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Fingolimod: Direct CNS effects of sphingosine 1-phosphate (S1P) receptor modulation and implications in multiple sclerosis therapy

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ABSTRACT

Fingolimod is the first oral disease-modifying therapy approved for relapsing forms of multiple sclerosis (MS). Following phosphorylation *in vivo*, the active agent, fingolimod phosphate (fingolimod-P), acts as a sphingosine 1-phosphate (S1P) receptor modulator, binding with high affinity to four of the five known S1P receptors ($S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$). The mechanism of action of fingolimod in MS has primarily been considered as immunomodulatory, whereby fingolimod-P modulates $S1P_1$ on lymphocytes, selectively retaining autoreactive lymphocytes in lymph nodes to reduce damaging infiltration into the central nervous system (CNS). However, emerging evidence indicates that fingolimod has direct effects in the CNS in MS. For example, in the MS animal model of experimental autoimmune encephalomyelitis (EAE), fingolimod is highly efficacious in both a prophylactic and therapeutic setting, yet becomes ineffective in animals selectively deficient for $S1P_1$ on astrocytes, despite maintained normal immunologic receptor expression and functions, and S1P-mediated immune activities. Here we review S1P signaling effects relevant to MS in neural cell types expressing S1P receptors, including astrocytes, oligodendrocytes, neurons, microglia and dendritic cells. The direct effects of fingolimod on these CNS cells observed in preclinical studies are discussed in view of the functional consequences of reducing neurodegenerative processes and promoting myelin preservation and repair. The therapeutic implications of S1P modulation in the CNS are considered in terms of the clinical outcomes of MS, such as reducing MS-related brain atrophy, and other CNS disorders. Additionally, we briefly outline other existing and investigational MS therapies that may also have effects in the CNS.

Contents

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1. Introduction

Sphingosine 1-phosphate (S1P), a naturally occurring lipid mediator and part of the larger family of lysophospholipids, can act as a regulator of diverse physiological and pathophysiological processes, including those involved in the pathogenesis of multiple sclerosis (MS) [1–3]. S1P is produced from sphingolipids present in the cell membrane, which are in part defined by the constituent presence of the amino alcohol sphingosine. A prominent sphingolipid is sphingomyelin, from which sphingosine is liberated through a series of reactions catalyzed by metabolic enzymes, including sphingomyelinase and ceramidase [4,5]. Sphingosine can then be phosphorylated to produce S1P by sphingosine kinase 1 (SphK1) or 2 (SphK2). Both of these enzymes have fairly broad tissue distribution, with SphK1 predominating in the lungs and spleen, and SphK2 predominating in the heart, brain and liver [6,7]. Extracellular S1P acts in both autocrine and paracrine fashions by binding to five cell-surface S1P receptor subtypes named $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ [8], which belong to the G protein-coupled receptor (GPCR) super family [3,9]. $S1P_1$, $S1P_2$ and $S1P_3$ show broad tissue gene expression, while $S1P_4$ shows gene expression primarily in immune system cells, and $S1P_5$ is primarily expressed in the spleen (on natural killer cells and other lymphocytes) and central nervous system (CNS; mainly on oligodendrocytes) [3]. These receptors can, therefore, function in multiple organ systems, such as the immune, cardiovascular, and respiratory systems, as well as in the CNS. Precedence for CNS functions of S1P receptors can be seen through their relationships to known activities of the closely related lysophospholipid receptors for lysophosphatidic acid (LPA). The first lysophospholipid receptor, now known as LPA_1, was identified through studies of the CNS [10]. This led to the deorphanization of homologous putative receptors in genomic databases resulting in the discovery of new receptors for LPA and S1P that shared homology despite recognizing distinct ligands [11–13]. Indeed, early studies identified the S1P receptor known as $S1P_1$, which plays a key role in the actions of fingolimod, as a receptor for LPA [14,15]. The neurobiological effects of LPA receptors have a remarkable range of activities that affect most CNS cell types at some time during their developmental history, covering the gamut of processes from neurogenesis and differentiation to survival and cell death [10,16–36]. Neurological disorders may also be impacted by LPA receptor signaling, as reported for neuropathic pain [30,37], hypoxic insults [38] and hydrocephalus [39]. Evidence has further highlighted S1P receptors as a potential target for the treatment of pain [40] and stroke *via* neuroprotection [41]. Furthermore, S1P receptor modulation has been shown to decrease vascular permeability and astrocyte accumulation in spinal cord injury [42].

These examples underscore the likelihood that S1P signaling, as part of the larger field of lysophospholipid signaling, will have functions through direct CNS activities as is known to occur for the signaling activities of LPA.

Fig. 1 provides a composite picture of S1P receptor gene expression reported in the literature for neurons and glia [8,43,44]. Binding of S1P to each of the S1P receptor subtypes activates a range of different intracellular signaling pathways mediated by distinct heterotrimeric G proteins [3,45–49]. Fingolimod (FTY720; GILENYA™, Novartis Pharma AG, Basel, Switzerland) is a modulator of S1P receptors and is the first oral disease-modifying therapy to be approved for relapsing forms of MS. Fingolimod is phosphorylated *in vivo* by sphingosine kinase, particularly SphK2, to produce the active metabolite fingolimod phosphate (fingolimod-P). Fingolimod and fingolimod-P are structural analogs of sphingosine and S1P, respectively. Being a structural analog of S1P enables fingolimod-P to bind to and activate four of the five S1P receptor subtypes. Receptor studies have shown that fingolimod-P activates $S1P_1$, $S1P_4$, $S1P_5$ (half maximal effective concentration [EC_{50}] values of ~0.3–0.6 nM) and $S1P_3$ (EC_{50} values of ~3 nM), but has shown essentially no activity at $S1P_2$ (EC_{50} values of >10 μM) [50,51].

Modulation of $S1P_1$ on lymphocytes by fingolimod is thought to retain circulating pathogenic lymphocytes in the lymph nodes, thereby preventing their infiltration into the CNS where they would promote pathological damage [52–54]. Fingolimod-P initially acts as an $S1P_1$ agonist [50,51]; however, chronic exposure to fingolimod-P leads to irreversible receptor internalization resulting in 'functional antagonism' of $S1P_1$-mediated S1P signaling [55–57]. Circulating T cells express $S1P_1$ and lower levels of $S1P_4$ and $S1P_3$ [56,58], and the interaction of extracellular S1P with $S1P_1$ is thought to initiate lymphocyte egress from lymph nodes by overcoming retention signals

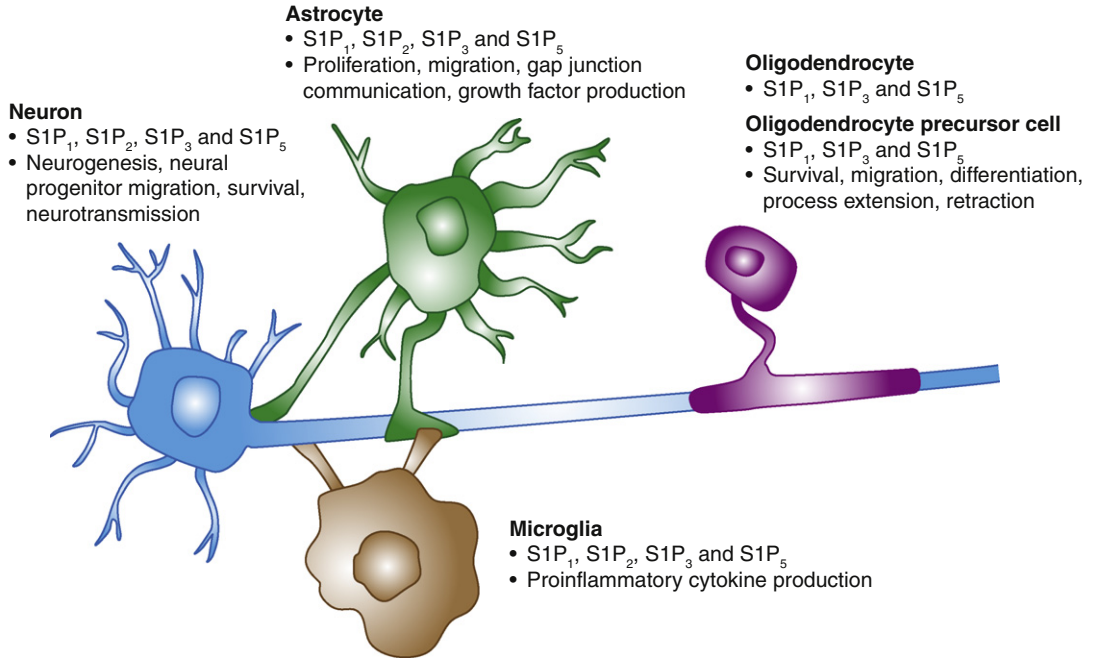

Fig. 1. Distribution and functions of sphingosine 1-phosphate (S1P) receptor subtypes in cells resident in the central nervous system from a composite review of the literature covering many different growth conditions in culture, developmental stages, disease states or models and species. For example, S1P receptor expression on microglia varies according to the activation state of these cells and in the figure is shown for microglia in an inactive state isolated acutely from rat brain [43].

mediated by chemokine (C – C motif) receptor 7 (CCR7) expressed on B cells and naïve and central memory T cells. In the presence of fingolimod-P, functional antagonism of $S1P_1$ prevents the egress of CCR7-positive naïve and central memory T cells from lymph nodes [52,59], consistent with experimental data produced using S1P receptor knockout mice to study lymphocyte circulation [55,60]. Importantly, fingolimod does not significantly affect activation and proliferation of redistributed naïve and central memory T cells, and does not block the egress from lymph nodes of effector memory T cells that are CCR7-negative, a distinct subpopulation of T cells that are important for immunosurveillance [59]. Thus, fingolimod has a targeted mechanism of action, selectively affecting lymphocyte subsets.

In addition to these immunologic actions, and in view of the general actions of lysophospholipid receptors in the CNS and a growing literature that has identified S1P signaling effects on neural cells, fingolimod would be expected to have direct effects on CNS cells that express S1P receptors. Indeed, fingolimod, which is lipophilic, is able to cross the blood–brain barrier into the CNS and, following oral administration of fingolimod, fingolimod-P has been detected in the cerebrospinal fluid at subnanomolar levels [61], which are sufficient for modulating human CNS cell properties *in vitro* [62,63]. In addition, recent data utilizing conditional knockout of $S1P_1$ from neural lineages have identified key roles for astrocytes in reducing the severity of pathological changes in an animal model of MS, experimental autoimmune encephalomyelitis (EAE). Moreover, the astrocytic loss of $S1P_1$ also prevents the efficacy of fingolimod in this model [64]. Here, we discuss the emerging evidence for direct CNS effects of fingolimod through alteration of S1P signaling and the implications for MS therapies.

2. S1P signaling in MS

S1P receptors have been reported to be expressed on oligodendrocytes, astrocytes, neurons, and microglia in a range of experimental and growth conditions that encompass cellular expression rather than actual *in vivo* CNS expression. This issue is particularly important in determining S1P signaling alterations that may exist at different stages of MS. Findings from some studies suggest that S1P signaling is disrupted in MS. Compared with control individuals, patients with MS have been reported to have a lower content of sphingomyelin (from which endogenous sphingosine and S1P are derived) in their white matter [65] but an increased level of S1P in their cerebrospinal fluid [66]. S1P levels have been found to be lower and sphingosine levels higher in the white matter and lesions of patients with MS compared with white matter from control individuals [67]. In active and chronic inactive MS lesions, reactive astrocytes have been reported to show high expression of $S1P_1$ and $S1P_3$ [68]. In addition, under proinflammatory conditions, $S1P_3$ and SphK1 have been shown to be upregulated on astrocytes [69]. These combined reports suggest that S1P signaling in the CNS is altered in patients with MS. This conclusion has received recent support in experimental models of MS, whereby removal of $S1P_1$ from astrocytes produced a reduction of the elevated S1P levels occurring in animals challenged by EAE [64].

3. S1P signaling in CNS cells and effects of fingolimod

3.1. Astrocytes

Astrocytes are the most abundant cells in the human CNS and have an extremely diverse and important range of roles that are relevant to normal brain activity and its alteration in disease states [70–78]. In MS, evidence suggests that astrocytes have a dual, paradoxical role. At sites of demyelination in MS lesions, reactive astrocytes form a glial scar that impairs remyelination [79,80]. However, astrocytes have also been shown to act as cellular mediators of CNS myelination by promoting oligodendrocyte progenitor migration, proliferation, and differentiation [80]. Indeed, astrogliosis appears to be an early CNS response to MS-related insults [81]. Astrocytes preferentially express $S1P_3$ and $S1P_1$ and can express $S1P_2$ at a low level; $S1P_5$ expression is not detectable under basal conditions, but can be upregulated by astrocytes grown in culture [29,31,82,83]. Injection of S1P into the striatum of mice induced astrogliosis [84]. A mouse model of Sandhoff disease, another neurodegenerative disease associated with astrogliosis, was attenuated by genetic deletion of either SphK1 or $S1P_3$ [85]. Critically, selective removal of $S1P_1$ from astrocytes attenuated EAE severity and reduced histological sequelae of EAE challenge in the CNS [64].

Fingolimod-P treatment of cultured human astrocytes has been shown to inhibit production of inflammatory cytokines [68]. In cultured rat astrocytes, fingolimod-P stimulated extracellular signal-regulated kinase (ERK) phosphorylation and cell migration; these effects were also seen with selective $S1P_1$ agonists, suggesting that fingolimod-P acted as a functional agonist of $S1P_1$ in these *in vitro* experiments [86,87]. In contrast, results from an *in vivo* study performed using a mouse EAE model supported functional antagonism of astrocyte $S1P_1$ rather than forms of agonism as the predominant receptor mechanism (with regard to CNS cells) for fingolimod efficacy [64]. In this study, inflammatory cytokine levels, as well as disease-associated increases in S1P levels, were reduced in animals lacking $S1P_1$ on astrocytes. All conditional null mutants lacking $S1P_1$ in CNS cell lineages displayed wild-type lymphocyte trafficking that responded normally to fingolimod treatment. EAE severity was attenuated in mutants lacking $S1P_1$ on glial fibrillary acidic protein-expressing astrocytes, compared with unrecombined littermate controls [64]. Reductions in EAE severity were accompanied by reductions in demyelination, axonal loss, and astrogliosis. If lymphocyte depletion was solely responsible for fingolimod efficacy, then EAE severity in $S1P_1$ null mutants should have been further reduced with fingolimod treatment. However, this was not observed and clinical scores were refractory to fingolimod treatment, despite the maintained immunologic effects on peripheral blood lymphocyte depletion. Mutants lacking $S1P_1$ on neurons but not on astrocytes showed the same response to fingolimod treatment as littermate controls. These *in vivo* results were supported by experiments in astrocyte cultures, in which fingolimod treatment was found to induce rapid internalization of $S1P_1$ that was not followed by recycling of $S1P_1$ to the cell surface [64]. Overall, these findings identified functional antagonism of $S1P_1$ on astrocytes as a non-immunologic direct CNS effect of fingolimod necessary for its efficacy [64].

3.2. Oligodendrocytes

Oligodendrocytes are myelinating cells of the CNS. Demyelination and failure of remyelination by oligodendrocytes contribute to the progression of disease in MS. Therefore, targeting the oligodendrocyte is a potentially important therapeutic strategy [88]. Remyelination requires oligodendrocyte precursor cell (OPC) proliferation, migration to sites of demyelination and differentiation into mature myelin-forming oligodendrocytes. Mature oligodendrocytes preferentially express $S1P_5$ and may express $S1P_1$, $S1P_2$ and $S1P_3$ at lower levels, while OPCs show high levels of $S1P_1$ gene expression and lower levels of $S1P_5$ and $S1P_3$ expression [49,62,89–94]. The effects of S1P on oligodendrocyte lineages include differentiation, migration and survival, depending on the developmental stage [91,92]. However, in non-pathological conditions, mice deficient in $S1P_5$ do not show impaired myelination [91,92], suggesting at least some functional redundancy among S1P receptor subtypes in OPCs and oligodendrocytes.

Results from *in vitro* studies have shown that the effects of fingolimod-P on cultured oligodendrocyte lineage cells are diverse and are affected by developmental stage, treatment dose, and duration [49,62,91–95]. Fingolimod has been shown to protect cultured rodent OPCs from apoptosis induced by inflammatory cytokines and microglial activation (both of which have been implicated in the pathogenesis of MS), *via* apparent activation of ERK 1/2 and Akt signaling [95].

Additionally, activation of $S1P_5$ by fingolimod impeded spontaneous migration of cultured neonatal rat OPCs [49], but fingolimod did not inhibit OPC migration when platelet-derived growth factor was used as a chemoattractant [92]. The differentiation of OPCs was stimulated by fingolimod at low nanomolar doses [92], but was inhibited at higher concentrations [92,94,95]. Similarly, the effects of fingolimod on process dynamics in mature oligodendrocytes depended on both dose and treatment duration [62].

3.3. Neurons

Neural progenitor cells can express $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_5$ [27,96], while neurons predominantly express $S1P_3$ and $S1P_1$ [96,97]. Genetic deletion of $S1P_1$ or deletion of both SphK1 and SphK2 in mice caused severe defects of neurogenesis [98]. $S1P_2$ knockout mice showed defects in the inner ear that are associated with neurodegeneration and can result in loss of hearing and balance [34,99,100]. In addition, while phenotypes of $S1P_2$ deletion mutants appeared relatively normal [99,101], some background strains promoted increased excitability [99] and seizure activity [102].

In primary cultures of neural progenitor cells, S1P produced many similar effects to those reported for LPA, including induced proliferation, morphologic changes, and enhanced survival [96,103,104]. S1P modulated neurite extension in cultured PC12 cells and dorsal root ganglion neurons [104], and enhanced nerve growth factor-induced excitability of adult sensory neurons [105]. In primary hippocampal neurons, S1P acted both as a secretagogue, triggering glutamate secretion, and as an enhancer, potentiating depolarization-evoked glutamate secretion [97]. In cultured cortical neurons, fingolimod and S1P have been shown to protect against excitotoxic death [106]. These cell culture phenomena require further examination *in vivo*.

As described above, in the study by Choi et al. [64], neuronal $S1P_1$ mutants responded to fingolimod treatment in the same way as littermate controls, indicating that $S1P_1$ on astrocytes, but not on neurons, is a major locus for direct CNS effects of fingolimod [64]. In a rat model of optic neuritis, fingolimod treatment reduced inflammation, demyelination and axonal damage, but did not prevent apoptosis of retinal ganglion cells, the neurons that form the axons of the optic nerve [107]. Rossi et al. used electrophysiological recordings to investigate whether fingolimod could ameliorate synaptic defects in EAE mice and found that oral fingolimod prevented and reversed the presynaptic and postsynaptic alterations of glutamate transmission [108]. These effects were associated with reduced clinical deterioration. In addition, prophylactic fingolimod treatment significantly reduced the dendritic spine loss observed during the acute phase of EAE. In model systems, fingolimod did not alter the spontaneous excitatory postsynaptic currents in neurons from healthy control mice, indicating that fingolimod does not interfere with physiological synaptic transmission [108].

3.4. Microglia

Microglia are involved in both innate and adaptive immunity in the CNS. Microglial activation seems to be critical for MS pathogenesis [109] and inhibition of activation suppressed relapsing paralysis in EAE [110]. Activated microglia have been shown to differentiate into M1 and M2 microglia that contribute to both protective and detrimental aspects of the inflammatory process through antigen presentation, cytokine release and phagocytosis [109]. S1P receptor expression on microglia varies according to the activation state of these cells [43]. Microglia in an inactive state isolated acutely from rat brain showed gene expression for $S1P_1$ and $S1P_3$ that was higher than $S1P_2$, and much higher than $S1P_5$ [43]. *In vitro*, S1P increased the release of proinflammatory cytokines from activated microglia [111]. Fingolimod-P has been reported to have no effect on cytokine production by cultured human microglia [112]. Non-phosphorylated fingolimod, but not fingolimod-P, induced apoptosis of a human microglia cell line by activating sterol regulatory element-binding protein-2 [113]. In rats, fingolimod treatment attenuated infiltration of reactive macrophages/microglia into lesions produced by traumatic brain injury [114]. Fingolimod also reduced microglial activation in cerebral ischemic lesions in mice [115].

Jackson et al. examined the effects of fingolimod on remyelination in rat telencephalic neurospheres [116]. The absence of blood-borne immune cells in this model allowed the direct CNS effects of fingolimod to be assessed. Following lysophosphotidyl choline-induced demyelination, fingolimod treatment significantly augmented expression of myelin basic protein, a marker of remyelination; in addition, fingolimod downregulated ferritin, a marker of microglial activation. Fingolimod also downregulated tumor necrosis factor-α and interleukin (IL)-1b; these cytokines are produced by activated microglia and astrocytes [116]. The $S1P_1$/$S1P_5$-selective receptor modulator BAF312 (siponimod), but not the $S1P_1$-selective receptor modulator AUY954, also increased levels of myelin basic protein in this model, indicating that $S1P_5$ is, in some way, involved in promoting remyelination *in vitro*. Overall, these results indicate that fingolimod can modulate microglial activation and actively promote remyelination *via* direct interaction with microglia, oligodendrocytes, and/or astrocytes [116].

3.5. Dendritic cells

Dendritic cells are a class of antigen-presenting cell that are able to prime naïve T cells and regulate adaptive immune responses [117,118]. At least four subtypes of dendritic cell exist: plasmacytoid, migratory myeloid, secondary lymphoid tissue resident myeloid and inflammatory, each having different functional properties [117]. The role of dendritic cells in MS may be dependent on the subtype of the cell. For example, peripheral myeloid cells can contribute to autoimmune CNS inflammation in EAE [117]. In contrast, plasmacytoid cells have been shown to have anti-inflammatory properties in EAE and limit the severity of the condition [119]. Patients with MS have been found to have higher levels of myeloid dendritic cells that secrete higher levels of proinflammatory cytokines than healthy individuals [117] and have functionally abnormal plasmacytoid cells [120]. Therefore, depending on the cell subtype, dendritic cells may contribute to, and also prevent CNS autoimmune inflammation. All five S1PR subtypes are expressed on dendritic cells in animals [121]; however, the effect of fingolimod on dendritic cells in humans or individuals with MS has not been investigated. In mice, fingolimod enhanced retention of plasmacytoid cells in the lymph nodes, possibly *via* $S1P_4$ [122], although in another study fingolimod increased dendritic cell levels in the blood [121]. It has been recently reported that the efficacy of $S1P_1$ treatment in reducing CNS inflammation in EAE correlates with the presence of plasmacytoid cells in the CNS [119]. These studies suggest that dendritic cells play diverse roles in MS pathology and CNS inflammation, although their exact roles are yet to be fully characterized.

3.6. Functional effects of S1P signaling and fingolimod

3.6.1. Blood–brain barrier

Penetration of lymphocytes into the CNS across endothelial cells of the blood–brain barrier is a critical event in the pathogenesis of MS [123]. Vascular endothelial cells can express $S1P_1$ and $S1P_3$ [124]; hence, the S1P signaling pathway might influence blood–brain barrier function [125]. Fingolimod can induce adherens junction assembly in human umbilical vein endothelial cells *in vitro* and can reduce vascular leakage induced by vascular endothelial cell growth factor or lipopolysaccharide-mediated acute lung injury in mice *in vivo* [126,127]. Fingolimod also enhanced human pulmonary endothelial cell barrier function *in vitro* [128]. Enhancement of barrier function in this model appeared to be independent of $S1P_1$ binding and did not require phosphorylation of fingolimod, indicating a non-$S1P_1$ mechanism of action [128]. Importantly, heterogeneity of vascular beds leaves open the question of whether S1P signaling and prolonged fingolimod exposure actually

alters the blood–brain barrier. This issue is relevant to fingolimod acting as a functional antagonist of $S1P_1$ on astrocytes [64]. Some models implicate astrocyte end-feet as an integral component of the blood–brain barrier [129]; therefore, it is possible that astrocyte-mediated effects of fingolimod might also influence some aspects of normal blood–brain barrier function.

Lymphocyte penetration of the blood–brain barrier is dependent on vascular cell adhesion molecules and matrix metalloproteinases (MMPs), which degrade the endothelial basement membrane [130,131]. In a rat EAE model, both prophylactic and therapeutic treatment with fingolimod suppressed/reversed neurological deficits and normalized upregulated gene expression of vascular cell adhesion molecules and MMP-9 in the spinal cord [132]. These effects may in part be caused by direct effects of fingolimod on microvascular and/or glial cells in the CNS [132].

4. Preservation of CNS tissue integrity and functional recovery in animal models and organotypic cultures

Overall, the *in vitro* and *in vivo* studies described above suggest that fingolimod could directly affect CNS resident cells in ways that could potentially prevent demyelination or promote myelin repair in MS lesions (Fig. 2). In a relapsing–progressive EAE model in mice, prophylactic and therapeutic fingolimod treatment during relapsing EAE inhibited subsequent relapses and axonal loss in the spinal cord, and facilitated motor recovery. This was not observed when fingolimod was initiated at a very late stage of the model (after 4 months), during the non-relapsing, secondary advanced progressive stage, after accumulation of significant neurological deficits [133]. In the dark agouti (DA) rat model of EAE, prophylactic fingolimod therapy protected against the presentation of EAE symptoms and disturbances in neuronal function; therapeutic treatment decreased demyelination in the brain and spinal cord, correlating with reversed paralysis and restored neuronal function [134]. In another study in the DA rat model of EAE, fingolimod reversed blood–brain barrier leakiness, reduced demyelination and also improved neurological function [132]. Administration of fingolimod was also found to reduce the area of demyelination in the spinal cord in other EAE studies [135,136]. Fingolimod did not promote remyelination [137,138] but attenuated injury to oligodendrocytes, myelin, and axons in the corpus callosum during cuprizone-induced demyelination in mice [138], suggesting a protective effect of fingolimod that is independent of the effect on peripheral lymphocytes. The protective effect of fingolimod was also associated with decreased IL-1β and chemokine (C–C motif) ligand 2 levels in the corpus callosum and altered $S1P_1$ expression [138].

Anthony et al. investigated fingolimod in a focal delayed-type hypersensitivity (DTH) model of MS in rats. DTH lesions are initially characterized by breakdown of the blood–brain barrier, macrophage and lymphocyte infiltration, and tissue damage, including myelin loss. Fingolimod treatment during the active phase (when the blood–brain barrier is disrupted) reduced blood–brain barrier breakdown, inflammatory cell infiltration, and tissue damage [139]. During the remission phase of the DTH model, when the blood–brain barrier was functionally intact, fingolimod treatment reduced demyelination and microglial activation without a corresponding reduction in lymphocytes [140]. These results provide evidence of direct effects of fingolimod in the CNS that are independent of the effects on lymphocyte infiltration. One possible mechanism of CNS direct protective effects was recently demonstrated by Deogracias et al. using fingolimod in a mouse model of Rett syndrome. Fingolimod increased brain-derived neurotrophic factor (BDNF) levels in the cortex, hippocampus and striatum, and also improved motor functioning [141]. The precise mechanism for these effects requires further investigation; however, these changes suggest that fingolimod may promote neuronal repair and improve CNS function through the effects of BDNF.

Jackson et al. found that fingolimod promoted remyelination in rat telencephalic neurospheres (see earlier Microglia section) [116]. In rat organotypic cerebellar slice cultures, both fingolimod-P and the $S1P_1$-selective agonist, SEW2871, inhibited lysolecithin-induced demyelination, upregulated $S1P_1$ expression on astrocytes and inhibited the release of several chemokines, including lipopolysaccharide-induced CXC chemokine (CXCL5), macrophage inflammatory protein (MIP)-1α, and MIP-3α [142]. Fingolimod may therefore attenuate demyelination not only by preventing S1P-receptor-mediated T-cell migration into the CNS, but also *via* a mechanism that includes an S1P-receptor-mediated reduction of cytokine/chemokine release in the CNS [142]. Fingolimod also enhanced remyelination and process extension by OPCs and

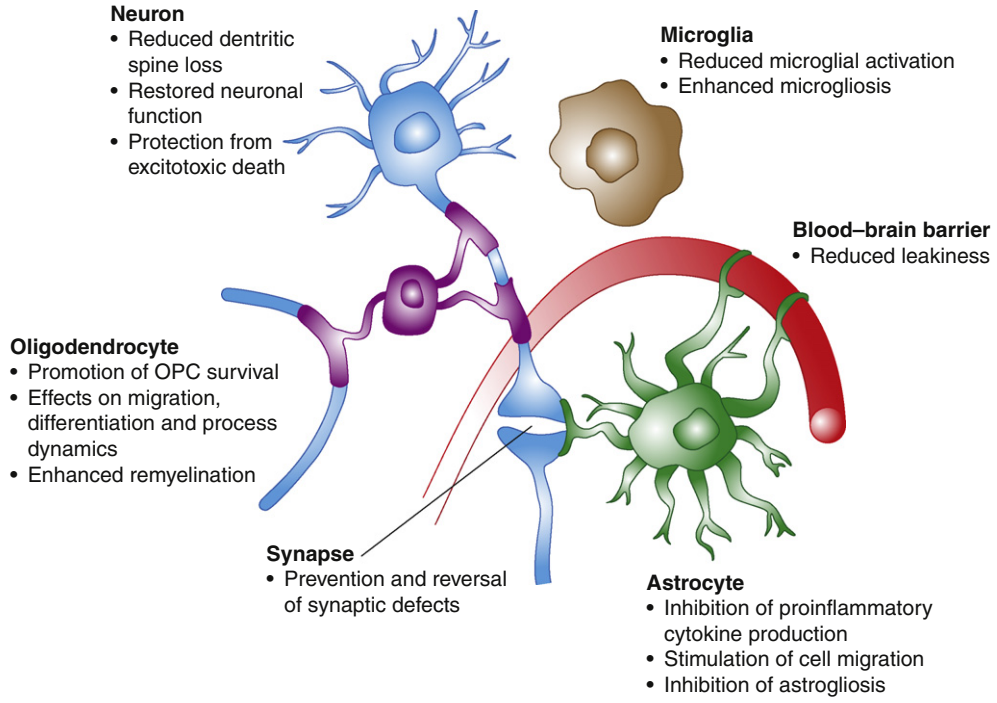

Fig. 2. Summary of the effects of fingolimod treatment on different cells in the central nervous system.

mature oligodendrocytes in neonatal mouse organotypic cerebellar slice cultures following lysolecithin-induced demyelination [63]. Increased numbers of microglia and astrocytes were also observed with fingolimod treatment. In addition, selective removal of $S1P_1$ from astrocytes also preserved myelin *in vivo* [64]. These data suggest that S1P receptor modulation in the CNS can potentially enhance remyelination or limit demyelination, although other data do not support remyelination with fingolimod treatment. Fingolimod did not promote myelin repair in cuprizone [137,138] and lysolecithin demyelination animal models [137]. However, because of the fast endogenous remyelination process in both models, it has been suggested that these models may be more appropriate to explore negative, rather than positive effects on myelin repair [143].

5. Clinical effects of S1P signaling altered by fingolimod in the CNS

Fingolimod (0.5 mg once daily) is approved for the treatment of relapsing forms of MS in many countries [144,145]. The clinical efficacy of fingolimod in relapsing–remitting MS (RRMS) was demonstrated in two randomized, double-blind, phase 3 clinical trials: FREEDOMS (FTY720 Research Evaluating Effects of Daily Oral Therapy in Multiple Sclerosis; a placebo-controlled trial of 1272 patients) and TRANSFORMS (Trial Assessing Injectable Interferon *versus* FTY720 Oral in Relapsing–Remitting Multiple Sclerosis; comparing fingolimod with an interferon in a total of 1292 patients) [146,147]. In TRANSFORMS, oral fingolimod 0.5 mg significantly reduced the annualized relapse rate (ARR) by 52% compared with intramuscular interferon beta-1a over 1 year (ARR 0.16 *versus* 0.33, respectively) [146]. In the 2-year FREEDOMS study, fingolimod 0.5 mg also significantly reduced the ARR ($p<0.001$) and significantly reduced the risk of disability progression confirmed at 3 and 6 months by 30% and 37%, respectively ($p=0.02$) [147]. In both TRANSFORMS and FREEDOMS, fingolimod was superior to placebo or active comparator with regard to magnetic resonance imaging (MRI) outcomes, including a reduction in the rate of brain volume loss [146,147]. A recent study that examined the relationship between interferon beta exposure and disease progression indicated that such treatments may not alter long-term disease progression [148]. Interferons, and perhaps other immunologically targeted therapies, might therefore have limited effectiveness in preventing long-term disability evolution. By contrast, agents like fingolimod that have dual activities on not only the immune system but also the CNS, may access novel brain mechanisms by preserving tissue integrity to reduce long-term disability, as suggested by experimental animal studies, the preservation of brain atrophy, and the reduced risk of disability progression observed in the FREEDOMS trial [147].

Imaging outcomes currently provide the best *in vivo* measures of neuroprotection and also possibly of repair in MS [149]. Percentage change in brain volume, a sensitive measure of neuroprotection over 1 year, was reported to correlate with physical disability, and to be a strong predictor of future disability [149,150]. Axonal loss and myelin damage result in brain volume reduction in MS [151]. In phase 3 studies, fingolimod 0.5 mg significantly reduced brain volume loss by 31% over 1 year compared with intramuscular interferon-beta 1a ($p<0.001$; TRANSFORMS) [146], and by 35% over 2 years compared with placebo ($p<0.001$; FREEDOMS) [147]. Subgroup analyses from FREEDOMS confirmed that these effects over 2 years were independent of the presence or absence of gadolinium (Gd)-enhancing lesions, T2 lesion load, previous treatment status, or level of disability [152]. Furthermore, the degree of brain volume loss with fingolimod differed from those observed with interferon beta or natalizumab [153], in which early acceleration of brain volume loss was seen (equal to or exceeding that of controls) with no treatment difference over 2 years. These findings suggest that, in addition to peripheral immunomodulatory actions, other effects of fingolimod, including direct CNS effects, could be related to reductions in brain atrophy observed with fingolimod that are not seen with other conventional immunomodulatory or immunosuppressant therapies.

Disease-modifying drugs such as interferon beta and glatiramer acetate are largely ineffective in primary–progressive MS (PPMS), which exhibits neurodegeneration with a relative lack of inflammatory lesion activity [154]. An ongoing study is evaluating whether fingolimod is effective in delaying MS disability progression compared with placebo in patients with PPMS [155]. If fingolimod is found to have efficacy in PPMS, this would be a major step forward in the treatment of this MS subtype, and could be consistent with the operation of direct CNS signaling mechanisms accessed by fingolimod treatment.

In addition to different subtypes of MS, fingolimod may potentially be useful in treating other autoimmune diseases and disorders involving other systems. For example, the effect of fingolimod as a therapeutic agent in a model of spontaneous autoimmune polyneuropathy has recently been investigated in mice. Animals treated with fingolimod showed reduced disease progression and demyelination compared with animals treated with water [156]. Treatment with fingolimod has demonstrated suppression of experimental autoimmune uveitis in mice [157,158]. Diabetes was prevented in non-obese diabetic mice with peripheral insulitis treated continuously with fingolimod; fingolimod treatment also reversed diabetes in mice that were diabetic [159]. Fingolimod also prevented autoimmune diabetes in diabetes-resistant biobreeding rats [160]. In mice deficient for apolipoprotein-E, oral administration of fingolimod significantly reduced atherosclerotic lesion formation compared with control mice [161]. S1P has been proposed to play a role in the pathogenesis of rheumatoid arthritis and therefore may represent a possible therapeutic target in the disease [162,163]. The actions of fingolimod on CNS astrocytes in EAE [64] have further suggested fingolimod actions in other CNS diseases such as amyotrophic lateral sclerosis, where astrocytes have also been implicated [164]. Taken together, these data suggest that fingolimod could potentially be beneficial in treating diseases other than MS.

Other S1P receptor modulators in clinical development include BAF312 [165], ONO-4641 [166], ponesimod (ACT-128800) [167], and CS-0777 [168]. Few clinical data have yet been published for these investigational drugs. In a phase 2 trial of MS, ponesimod reduced the cumulative number of new active MRI lesions during weeks 12–24 *versus* placebo [169]. In a phase 2 trial in patients with RRMS, BAF312 reduced MRI lesion numbers by up to 80% *versus* placebo and also improved relapse outcomes [170]. ONO-4641 significantly reduced the number of T1 Gd-enhancing lesions during weeks 10–26 compared with placebo in a phase 2 trial in patients with RRMS [171].

6. Other current or potential MS therapies

Interferon-β and glatiramer acetate are not thought to penetrate the blood–brain barrier, and therefore these drugs have no proven, direct neurobiologic effects in the CNS [172,173]. In addition to the investigational S1P receptor modulators mentioned in the previous section, other oral therapies in development for MS include teriflunomide, laquinimod, and BG-12 (dimethyl fumarate) [173]. Teriflunomide, a selective inhibitor of *de novo* pyrimidine synthesis, is thought to act mainly by exerting a cytostatic effect on proliferating T and B cells in the periphery [174]. However, teriflunomide has also been reported to increase the secretion of IL-10 by rat microglia *in vitro* [175]. In addition, teriflunomide also significantly reduced demyelination and axonal loss in a rat model of EAE [176]. There is some evidence that laquinimod [177–179] and BG-12 [180,181], both currently in phase 3 development for RRMS, may have some neuroprotective effects in the CNS. In animal models, laquinimod treatment reduced axonal damage [177], astrogliosis and demyelination [182]. This effect may be mediated by stimulating BDNF secretion in the periphery and CNS [178]. In phase 2 trials in RRMS, laquinimod significantly increased BDNF serum levels compared with placebo after 12 and 36 weeks of treatment [179]. Laquinimod may also exert neuroprotection through other mechanisms. Laquinimod prevented alterations of GABAergic synapses induced by EAE, preserved cannabinoid CB1 receptor sensitivity

(normally absent in EAE) and also regulated synaptic transmission [183]. Furthermore, laquinimod has been shown to prevent cuprizone-induced demyelination by reducing astrocytic nuclear factor-kB (NF-kB) activation [184]. Attenuation of the astrocytic proinflammatory response may be a mechanism of laquinomod's effects in the CNS, which occur independently of its immunomodulatory actions [184]. In EAE, BG-12 treatment led to reduced loss of neurons and glia in the CNS [180]. *In vitro*, monomethyl fumarate, the active metabolite of BG-12, protected cultured neurons and astrocytes from hydrogen-peroxide-induced cell death [180]. In an *in vitro* model of brain inflammation, BG-12 decreased the production of proinflammatory mediators in activated microglia and astrocytes [185]. It has been proposed that cytoprotective effects of BG-12 are dependent on anti-oxidative pathways mediated by NF-E2-related factor 2 [180,181]. NF-E2-related factor 2 has reported properties that include blood–brain barrier protection [186] and myelin maintenance [187] that may also contribute to the mechanism of action of BG-12 [188]. BG-12 has also been reported to show a limited treatment effect in EAE in a therapeutic setting [180]. The recent discontinuation of a NF-E2-related factor 2 activator compound in phase 3 clinical trials (Bardoxolone [Abbott], for kidney disease) underscores a need to better understand BG-12's mechanism of action, as several cellular targets may be involved [174,189].

7. Conclusions

As well as being the first oral MS disease-modifying therapy, fingolimod is the first human medicine to be approved that targets S1P receptors, and thus has a fundamentally different and validated molecular target compared with all previously approved MS therapies. Emerging evidence from preclinical studies demonstrates that mechanisms independent of peripheral immune effects contribute substantially to the efficacy of fingolimod in models of MS. Fingolimod readily crosses the blood–brain barrier into the CNS where it is phosphorylated to its active metabolite, fingolimod-P. Fingolimod-P then potentially interacts with S1P receptors that are expressed on oligodendrocytes, astrocytes, neurons, and microglia, as well as on vascular endothelial cells of the blood–brain barrier. Importantly, the cell-specific expression of defined receptor subtypes during the course of MS may have more restricted expression patterns. Several animal models and organotypic studies have provided evidence that fingolimod treatment can reduce demyelination and promote remyelination *via* direct effects in the CNS. Furthermore, deletion of $S1P_1$ or $S1P_5$ from CNS cells reduces EAE severity and fingolimod efficacy, again indicating direct CNS effects. Results from phase 3 trials of fingolimod suggest that the preservation of neural cells observed preclinically may be related to the efficacy on brain atrophy outcomes observed in patients with MS. In addition, fingolimod has been shown to ameliorate synaptic dysfunction in EAE, opening up the possibility that it may have efficacy in other neurodegenerative diseases. The capacity of fingolimod for direct CNS preservation effects also raises the possibility of efficacy in non-relapsing forms of MS, and results are awaited from an ongoing trial of fingolimod in PPMS, for which direct CNS activities provide a rationale for this form of MS that currently lacks specifically approved treatment.

Conflict of interest

JC has received honoraria, consulting fees and/or grant support from: Abbott, Amira Pharmaceuticals, Biogen-Idec, Celgene, GlaxoSmithKline, Johnson and Johnson, Merck, Mitsubishi Tanabe Pharma Corporation, Novartis, Ono Pharmaceutical Co., Pfizer and Taisho Pharmaceutical Co.

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