also effects on cell-cell interaction, in particular on cell adhesion molecules. However, several areas are identified that need more focus to understand how PAE affects these molecules.

Effects of Prenatal Alcohol Exposure Variables

Despite CAMs being expressed throughout the brain, the literature to date suggests that PAE modulates the protein level expression in a region-specific manner. NCAMs expressed in cortex are most consistently affected by alcohol exposure, although the dosage, duration and gestational timing of PAE models used all impact these effects. Since few studies have been performed so far, future investigation will be needed to evaluate the PAE's effects in rodent model using different doses, time of exposure and routes of administration. To date, the literature specific to dose and model suggests that both low, moderate and high alcohol exposure paradigms in rodent models lead to significant alterations in the protein expression of different CAMs, in particular NCAMs and N-cadherins. Given that recent studies have identified gestational day (G12) as a vulnerable stage during fetal development, especially for anxiety-like behavior in offspring (Rouzer et al., 2017), it would be interesting and useful to evaluate the impact on CAMs after a single day of alcohol exposure in rodent models, considering the limits described in the section about *in vitro* studies. Moreover, it is well established that single P7 alcohol exposure reduces total brain volume in adult animals P80 (Coleman et al., 2012). Considering these interesting experimental findings and the role played by the CAMs during the development, it would be appropriate to evaluate the possible PAE effects in relation to the time exposure.

Interactions of Sex and Prenatal Alcohol Exposure on Cell Adhesion Molecules

The collected data to date reveal an important gap present in literature concerning the role of sex on PAE effects on NCAMs, cadherins and integrins. At the time of this review none of the studies performed in rodent models discussed here have investigated the possible sex differences in expression of CAMs following PAE. Recent focus on sex specific effects in PAE models have revealed it to be a critical biological variable in several widely utilized exposure models. For example, in a model of third-trimester exposure (two injections of alcohol (20% w/v) 2 h apart on P7) adult hippocampal neurogenesis was shown to be altered in a sex-specific manner (Coleman et al., 2012). Similarly, "drinking in the dark exposure" during first and second trimester equivalent was found to impair visuospatial discrimination robustly in females, but not in males (Kenton et al., 2020). That same exposure model was recently shown to alter evoked *N*-methyl-D-aspartate (NMDA) currents in orbital frontal cortex pyramidal neurons in a sex specific manner (Licheri et al., 2021). Considering the important role played by CAMs in synaptogenesis, it might be possible that this sex-specific effect could be mediated by molecular mechanisms involving this class of molecules. Interestingly, recent studies describe sex-related changes in dendritic and synaptic architecture during human brain development (Duerden et al., 2020). In addition, sex differences were seen in the gene expression levels of postsynaptic cell-adhesion in rats between P5 and P7 days (Srancikova et al., 2021). Together, these studies underline the importance of investigating the role of sex in the effects of alcohol on cell-adhesion molecules in morphological, sensory and cognitive effects in FASD.

CONCLUSION

There is strong evidence across several preclinical models, and from limited clinical studies, for the involvement of CAMs in the development of neurobiological abnormalities and behavioral effects following PAE. While more work needs to be done to disentangle the role of specific CAMs in these processes, the potential for this class of proteins for developing pharmacological therapies makes this an important area of research focus going forward.

AUTHOR CONTRIBUTIONS

VL performed literature search and outline, and wrote the review. JLB contributed to the final draft. Both authors revised the final draft for important intellectual content.

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