



A_{2B} Adenosine Receptors and Sphingosine 1-Phosphate Signaling Cross-Talk in Oligodendroglogenesis

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Oligodendrocyte-formed myelin sheaths allow fast synaptic transmission in the brain. Impairments in the process of myelination, or demyelinating insults, might cause chronic diseases such as multiple sclerosis (MS). Under physiological conditions, remyelination is an ongoing process throughout adult life consisting in the differentiation of oligodendrocyte progenitor cells (OPCs) into mature oligodendrocytes (OLs). During pathological events, this process fails due to unfavorable environment. Adenosine and sphingosine kinase/sphingosine 1-phosphate signaling axes (SphK/S1P) play important roles in remyelination processes. Remarkably, fingolimod (FTY720), a sphingosine analog recently approved for MS treatment, plays important roles in OPC maturation. We recently demonstrated that the selective stimulation of A_{2B} adenosine receptors (A_{2BR}s) inhibit OPC differentiation *in vitro* and reduce voltage-dependent outward K⁺ currents (I_K) necessary to OPC maturation, whereas specific SphK1 or SphK2 inhibition exerts the opposite effect. During OPC differentiation A_{2BR} expression increases, this effect being prevented by SphK1/2 blockade. Furthermore, selective silencing of A_{2BR} in OPC cultures prompts maturation and, intriguingly, enhances the expression of S1P lyase, the enzyme responsible for irreversible S1P catabolism. Finally, the existence of an interplay between SphK1/S1P pathway and A_{2BR}s in OPCs was confirmed since acute stimulation of A_{2BR}s activates SphK1 by increasing its phosphorylation. Here the role of A_{2BR} and SphK/S1P signaling during oligodendrogenesis is reviewed in detail, with the purpose to shed new light on the interaction between A_{2BR}s and S1P signaling, as eventual innovative targets for the treatment of demyelinating disorders.

Keywords: adenosine, sphingosine kinase (SphK), remyelination, K⁺ channels, oligodendrocyte differentiation, sphingosine-1-phosphate, oligodendrocyte progenitor cells (OPCs), A_{2B} receptors

INTRODUCTION

In the central nervous system (CNS), oligodendrocytes (OLs) are responsible for myelin production, which allows fast signal transmission and provides metabolic support to axons (Nave, 2010; Saab et al., 2016). During development, OLs are generated in the germinal zones of the brain, i.e., the subventricular zone (SVZ) (Yu et al., 1994), from migratory bipolar oligodendrocyte precursor cells (OPCs), which are renowned for the expression of the proteoglycan nerve-glial antigen 2 (NG2) (Grinspan, 2002; Brazel et al., 2004). Thanks to their migratory ability, OPCs spread and populate the embryonic brain and spinal cord (Emery, 2010) to differentiate into myelinating OLs. However, a pool of immature OPCs, comprising the 5–8% of total glial cells (Levine et al., 2001), persists within the adult CNS where they represent the major population of cycling cells (Dawson et al., 2003). This process guarantees, under physiological conditions, myelin turnover and remodeling in response to life experience (Malerba et al., 2015; Bergles and Richardson, 2016) and, in conditions of tissue damage, the generation of new OLs able to remyelinate the brain after a lesion or injury, an ability that might be lost through normal aging or chronic diseases. However, under pathological conditions such those characterized by chronic neuroinflammation and neurodegeneration, this process fails, leading to improper nerve conduction due to discontinuities in the myelin sheet (Nave, 2010; Franklin and Ffrench-Constant, 2017). For this reason, it is of critical relevance to identify innovative targets able to encourage OPC differentiation toward the mature, myelin-producing OL phenotype.

Among the factors influencing oligodendroglogenesis, interest has been focused in the last years on the neuromodulator adenosine (Coppi et al., 2021), on one hand, and on the pleiotropic signaling molecule sphingosine 1-phosphate (S1P).

This review will focus on S1P and adenosine receptor-mediated effects in oligodendrocyte progenitors relevant for oligodendrogenesis and their possible functional interplay with the purpose to deepen the knowledge of molecular mechanisms involved in A_{2B} receptor- ($A_{2B}R$ -) or S1P-mediated effects and their cross-talk in oligodendrocyte biology.

Oligodendrocyte Differentiation

Before being able to produce myelin, oligodendroglial cells progress through a series of highly regulated steps of differentiation from OPCs to mature OLs (de Castro and Bribian, 2005; Barateiro and Fernandes, 2014). This process is characterized by the loss of proliferative and migratory activity and the acquisition of an elaborate and highly ramified morphology (de Castro and Bribian, 2005). Oligodendroglogenesis implicates a sequence of distinct maturational stages each of them identified by distinct morphological changes and by the expression of specific antigens (Levi et al., 1986; Gard and Pfeiffer, 1990; Warrington et al., 1992; Jung et al., 1996). On these bases, three major stages of differentiation have been proposed: a proliferating, bipolar OPC phase, characterized by the expression of platelet-derived growth factor (PDGF) receptor alpha (PDGF α), NG2 and the transcription factor Olig2 (Pringle et al., 1992; Ligon et al., 2006),

a post-mitotic, moderately ramified pre-oligodendrocyte phase (pre-OL), positive for the markers O4 (Szuchet et al., 2011) and the recently deorphanized (Ciana et al., 2006) P2Y-like GPR17 receptor (Lecca et al., 2008; Emery, 2010; Fumagalli et al., 2011; Coppi et al., 2013b), and a mature myelinating OL phase, characterized by a highly ramified morphology and by the expression of galactocerebroside (GC), a specific marker for the oligodendrocyte membrane, myelin specific structural proteins such as 2,3'-Cyclic-nucleotide-3'-phosphodiesterase (CNPase), myelin associated glycoprotein (MAG) and myelin basic protein (MBP) (Scolding et al., 1989; Zhang, 2001; Szuchet et al., 2011; Coppi et al., 2013a, 2015). Mature OLs synthesize large amounts of myelin, giving rise to multilamellar myelin sheaths that wrap and insulate neuronal axons which allow electrical isolation and saltatory conduction of electric impulses.

It is known that, during their maturation, oligodendroglial cells display functional voltage-gated ion channels whose expression changes during differentiation (Sontheimer et al., 1989; Barres et al., 1990; Williamson et al., 1997; Spitzer et al., 2019) including either inward or outward rectifying K^+ channels (K_i and K_v , respectively), Na^+ channels (Na_v) and different subtypes of Ca^{2+} channels (Ca_v) (Verkhratsky et al., 1990).

During mouse brain development, the first ion channel subtype detected in OPCs is the K_v voltage-dependent, outwardly rectifying K^+ conductance, at embryonic day 18 (E18) (Spitzer et al., 2019). However, a fraction of OPCs are described to express also Na_v currents that sharply increase around birth. This OPC population, with high Na_v and K_v densities, reflect a proliferative and migratory state during myelin generation (Gautier et al., 2015; Spitzer et al., 2019). Intriguingly, not all OPCs express Na_v , but only a fraction, described to be around 60% (Kettenmann et al., 1991). Of note, a subpopulation of electrically excitable, spiking, Na_v OPCs, able to generate full action potentials when stimulated by depolarizing current injection, have been described in brain slices (Karadottir et al., 2008), but the functional role of this “electrically excitable” OPC subpopulation is still unknown. Of note, single action potentials have also been detected in a minority of cultured OPCs (Barres et al., 1990).

The functional role of action potentials (APs) in OPC is still a matter of debate since, up to now, no functional differences have been detected between firing and not-firing OPCs (Bonetto et al., 2020). As long as OPCs undergo functional maturation, the density of Na_v currents decreases as well as the expression of outward K_v conductances. In this phase, OPC differentiation potential declines and thus it can be considered a “quiescent” OPC state.

Among K_v currents, OPCs show outward conductances mainly composed by tetraethylammonium (TEA)-sensitive, delayed rectifying K^+ currents (I_K) (Sontheimer and Kettenmann, 1988) characterized by scarce time- and voltage-dependent inactivation and by a threshold for activation around -40 mV (Gutman et al., 2005). They also express a transient outward K^+ current, or I_A (Gallo et al., 1996; Coppi et al., 2013b; Coppi et al., 2015), characterized by a rapid time-dependent inactivation (approximately 50 ms) and a voltage-dependent inactivation at potentials above -80 mV.

During maturation, outward K^+ conductances (both I_K and I_A), as well as I_{Na} , undergo a strong downregulation up to almost completely disappearance in mature OLs (Sontheimer and Kettenmann, 1988; Sontheimer et al., 1989; Barres et al., 1990; Coppi et al., 2013a). Concomitantly, a gradual increase in the expression of inwardly rectifying K^+ currents (Kir), activated at potentials lower than -100 mV, appears. Indeed, Kir currents are the main conductance observed in mature OLs (Knutson et al., 1997). Among the mentioned currents, TEA-sensitive I_K are crucially linked to cell cycle regulation and hence to myelin formation (Chittajallu et al., 2005), as demonstrated by the fact that when OPC cultures are grown in the presence of TEA, a significant inhibition of their proliferation and differentiation is observed (Gallo et al., 1996; Knutson et al., 1997; Chittajallu et al., 2005; Coppi et al., 2013b). Hence, compounds that modulate these currents may affect oligodendrocyte proliferation and myelination, as well as neurotransmitters, cytokines and growth factors acting on specific metabotropic receptors described to modify K^+ current expression in OPCs (Stellwagen and Malenka, 2006; Zonouzi et al., 2011; Lundgaard et al., 2013; Malerba et al., 2015; Spitzer et al., 2019).

Adenosine as a Neuromodulator

Adenosine is an intermediary metabolite acting as a building molecule for nucleic acids and a component of the biological energy currency ATP. In addition, adenosine is one of the most evolutionarily ancient signaling molecules (Verkhratsky and Burnstock, 2014), acting through the stimulation of four distinct adenosine-sensitive metabotropic P1 receptors denoted as A_1 , A_{2A} , A_{2B} and A_3 adenosine receptors (A_1Rs , $A_{2A}Rs$, $A_{2B}Rs$ and A_3Rs) (Fredholm et al., 2011). These receptors are widely expressed both in the periphery or in the central nervous system (CNS) and have been implicated in a myriad of biological functions (Pedata et al., 2007; Coppi et al., 2012).

High extracellular concentrations of the nucleoside adenosine are found under conditions of tissue damage or when an imbalance in oxygen supply occurs (Latini et al., 1999; Pedata et al., 2014). Adenosine is short-lived in the extracellular space due to enzymatic degradation by adenosine deaminase (ADA) or adenosine kinase (AK) (Chen et al., 2013) or re-uptake operated by the equilibrative nucleoside transporters (ENT) isoforms ENT1 and ENT2 (Inoue, 2017), so its effects in the CNS are mainly described as autocrine and/or paracrine.

Among the four different P1 receptor subtypes, different affinities have been shown for the endogenous ligand. The activation of A_1Rs is achieved as long as extracellular adenosine falls in the low nanomolar range (1–10 nM), a concentration generally present in most tissues and organs throughout the body (Latini and Pedata, 2001). A_1R -mediated signal activates $G_{i/o}$ proteins leading to the inhibition of adenylyl cyclase (AC) and to a decrease in intracellular cAMP levels (Antonioli et al., 2019). A_1Rs are the predominant P1 receptor subtype in the CNS, with high levels of expression reported in the cerebral cortex, hippocampus, cerebellum, thalamus, brainstem and spinal cord. It is well known that A_1R activation inhibits synaptic transmission (Corradetti et al., 1984; Dunwiddie, 1984)

leading to sedative, anticonvulsant (Muzzi et al., 2013) and anxiolytic (Vincenzi et al., 2016) effects in the CNS whereas, at cardiovascular levels, they are potent bradycardic agents (Jacobson and Gao, 2006).

The $A_{2A}R$ subtype is known to stimulate AC by G_s protein activation, leading to the production of cAMP which acts as a second messenger by activating protein kinase A (PKA) (Antonioli et al., 2019). Within the brain, this receptor subtype is widely expressed with particularly high levels found in the striatum/caudate-putamen nuclei (Peterfreund et al., 1996). In the periphery, its expression is abundant on blood vessels and inflammatory/immune cells (Yu L. Q. et al., 2004). The functional effect of $A_{2A}Rs$ in the brain is at variance from A_1R subtype, as they enhance glutamate release and promote cell excitability (Goncalves and Ribeiro, 1996; Lopes et al., 2002). In accordance, $A_{2A}R$ activation participates to excitotoxic damage due to extracellular glutamate overload during an ischemic-like insult obtained *in vitro* by oxygen and glucose deprivation (OGD) (Colotta et al., 2012; Maraula et al., 2013; Maraula et al., 2014). Concerning peripheral functions of $A_{2A}Rs$, it is worth to note that adenosine, thanks to its actions on this receptor subtype, is one of the most powerful endogenous anti-inflammatory agents (Antonioli et al., 2019). Indeed, $A_{2A}Rs$ are highly expressed in inflammatory cells including lymphocytes, granulocytes and monocytes/macrophages, where their activation reduces pro-inflammatory cytokines, i.e., tumor necrosis factor-alpha (TNF α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) (Varani et al., 2011) and enhances the release of anti-inflammatory mediators, such as interleukin-10 (IL-10) (Bortoluzzi et al., 2016).

The $A_{2B}R$ subtype is somewhat the most enigmatic and less studied among the four P1 receptors as its pharmacological and physiological characterization has long been precluded by the lack of suitable ligands able to discriminate among the other adenosine receptor subtypes (Popoli and Pepponi, 2012; Chandrasekaran et al., 2019). The central distribution of $A_{2B}Rs$ on neurons and glia is scarce but widespread, whereas in the periphery abundant levels of $A_{2B}Rs$ are observed in bronchial epithelia, smooth muscles, inflammatory cells such as mast, neutrophils and monocytes, vasculature and digestive tracts like ileum and colon (Feoktistov et al., 1998; Chandrasekaran et al., 2019). It is reported that $A_{2B}R$ activation stimulates G_s and, in some cases, also $G_{q/11}$ proteins, thus activating either or both cAMP-pathway and IP $_3$ -related pathways. In this second case, the activation of phospholipase C (PLC) leads to the production of inositol-(1,4,5)-trisphosphate (IP $_3$) and diacylglycerol (DAG), that increases the intracellular levels of Ca^{2+} and activates protein kinase C (PKC), respectively (Antonioli et al., 2019).

Differently from high affinity A_1Rs and $A_{2A}Rs$, activated by physiological levels of extracellular adenosine (low nM and high nM, respectively (Coppi et al., 2020b)), the $A_{2B}R$ needs much higher adenosine concentrations (in the μ M range) reached only in conditions of tissue damage or injury (Chandrasekaran et al., 2019). Such a low affinity of $A_{2B}Rs$ for the endogenous agonist implies that they represent a good therapeutic target, since they are activated only by high adenosine efflux reached under pathological conditions or injury, when a

massive release of adenosine occurs (Popoli and Pepponi, 2012) or that they can be driven to function by selective agonists (Coppi et al., 2020b). All the adenosine receptors mentioned above are also associated with mitogen-activated protein kinase (MAPK) pathways, which involves the activation of extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2), whose action can mediate numerous cellular responses, and of JUN N-terminal kinase (JNK) and p38 MAPK (Melani et al., 2009). In particular, A_1 Rs and A_{2B} Rs promote the activation of ERK1, ERK2, p38 MAPK and JNK, instead A_{2A} Rs and A_3 Rs are involved only in the stimulation of ERK1 and ERK2 signaling (Antonioli et al., 2013).

As mentioned above for the cognate A_{2A} R subtype, A_{2B} R activation within the CNS is reported to increase glutamate release (Goncalves et al., 2015; Fusco et al., 2019) but the mechanism is at variance from the former. In fact, Cunha and co-workers demonstrated that the A_{2B} R selective agonist BAY60-6583 attenuates the predominant A_1 R-mediated inhibitory control of synaptic transmission in the CA1 hippocampus (Goncalves et al., 2015). These data are consistent with the relatively abundant expression of A_{2B} Rs in hippocampal presynaptic sites, demonstrated by means of synaptosome preparation, reported by the same authors (Goncalves et al., 2015). The facilitatory effect of A_{2B} Rs on glutamatergic neurotransmission was confirmed by us in acute hippocampal slices by using the electrophysiological protocol of paired pulse facilitation (PPF) (Fusco et al., 2019). We reported that A_{2B} R activation decreases PPF in the CA1 hippocampus, an effect known to be ascribed to enhanced glutamate release (Zucker and Regehr, 2002). Furthermore, the effect of BAY60-6583 was prevented not only by the A_{2B} R antagonists MRS1754 and PSB-603, but also by the A_1 R blocker DPCPX (Fusco et al., 2019), confirming the fact that A_1 R activation is necessary for the enhancing effect of A_{2B} Rs on glutamate release. Furthermore, we extended results to a newly synthesized A_{2B} R-selective agonist, the recently described BAY60-6583-analog P453 (Betti et al., 2018) which proved higher affinity for the A_{2B} R (50 nM vs. 200 nM, respectively) than the commercially available BAY 60-6583. In addition, as reported for A_{2A} Rs, also A_{2B} R activation participates to OGD-induced synaptic failure in the hippocampus (Fusco et al., 2018).

In the periphery, A_{2B} Rs are present on hematic cells, such as lymphocytes and neutrophils, with the highest expression levels found on macrophages (Gessi et al., 2005; Yang et al., 2006). Here, A_{2B} Rs in most cases are co-expressed with A_{2A} Rs and their activation exert anti-inflammatory effects by inhibiting migration and vascular adhesion (Yang et al., 2006) of inflammatory cells (Wakai et al., 2001; Eckle et al., 2008).

The A_3 R subtype is known to couple to $G_{i/o}$ proteins and to inhibit AC but, under particular conditions or in different cell types, activation of $G_{q/11}$ by A_3 R agonists has also been reported (Antonioli et al., 2019). This receptor subtype presents large interspecies differences, with only 74% sequence homology between rat and human (Koscso et al., 2011). Its expression is not uniform throughout the body: low levels are found in the brain and spinal cord whereas a predominance of this receptor subtype is described in peculiar regions at the periphery, i.e., in the testis, lung, kidneys, placenta, heart, brain, spleen and liver

(Gessia et al., 2008). Interestingly, most of the cell types of the immune system express functional A_3 Rs on their surface (Hasko et al., 2008) and its activation is one of the most powerful stimuli for mast cells degranulation (Ramkumar et al., 1993).

Adenosine in Oligodendroglogenesis

Oligodendrocyte turnover is rather slow under physiological conditions and guarantees myelin turnover and remodeling in response to life experience (Bergles and Richardson, 2016). However, a disruption in this process, for example in case of a maturation block, could have devastating consequences during aging and in pathological conditions, such as multiple sclerosis (MS).

Recruitment of OPCs to injured areas is in fact one of the most important events to promote remyelination after CNS injury (Simon et al., 2011; Neumann et al., 2019). Unfortunately, it is still not clear why this process fails, or is insufficient to provide myelin repair, during chronic demyelinating diseases (Romanelli et al., 2016). In fact, most OPCs fail to mature into myelin-producing OLs in MS, indicating that remyelination by adult OPCs is hindered principally due to a failure of OPC differentiation into mature OLs rather than a failure of repopulation or migration of OPCs (Chang et al., 2002; Patel and Klein, 2011).

A deep knowledge of the intricate processes regulating OPC maturation to OLs is mandatory to investigate new therapeutic targets aimed at counteracting demyelinating diseases and repair myelin damage in the long run.

Among the neuromodulators contributing to the balance between proliferating, immature OPCs and myelinating, mature OLs there are purines (Stevens et al., 2002; Fields, 2004; Fields and Burnstock, 2006) and, in particular, adenosine (Coppi et al., 2020b).

All P1 receptors are expressed by maturing oligodendroglial progenitors as well as by mature OLs (Stevens et al., 2002; Coppi et al., 2020a) and exert a key role in cell development (Coppi et al., 2015, 2020b). Furthermore, the expression by oligodendrocytes of the nucleoside transporters ENT1 and ENT2, as well as adenosine degrading enzymes ADA and AK, suggests that these cells are able to sense and finely tune extracellular adenosine levels (Gonzalez-Fernandez et al., 2014), thus supporting the notion that purinergic signaling exerts a prominent role in these cells (Burnstock et al., 2011).

Indeed, our research group contributed to demonstrate that adenosine can affect numerous OPC functions such as migration, proliferation and maturation (Fields and Stevens-Graham, 2002; Stevens et al., 2002; Coppi et al., 2013a,b, 2015; Cherchi et al., 2021), with distinct effects mediated by different receptor subtypes, as summarized in **Figure 1**.

It has been demonstrated that tonic electrical stimulation of co-cultures of OPCs with dorsal root ganglion neurons induces the release of adenosine that inhibits OPC proliferation and promotes their maturation toward pre-myelinating OLs, an effect blocked by a cocktail of A_1 R, A_{2A} R and A_3 R antagonists (Stevens et al., 2002), suggesting that endogenous adenosine released in response to impulse activity promotes oligodendrocyte development and myelination. Furthermore,

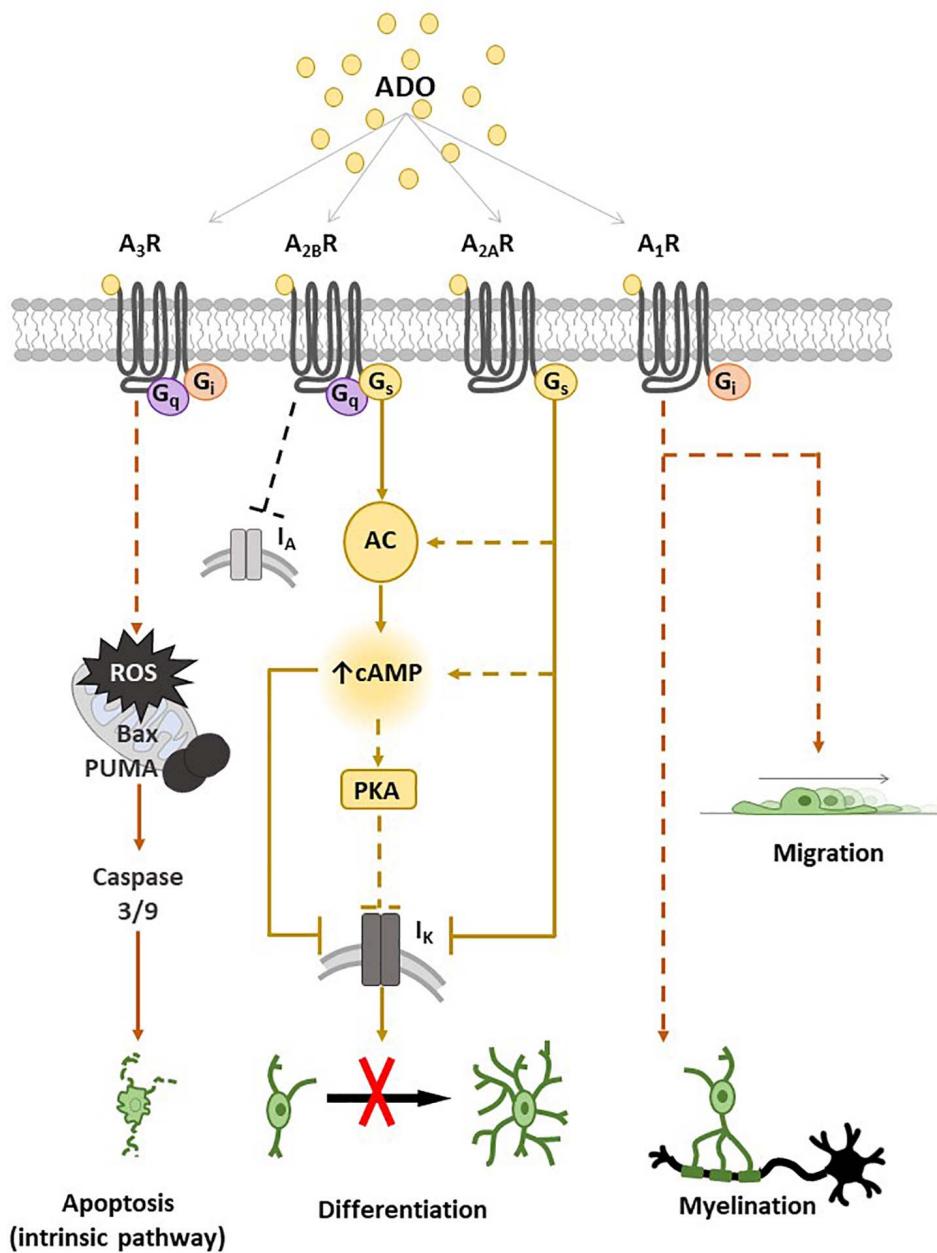


FIGURE 1 | Adenosine receptor expression and main transduction pathways activated in oligodendrocyte progenitor cells (OPCs) and oligodendrocytes (OLs). Schematic representation of A_1 , A_{2A} , A_{2B} and A_3 receptor (A_1R , $A_{2A}R$, $A_{2B}R$ and A_3R) effects on OPCs and main intracellular pathways involved. The activation of A_1R by adenosine (ADO) or other selective receptor agonists promotes myelination and migration. The stimulation of G_s -coupled $A_{2A}R$ and/or $A_{2B}R$ leads to adenyl cyclase (AC) activation with a consequent increase in intracellular cyclic adenosine monophosphate (cAMP), which closes I_K channels and inhibits OPC differentiation, probably by a mechanism involving protein kinase A (PKA). A_3R stimulation induces OPC apoptosis by the activation of an intrinsic pathway, i.e., through reactive oxygen species (ROS) production and activation of Bcl-2-associated X (Bax), p53-upregulated modulator of apoptosis (PUMA) and caspase 3/9. Dotted lines are used when the intracellular pathway/s have not been described.

A_1R agonists have been reported to stimulate OPC migration (Figure 1; Othman et al., 2003).

Concerning the $A_{2A}R$ subtype, our group of research demonstrated that the selective $A_{2A}R$ agonist CGS21680 inhibits TEA-sensitive I_K currents in cultured OPCs and delays *in vitro* cell differentiation without affecting neither cell

viability nor proliferation (Coppi et al., 2013a). These effects were completely prevented in the presence of the selective $A_{2A}R$ antagonist SCH58261 (Coppi et al., 2013a). In keeping with data demonstrating that TEA impairs OPC maturation (Attali et al., 1997; Gallo et al., 1996; Coppi et al., 2013b) and blocks myelin deposition in the embryonic spinal cord

(Shrager and Novakovic, 1995) and ovine OPCs (Soliven et al., 1988), it appears that the G_s -coupled A_{2A} R inhibits OPC differentiation by reducing I_K currents. In line with this assumption is the observation that selective activation of GPR17, a G_i -coupled P2Y-like receptor, enhances TEA-sensitive I_K and improves OPC differentiation (Coppi et al., 2013b).

The less known adenosine receptor in OPCs is the A_3 R subtype. The only paper available in the literature demonstrates that the A_3 R agonist 2-CI-IB-MECA induces apoptosis of cultured oligodendroglial cells isolated from rat optic nerve (Gonzalez-Fernandez et al., 2014) and induces myelin loss in an *ex vivo* preparation of optic nerve, an effect prevented by the A_3 R antagonist MRS1220 (Gonzalez-Fernandez et al., 2014). Moreover MRS1220 also prevented optic nerve demyelination induced by *in vitro* ischemic-like conditions, i.e., OGD (Gonzalez-Fernandez et al., 2014). Thus, data suggest that adenosine, via activation of A_3 Rs, triggers oligodendrocyte death (Figure 1) and contributes to white matter ischemic damage.

Role of A_{2B} Rs in OPCs and Oligodendroglogenesis

Current research in the field of adenosine is ongoing thanks to the growing interest on this receptor subtype. The pharmacological and physiological characterization of A_{2B} Rs has long been precluded by the lack of suitable ligands (Popoli and Pepponi, 2012).

Very few data are available to date about the role of A_{2B} Rs in oligodendroglial cells, as this adenosine receptor subtype is somewhat the most enigmatic and less studied among the four P1 receptors. We recently found that A_{2B} Rs are crucially involved in OPC maturation by demonstrating that the selective A_{2B} R agonist BAY60-6583, and its recently synthesized analog P453 (Betti et al., 2018), inhibited *in vitro* OPC differentiation, as demonstrated by the reduced expression of myelin-related proteins such as MBP or MAG in primary purified OPC cultures (Coppi et al., 2020a). We also demonstrated that A_{2B} R activation reversibly inhibits TEA-sensitive, sustained I_K , and 4-aminopyridine- (4-AP) sensitive, transient I_A , conductances (Coppi et al., 2020a). As mentioned above, I_K are necessary to OPC maturation (Gallo et al., 1996), so this could be one of the mechanisms by which A_{2B} Rs inhibit myelin production. The AC activator forskolin mimicked BAY60-6583-mediated effect as it decreased I_K currents, in line with previous data (Soliven et al., 1988). Of note, a further application of BAY60-6583 in the presence of forskolin was devoid of effect, thus indicating that A_{2B} R activation inhibits I_K by increasing intracellular cAMP levels (Coppi et al., 2020a).

Data about an inhibitory role of A_{2B} R in myelin formation are consistent with recent findings from Manalo et al. (2020) who demonstrated that elevated cochlear adenosine levels in ADA $^{-/-}$ mice is associated with sensorineural hearing loss (SNHL) due to cochlear nerve fiber demyelination and mild hair cell loss. Intriguingly, A_{2B} R-specific antagonists administered in ADA $^{-/-}$ mice significantly restored auditory capacity, nerve fiber density and myelin compaction. The same authors also provided genetic

evidence for A_{2B} R upregulation not only in ADA $^{-/-}$ hear-impaired mice but also in age-related SNHL.

Adenosine and Multiple Sclerosis

Multiple sclerosis is a chronic demyelinating disease of the CNS that leads to progressive neurological disability (Courtney et al., 2009). Immune system and inflammatory niche react against the myelin sheath that covers axon fibers altering neuronal transmission leading to the onset of a permanent condition associated with nerve decline. Since the extent and severity of nerve damage is heterogeneous, MS clinical symptoms may differ upon patients depending on the type and amount of the affected nerves. The treatments available for MS are almost limited aiming at reducing relapsing frequency or increasing the remitting speed of the disease with the major aim to manage symptoms.

Adenosine receptors are involved in inflammation and oligodendroglogenesis, as reported above, and may represent potential therapeutic targets in the treatment of MS (Johnston et al., 2001; Stevens et al., 2002; Coppi et al., 2015; Cherchi et al., 2021). Compounds used in the treatment of MS, such as methotrexate and cladribine, have been shown to act as ligands at adenosine receptors, to exert their anti-inflammatory activities (Montesinos et al., 2000). Therefore, the beneficial actions of these drugs may also involve their ability to activate adenosine A_2 R and A_3 R in a compensatory manner to regulate cytokine expression (Johnston et al., 2001).

Experimental autoimmune encephalomyelitis (EAE) is the most frequently used animal model to study the immunopathogenesis of MS and to test the therapeutic efficacy of novel agents for preclinical study (Constantinescu et al., 2011). EAE can be induced by inoculating the animal with whole myelin or defined myelin protein, as myelin oligodendrocyte glycoprotein (MOG), with adjuvants, which lead to activation of autoreactive peripheral CD4 T-cells and their subsequent trafficking to the CNS by crossing the blood brain barrier (BBB).

The A_1 R is expressed on cells of the monocyte/macrophage lineage and a downregulation of A_1 R was found in both blood and brain from MS patients (Mayne et al., 1999; Johnston et al., 2001). Accordingly, activation of A_1 R is reported to protect from EAE damage (Chen et al., 2010; Wang et al., 2014; Liu G. P. et al., 2018) and A_1 R knockout ($A_1R^{-/-}$) exacerbates MOG-induced EAE pathology by increasing demyelination, axonal injury and neuroinflammation (Tsutsui et al., 2004). Finally, A_1 R activation improves myelin repair by recruiting OPCs in an experimental model of rat optic nerve demyelination (Asghari et al., 2013).

The role of A_{2A} Rs in MS is controversial. This receptor subtype is upregulated in human lymphocytes (Vincenzi et al., 2013) and in the CNS (Rissanen et al., 2013) of MS patients, thus suggesting an involvement in demyelinating pathologies. However, A_{2A} R overexpression does not correlate with different forms of the disease nor is affected by MS pharmacological treatments (Vincenzi et al., 2013). Surprisingly, $A_{2A}R^{-/-}$ mice developed more severe EAE than wild type animals (Mills et al., 2012; Yao et al., 2012) and the adoptive transfer of peripheral blood cells lacking the A_{2A} R into wild type animals induced more severe EAE than both wild type and

$A_{2A}R^{-/-}$ mice (Mills et al., 2012). This suggests that $A_{2A}R^{-/-}$ mice are susceptible to a severe acute form of EAE due to the lack of $A_{2A}R$ -mediated anti-inflammatory effects. Indeed, in human lymphocytes, $A_{2A}R$ agonist inhibits the release of proinflammatory cytokines, cell proliferation, the expression of the adhesion molecule VLA-4, and the activation of the transcription factor NF- κ B; these effects were more evident in lymphocytes from MS patients in comparison to healthy subjects, in line with upregulation of $A_{2A}R$ s in MS lymphocytes (Vincenzi et al., 2013). Consistent with this, the activation of $A_{2A}R$ signaling by selective agonist inhibits the EAE progression by suppressing the specific lymphocyte proliferation, reducing the infiltration of CD4 $^{+}$ T lymphocytes, increasing intracellular Ca $^{2+}$ levels (Liu et al., 2016), and reducing the effects of Th1 stimulation on the BBB permeability (Liu Y. et al., 2018). Additional evidence demonstrates that $A_{2A}R$ agonism in EAE leads to prevention of the disease when used in early disease stage (Ingwersen et al., 2016) whereas in late-stage EAE the number of foci with marked amount of myelin debris was higher in $A_{2A}R^{-/-}$ mice than wild type (Ingwersen et al., 2016). These findings led to hypothesise that the relevance of $A_{2A}R$ for the pathogenesis of chronic autoimmune neuroinflammation may depend on the time point or the compartment, i.e., the systemic immune response vs. the CNS, an issue that has been elegantly addressed by Rajasundaram in a recent review (Rajasundaram, 2018).

Little is known about the role of $A_{2B}R$ in MS or EAE. Similarly to $A_{2A}R$ s, $A_{2B}R$ expression increases in peripheral lymphoid tissues of EAE mice. However, up to now, evidences converge on indicating that $A_{2B}R$ blockade is protective in EAE models. Indeed, Wei and colleagues demonstrated that $A_{2B}R$ -specific antagonists or genetic ablation of the receptor attenuated the clinical signs of EAE and protect the CNS from immune damage, probably by eliminating adenosine-mediated IL-6 production (Wei et al., 2013). Furthermore, the $A_{2B}R$ agonist BAY60-6583 reversed mesenchymal stem cells-induced downregulation of AQP4 expression in cultured astrocytes, that is protective for maintaining the integrity of BBB in EAE (Liu et al., 2020).

Sphingosine 1-Phosphate and Its Metabolism

Sphingosine 1-phosphate (S1P) is a natural sphingolipid present in plasma and tissues (Ksiazek et al., 2015) that acts as a modulator of different physiological and pathological processes, such as angiogenesis, vascular stability and permeability, T- and B-cell trafficking, as well as tumorigenesis (Hla et al., 2008). Circulating S1P is mainly produced and released by erythrocytes together with platelets and endothelial cells; it is mainly transported in association with apolipoprotein M (apoM) in high-density lipoprotein (HDL), while a small fraction is transported bound to albumin. ApoM can be considered a S1P chaperone protein (Obinata et al., 2019), which carries the sphingolipid in blood and interstitial fluids, facilitates S1P signaling in tissues and is responsible for physio-pathological effects different from that elicited by albumin-bound S1P. ApoM-bound S1P is able to exert multiple effects such as reduction of vascular inflammation, improvement of endothelial barrier

function, inhibition of oxidized low-density lipoprotein- (ox-LDL-) induced inflammation, modification of BBB permeability and protection from bacteria-induced inflammatory responses (Christoffersen et al., 2011; Galvani et al., 2015; Christensen et al., 2016; Zheng et al., 2019). Endothelial cells embracing the BBB are able to secrete S1P into the blood as well as into brain compartments (Hajny and Christoffersen, 2017). S1P concentration is much higher in blood and lymph at μ M range than in interstitial tissues due to the activity of S1P degrading enzymes. This concentration gradient, termed the “vascular S1P gradient” causes lymphocyte and hematic cell trafficking from lymph organs or bone marrow to the circulation (Schwab et al., 2005; Pappu et al., 2007; Hla et al., 2008; Cyster and Schwab, 2012). Enhanced vascular permeability, which occurs especially during inflammation, induces a burst of S1P that becomes available in the extravascular milieu, suggesting that the vascular S1P gradient may contribute to physiological and pathological conditions.

The catabolic pathway of plasma membrane-derived complex sphingolipids, mainly sphingomyelin, gives rise to different bioactive molecules, among others S1P. The first event is the sphingomyelinase-dependent hydrolysis of sphingomyelin, that leads to the production of the pro-apoptotic ceramide (Proia and Hla, 2015). Ceramidase deacylates ceramide to sphingosine, which in turn is converted into S1P by ATP-dependent phosphorylation catalyzed by two isoforms of the enzyme sphingosine kinase (SphK), namely SphK1 and 2 (Figure 2). These isozymes have been cloned and characterized, and their encoding genes are localized in different chromosomes, 17 and 19 respectively, known to produce multiple splicing variants (Imamura et al., 2001; Alemany et al., 2007). SphK1 and SphK2 differ for subcellular localization, showing partial overlapping but sometimes different biological functions (Liu et al., 2002; Pyne et al., 2016). In this regard, although knockout mouse models for either SphK1 or SphK2 have no gross phenotypic alterations, the double null mutation is lethal to embryos due to alterations in vasculogenesis and hemorrhages (Allende et al., 2004; Mizugishi et al., 2005; Michaud et al., 2006). SphK1 and SphK2 have structural homologies and share five conserved catalytic domains including an ATP-binding motif related to that of the diacylglycerol kinase family (Pitson et al., 2000; Liu et al., 2002). However, the sequence of SphK2 shows additional regions at the N-terminal and central proline-rich sequences which are absent in SphK1 (Igarashi et al., 2003; Gao et al., 2012). A nuclear localization sequence (NLS) and a nuclear exporting sequence (NES) are responsible for SphK2 major localization in the nucleus, since overexpression of SphK2 or SphK1 fused with NLS, but not wild-type SphK1, causes an inhibition of DNA synthesis (Igarashi et al., 2003). Indeed, it has been demonstrated that SphK2 has a role in epigenetic regulation of gene expression being involved in the suppression of histone deacetylase (HDAC) activity (Hait et al., 2009). Moreover, SphK2 is present in five different splicing variants that have been reported to localize into other different intracellular compartments such as the cytosol, mitochondria and the endoplasmic reticulum (ER) (Maceyka et al., 2005; Okada et al., 2005; Strub et al., 2011).

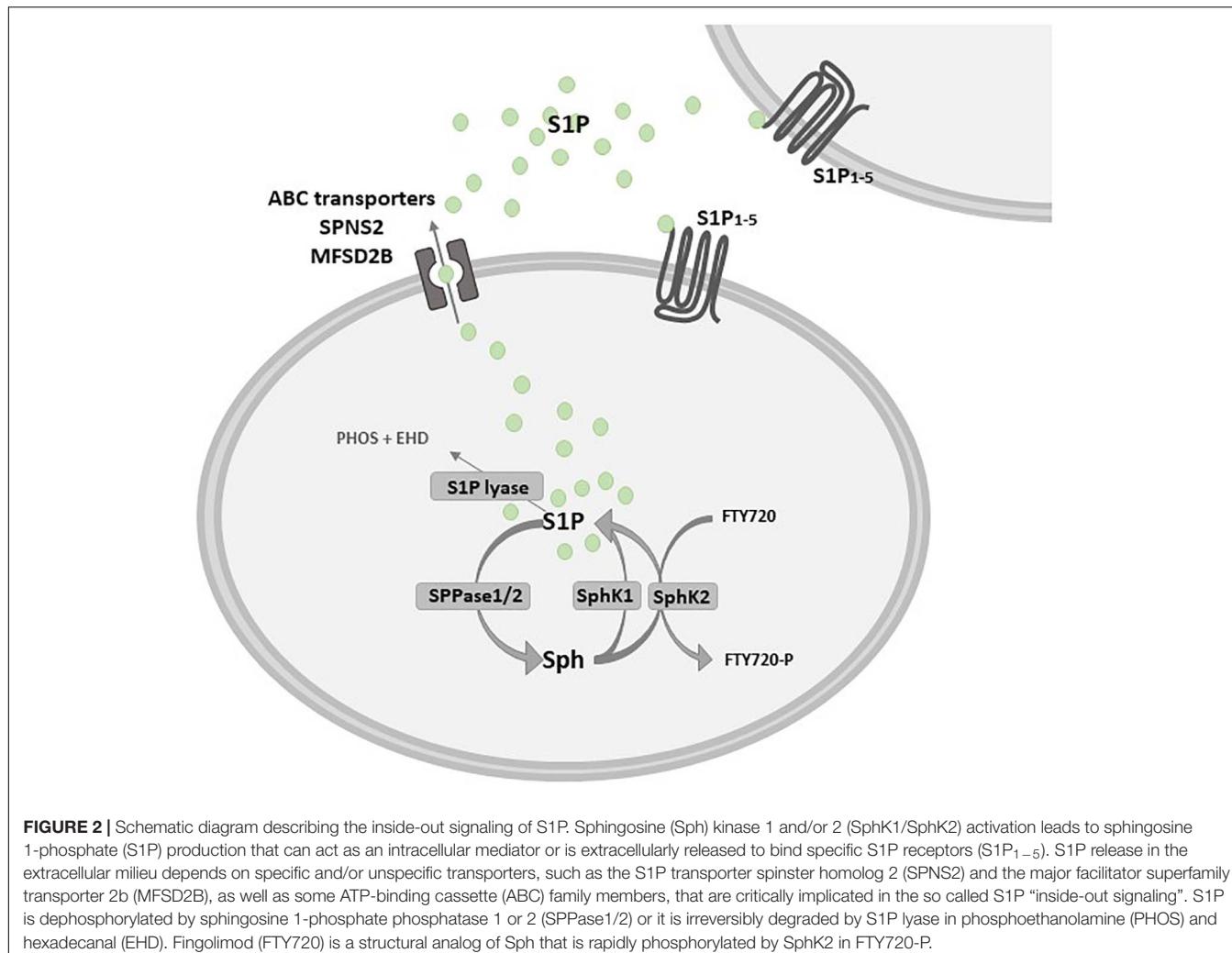


FIGURE 2 | Schematic diagram describing the inside-out signaling of S1P. Sphingosine (Sph) kinase 1 and/or 2 (SphK1/SphK2) activation leads to sphingosine 1-phosphate (S1P) production that can act as an intracellular mediator or is extracellularly released to bind specific S1P receptors (S1P₁₋₅). S1P release in the extracellular milieu depends on specific and/or unspecific transporters, such as the S1P transporter spinster homolog 2 (SPNS2) and the major facilitator superfamily transporter 2b (MFSD2B), as well as some ATP-binding cassette (ABC) family members, that are critically implicated in the so called S1P “inside-out signaling”. S1P is dephosphorylated by sphingosine 1-phosphate phosphatase 1 or 2 (SPPase1/2) or it is irreversibly degraded by S1P lyase in phosphoethanolamine (PHOS) and hexadecanal (EHD). Fingolimod (FTY720) is a structural analog of Sph that is rapidly phosphorylated by SphK2 in FTY720-P.

Intriguingly, SphK2 displays paradoxical effect on cell survival. Indeed, SphK2 shows a BH3 putative pro-apoptotic domain, although apoptosis is induced via SphK2-Bcl-xL-interaction after overexpression of the kinase (Liu et al., 2003). Moreover, knockdown of SphK2 also affects apoptosis induced by transforming growth factor beta (TGF β) in C2C12 myoblasts (Cencetti et al., 2013). Alternately, SphK2 is significantly elevated in a broad range of human cancers, including bladder, melanoma, breast, neuroblastoma and leukemia (Neubauer et al., 2016; Bruno et al., 2020); in agreement to SphK2 expression in cancer, SphK2 down-regulation has been demonstrated to decrease the proliferation of cancer cells (Van Brocklyn et al., 2005; Hait et al., 2005), and SphK2-deficient xenografts show a significantly delayed growth (Weigert et al., 2009), pointing at a crucial role of the kinase in carcinogenesis.

Concerning SphK1, its up-regulation is tumorigenic whereas down-regulation results in anti-cancer effects (Sarkar et al., 2005; Taha et al., 2005). The localization of SphK1 is mainly in the cytosol, but upon activation by several stimuli the kinase is recruited at the plasma membrane (Johnson et al., 2002). Moreover, although both SphKs can phosphorylate

the sphingosine analog, immunomodulatory drug FTY720 (Fingolimod), SphK2 appears to be more efficient in this activity than SphK1 (Billich et al., 2003; Paugh et al., 2003). Indeed, in mice lacking SphK2, but not SphK1, lymphopenia and lymphocyte retention into lymphoid organs induced by FTY720 are completely lost (Allende et al., 2004; Kharel et al., 2005).

The levels of S1P are tightly regulated by modulation of both anabolic and catabolic pathways. Breakdown of S1P to sphingosine is reversibly triggered by S1P phosphatases (SPP1 and 2), as well as unspecific lipid phosphate phosphatase (LPP), otherwise S1P is irreversibly degraded to hexadecenal and phosphoethanolamine by S1P lyase (Hannun and Obeid, 2008; **Figure 2**). *De novo* sphingolipids pathway alternatively accounts for the production of ceramide, which is the main hub of the sphingolipid metabolism (Hannun and Obeid, 2008): this anabolic pathway is initiated by serine palmitoyl transferase that catalyzes the condensation of serine with palmitoyl-CoA to produce 3-keto-dihydrosphingosine, which is consequently converted into dihydrosphingosine (Hanada, 2003). Ceramide synthases catalyze the acylation of dihydrosphingosine in dihydroceramide and finally dihydroceramide desaturase is

involved in the final step of the *de novo* synthesis by producing ceramide; besides representing a substrate for sphingomyelin synthase giving rise to sphingomyelin and for other complex sphingolipid biosynthetic pathways, also *de novo* synthesized ceramide can fuel the activity of ceramidases and then S1P production. Ceramide is then transported to the Golgi complex, where it serves as substrate for production of complex sphingolipids (Gault et al., 2010).

Sphingosine 1-Phosphate Signaling

Extracellular stimuli including growth factors and cytokines such as epidermal growth factor (EGF), PDGF, vascular endothelial growth factor (VEGF), insulin-like growth factor- 1 (IGF-1), TNF α and TGF β can induce the activation of SphK1 that translocates from cytosol to the plasma membrane to produce S1P (Donati et al., 2007; Bernacchioni et al., 2012; Bernacchioni et al., 2021). The bioactive sphingolipid can act in an autocrine or paracrine manner after its release in the extracellular milieu (called “inside-out signaling”), depending on specific and/or unspecific transporters, such as the S1P transporter spinster homolog 2 (SPNS2) (Hisano et al., 2012; Spiegel et al., 2019), the major facilitator superfamily transporter 2b (MFSD2B) (Kobayashi et al., 2018) and some, unspecific, ATP-binding cassette (ABC) transporters (Mitra et al., 2006; Figure 2).

The bioactive S1P selectively binds to high affinity cell surface G protein-coupled receptors (GPCRs; S1PRs), named S1P₁, S1P₂, S1P₃, S1P₄, and S1P₅ (Ishii et al., 2004) that have been involved in the majority of physiological and pathological actions evoked by S1P such as immune response, cardiovascular functions, cancer, atherosclerosis (Cartier and Hla, 2019) as well as MS (Strub et al., 2010; Maceyka et al., 2012). While S1P₁, S1P₂ and S1P₃ show broad tissue expression, S1P₄ displays quite selective localization in immune system and S1P₅ is primarily expressed in the spleen, on natural killer cells and other lymphocytes, and in CNS, mainly in oligodendrocytes (Mutoh et al., 2012). S1PRs exhibit a distinct capacity to couple to different G proteins thus activating different patterns of intracellular signaling cascades (Figure 3). S1P₁ exclusively activates members of the G_i family, whereas S1P₂ and S1P₃ have a broader coupling profile and not only activate G_i but also G_q and G_{12/13}. Moreover, activation of Rho, Rac and other small GTPases induces downstream signaling pathways including MAPK, phosphoinositide 3-kinase/Akt, Rho-associated protein kinase (ROCK). Further downstream effectors of S1PRs include AC, PLC, PKC and intracellular Ca²⁺ transients (Windh et al., 1999; Maceyka et al., 2012; Figure 3). In addition, several lines of evidence show that S1P can also act as an intracellular messenger (Kohno et al., 2006; Olivera and Spiegel, 2001; Strub et al., 2010; Maceyka et al., 2012) regulating fundamental biological processes, such as gene expression, mitochondrial functions and inflammation by interacting with intracellular targets, including HDACs, E3 ubiquitin ligases and prohibitin 2. Indeed, it has been shown that SphK2 is associated with histone H3, and regulates histone tail acetylation via S1P-mediated inhibition of HDAC, thus inducing chromatin remodeling and gene transcription (Hait et al., 2009). Interestingly, TNF α -induced activation of NF- κ B requires

SphK1 activity and S1P production. In particular, pro-survival TNF signaling requires TRADD-mediated recruitment of TNF receptor-associated factor 2 (TRAF2), which is the prototypical member of E3 ubiquitin ligase (Alvarez et al., 2010). Recruitment of TRAF2 to the TNF α -induced signaling complex results in the polyubiquitination of receptor interacting protein 1 (RIP1) preventing procaspase 8 cleavage that leads to apoptosis. Instead, polyubiquitinated RIP1 is capable of binding and activating I κ B kinase (IKK), that phosphorylates the inhibitor of NF- κ B (I κ B), thus releasing the NF- κ B dimer that translocates to the nucleus to exert transcriptional control. The RING domain of E3 ubiquitin ligase TRAF2 binds and activates SphK1, responsible for increased S1P levels essential for NF- κ B-dependent p21/cip1 and c-fos gene expression that counteract caspase-8 activation and apoptosis induction. This peculiar molecular mechanism that accounts for S1P pro-survival effects relies exclusively on intracellular effects, since agonists of S1PR (such as dihydro S1P) unable to interact with TRAF2 do not possess anti-apoptotic effects. Moreover, S1P is able to specifically bind to prohibitin 2 (PHB2), regulating mitochondrial respiratory chain assembly. PHB2 is a highly conserved, ubiquitously expressed protein that forms large complexes in the inner mitochondrial membrane composed of heterodimers of PHB2/PHB1. SphK2 located in the mitochondria produces S1P that interacts with PHB2 and affects the assembly of complex IV (COX) respiratory chain activity and oxygen consumption (Strub et al., 2010). Finally, it was reported that both pharmacological inhibition/knockdown of SphK2, and overexpression of S1P lyase/SPP1 in neuroblastoma cells abrogate BACE1-mediated A β production (Takasugi et al., 2011) whereas exogenous S1P failed to increase amyloid-beta (A β) production. On the other hand, SphK2 overexpression increased the amount of secreted A β by producing S1P that interacts with BACE1, suggesting that SphK2-generated S1P regulates A β production via BACE1 activation.

Nevertheless, it has been extensively demonstrated that S1PRs have a major role in S1P-dependent biological effects. Importantly depending on the expressed profile of S1PRs, S1P appears capable of differently affecting key cellular events such as proliferation, survival, motility and differentiation in different cell types (Chun et al., 2000; Spiegel and Milstien, 2003; Blaho and Hla, 2011; Soliven et al., 2011). Indeed, S1PR have a wide variety of biological effects in multiple organs and tissues, such as immune, cardiovascular and respiratory systems, as well as in the CNS. Experimental evidence has further highlighted S1P receptors as a potential targets for the regulation of vascular permeability and neuroprotection in different conditions such as pain, stroke and demyelinating diseases (Fyrst and Saba, 2010).

Multiple Sclerosis and Fingolimod

Myriocin-derivative FTY720 (fingolimod), the active substance of Gilenya®, has been authorized by Food and Drug Administration (FDA) and European Medicines Agency (EMA) as the first oral treatment for relapsing-remitting MS, based on extensive clinical trials (Kappos et al., 2010; Cohen et al., 2010). FTY720 is a sphingosine analog prodrug phosphorylated by SphK2 isozyme to produce the active form FTY720-phosphate (FTY720-P) (Paugh et al., 2003; Billich et al.,

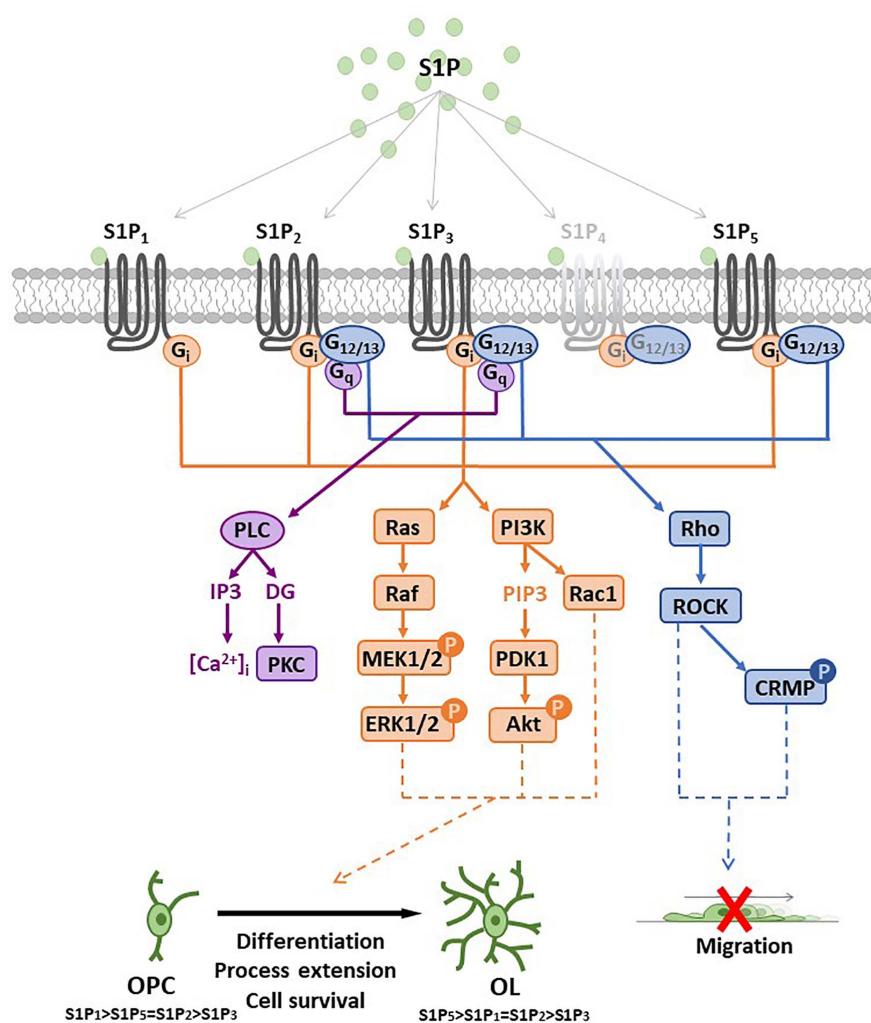


FIGURE 3 | Sphingosine 1-phosphate receptor (S1PR) expression and main signal transduction pathways activated in oligodendrocyte progenitor cells (OPCs) and oligodendrocytes (OLs). Schematic representation of sphingosine 1-phosphate (S1P) G-protein coupled receptors (S1P_{1–5}) and main signaling pathways involved after receptor activation. All S1P_{1–5} are coupled to G_i protein that activates Ras/PI3K pathways (in orange) promoting oligodendrocyte differentiation, process extension or cell survival. S1P_{2/5} are also coupled to G_{12/13} protein that activates Rho pathway (in blue) and reduces OPC migration or induces process retraction. S1P_{2/3} are coupled also with G_q protein that activates PLC leading to intracellular calcium increase and PKC activation. Abbreviation: mitogen-activated protein kinase (MEK); extracellular signal-regulated kinases (ERK); phosphatidylinositol 3-kinase (PI3K); phosphatidylinositol (3,4,5)-trisphosphate (PIP3); 3-phosphoinositide dependent protein kinase-1 (PDK1); protein kinase B (Akt); phospholipase C (PLC); protein kinase C (PKC); Rho-associated protein kinase 1 (ROCK); collapsin response mediator protein (CRMP); inositol triphosphate (IP3); diacylglycerol (DG).

2003; Zemann et al., 2006; Figure 2), that in turn activates four out of five S1PR receptor subtypes, except S1P₂, in the range of sub-nanomolar concentrations (Brinkmann and Lynch, 2002; Mandala et al., 2002). As initially reported, FTY720 was found to act as immunomodulator capable of depleting mature T cells in allograft models (Chiba et al., 2005). Although early studies addressed FTY720 as a low-efficacy suppressor of transplantation rejection (Budde et al., 2006), its successful employment in EAE supported the therapeutic action for MS treatment (Brinkmann and Lynch, 2002; Fujino et al., 2003; Webb et al., 2004; Kataoka et al., 2005; Papadopoulos et al., 2010).

The main recognized mechanism by which FTY720 improves MS disease is by affecting immune responses, specifically

regulating lymphocyte trafficking. Circulating T lymphocytes express S1P₁ and lower levels of S1P_{3/4} (Graeler and Goetzl, 2002) receptors, and the interaction of exogenous S1P with S1P₁ is capable of initiating lymphocyte egress from lymph nodes by overcoming retention signals (Brinkmann et al., 2010). Although acute administration of FTY720-P activates S1P₁ (Camm et al., 2014), chronic exposure to the S1P analogue leads to irreversible receptor internalization resulting in ‘functional antagonism’ of S1P₁ signaling (Graeler and Goetzl, 2004; Brinkmann, 2009; Gergely et al., 2012). FTY720 has a selective mechanism of action, targeting specific subclasses of lymphocytes: FTY720 treatment negatively modulates S1P₁, thus causing a retention of circulating pathogenic lymphocytes (naive and central memory T

cells positive for the chemokine receptor 7, the CCR7) back into the lymph nodes (Matloubian et al., 2004), thereby preventing their infiltration into the CNS, where they exert pathological effects (Cohen and Chun, 2011). However, FTY720 does not significantly affect activation or proliferation of redistributed naïve and central memory T cells and does not block the egress of CCR7-negative effector memory T cells from lymph nodes, preserving immunosurveillance (Mehling et al., 2008).

Intriguingly, differently from FTY720, apoM-S1P is unessential for lymphocyte trafficking although limits lymphopoiesis by activating the S1P₁ receptor on lymphocyte progenitors. Indeed, the effect exerted by apoM-bound S1P has been investigated in EAE mice lacking apoM, who developed more severe disease with increased lymphocytes in the CNS and breakdown of the BBB. Moreover, apoM-bound S1P, but not albumin-, inhibits lymphopoiesis *in vitro* and overexpression of apoM in rodents decreases endothelial inflammation and EAE manifestation exerting a protective function against autoimmune inflammation (Blaho et al., 2015; Wang et al., 2019).

In addition to immunological actions, FTY720 can penetrate the BBB acting on different S1P receptors that are expressed in brain resident cells, such as astrocytes and oligodendrocytes, claiming the possibility that FTY720 may elicit direct actions on CNS. Indeed, after the treatment with the pro-drug FTY720, FTY720-P has been detected in the cerebrospinal fluid at sub-nanomolar levels (Foster et al., 2007), which are sufficient to modulate human CNS cell properties *in vitro* (Miron et al., 2008c). This approach is consistent with multiple actions of the lysosphingolipid in the CNS, in line with growing literature describing direct effects of S1P signaling on brain cells. Using S1P receptor specific agonists and antagonists, S1P₃ and S1P₅ were involved in FTY720-induced effects for the remyelination and astrogliosis, whereas S1P₁ and S1P₅ affected microgliosis (Miron et al., 2010). Therapeutic interventions that affect oligodendrocyte remyelination processes could be critical factors for long-term functional recovery in MS. In particular, in organotypic cerebellar slices (Miron et al., 2010), the S1P analog was found to enhance remyelination which occurs subsequently to a demyelinating insult, this event being mediated at least in part by OPC differentiating into myelinating OLs.

Although FTY720 treatment has no positive impact on myelin content under basal conditions, FTY720 administration increases both remyelination score subsequent to lysolecithin-induced damage, and the number of endogenous OPCs within the lesion by facilitating migration, recruitment and proliferation of these cells. Furthermore, it enhances OPC process extension and differentiation into mature OLs (Yazdi et al., 2015), as well as amplifies the number of axons with remyelinated myelin sheaths. Despite these findings, in cuprizone model of MS, FTY720 treatment do not enhance remyelination while decreasing severity of demyelination and promoting OPC proliferation (Kim et al., 2011; Alme et al., 2015). However, in this MS model, fingolimod induces higher number of mature OLs near demyelinated areas, indicating a potential effect on differentiation and/or migration without apparent effects on remyelination (Hu et al., 2011; Alme et al., 2015; Nystad et al., 2020). Nevertheless, another research group reported that

fingolimod treatment (0.3 mg/kg) causes remyelination in acute cuprizone-induced demyelination model (Slowik et al., 2015). Hypothesis on such inconsistencies can be made regarding the concentration of fingolimod as well as time-dependence that may cause different effects in cuprizone model. Fingolimod (1 mg/kg) treatment after 3 days from cuprizone administration induces survival of mature OLs whereas later administration of the drug (started at 10 days after cuprizone treatment) lacks cytoprotective effect (Kim et al., 2018), claiming the possibility that early intervention appears to be required to prevent demyelination. In cuprizone model of demyelination, FTY720 (0.3 mg/kg), co-administered with transplanted neural progenitor cells derived from induced pluripotent cells (iPS-NPC), increases OPC survival and proliferation. Moreover, the authors assess that differentiation of transplanted iPS-NPC into oligodendroglial lineage may occur (Yazdi et al., 2015).

Treatment with FTY720 at the onset of EAE reduces clinical symptoms and decreases demyelination by blocking the Akt/mTOR signaling pathway (Hou et al., 2016). In later stage of EAE disease, FTY720 treatment increases MBP level and promotes the appearance of newly generated myelinating OLs, via Sonic Hedgehog signaling pathway, decreases EAE clinical manifestation, and improves neurological functions (Zhang et al., 2015). Furthermore, FTY720 administration (1 mg/kg) during EAE induction in female mice reduces demyelinated area, axonal damage, brain atrophy while increasing brain-derived neurotrophic factor (BDNF) level and clinical scores (Fukumoto et al., 2014; Smith et al., 2018), underling the positive effect of FTY720 on regeneration in different types of demyelination models.

Finally, conditional knockout of S1P₁ in neural lineages have been used to identify a key role for astrocytes in reducing the severity of pathological changes in EAE. Indeed, the efficacy of fingolimod is lost by astrocytic deletion of S1P₁, highlighting that the main protective effect of this compound in EAE comprises the modulation of astrocyte function by S1P₁ (Choi et al., 2011).

Sphingosine 1-Phosphate Receptor Signaling in Oligodendrocytes and Interaction With A_{2B} Rs

OPC availability, recruitment and differentiation into mature oligodendrocytes are pivotal aspects involved in remyelination. Experimental evidence demonstrates that S1PRs display different biological effects depending on oligodendrocyte differentiation state. Oligodendroglial cells express four out of five S1PRs, S1P₁, S1P₂, S1P₃, and S1P₅, whose expression profiles change and are selectively controlled during oligodendrogenesis. Indeed, the predominant isoform in immature OPC is S1P₁ whereas S1P₂, S1P₃ and S1P₅ are present at much lower levels (Im et al., 2000; Yu N. C. et al., 2004; Jaillard et al., 2005; Novgorodov et al., 2007). During differentiation from OPC into mature OLs, S1P₅ and S1P₁ are reciprocally regulated, the first being augmented while the latter decreases (Jung et al., 2007; Coelho et al., 2007; Miron et al., 2008c; van Doorn et al., 2012). Indeed, human mature OLs express S1PR transcripts in relative abundance of S1P₅ > S1P₃ > S1P₁ (Miron et al., 2008a). In particular, the

modulation of S1PR expression seems to be mediated by PDGF that is produced by OPCs. Indeed, S1PRs are differentially modulated by PDGF resulting in downregulation of S1P₅ and upregulation of S1P₁ in OPCs. Downregulation of S1P₁ by RNA interference affects PDGF-induced proliferation of OPCs (Jung et al., 2007) whereas, in mature OLs, S1P signaling promotes cell survival, suggesting that S1PRs may exert different functions during oligodendroglial development (Okada et al., 2009). The effects of fingolimod on oligodendrocyte process dynamics depended on both dose and duration of treatment, since S1PR activation by fingolimod induces process retraction in O4-positive pre-OLs, that appears to be transient and restricted to immature cells, not observed at later and mature developmental stages (Miron et al., 2008b). Process retraction is mediated by S1P₅ via a ROCK/collapsin response-mediated signaling pathway, whereas the effect is abrogated in S1P₅^{-/-} mice derived oligodendrocytes. Prolonged treatment with higher doses of fingolimod induces process extension associated with Rac1-linked cytoskeletal signaling cascades mimicked by a S1P₁ agonist SEW2871 (Miron et al., 2008b). Similarly, these results can be corroborated by the fact that, depending on the dose, FTY720-P exerts opposite actions in rat OPC cultures (Jung et al., 2007). Indeed, high concentration of FTY720-P (1 μ M) inhibits, OPC differentiation, whereas low concentration (10 nM) enhances both the percentage of mature OLs and MBP expression.

Furthermore, S1P-induced survival of mature OLs is mediated through a pertussis toxin- (PTx) sensitive, Akt-mediated pathway, since the molecular mechanism activated by S1PRs in oligodendrocytes specifically involves the phosphorylation of ERK1/2 and Akt, that subsequently promotes cell survival (Jaillard et al., 2005; van Doom et al., 2012). Noteworthy, the pro-survival effect of S1PR in human mature OLs is mimicked by the administration of S1P₅ agonist. Interestingly, S1PR agonists affect oligodendrocytes differentiation stages depending on the molecular mechanisms evoked and in a concentration-dependent manner. In particular, human OPC cultures treated with low nanomolar concentrations of either FTY720-P or S1P present enhanced differentiation into both pre-OLs (O4-positive) and mature (GC-positive) OLs. Of note, ERK1/2 pathway is involved in the differentiation of OPC into O4 positive-cells, since inhibition of MAPK pathway by U0126 prevented pre-OLs generation. However, the transition to mature OLs is mediated by p38MAPK signaling, since PD169316 administration blocks the progression of O4 positive into mature stages of differentiation (Cui et al., 2014).

Notably, experimental evidences highlight that activation of S1P₅ induces process retraction whereas activation of S1P₁ enhances process extension of OPCs (Miron et al., 2008b), and coherently during early myelination S1P₁ abrogation in the oligodendroglial cells delays OPC differentiation into mature OLs (Kim et al., 2011). In agreement, S1P₁^{-/-} mice show decreased myelin protein expression, thinner myelin and susceptibility to demyelination induced by cuprizone, that is conceivable with the major expression of S1P₁ in OPCs that could play a pivotal role in early OPC differentiation stages. On the contrary, abnormalities in myelination are not evident in S1P₅^{-/-} mice; oligodendrocytes display a higher expression of S1P₅ compared

to other S1PRs where it may be involved in late myelination processes. Although S1P₅ is expressed at lower levels in OPCs compared to OLs and OPCs are devoid of pro-survival effects of S1P₅, this receptor subtype is activated by S1P to arrest OPC migration (Novgorodov et al., 2007). Indeed, S1P-induced decrease of chemotaxis is completely prevented by the specific downregulation of S1P₅, but not S1P₂, and is insensitive to PTx, suggesting that S1P₅-initiated signaling is not mediated by the G α_i -protein coupled pathway. The molecular mechanism that is responsible for the impairment of migratory capacity after S1P administration involves G_{12/13} that stimulates the Rho/ROCK signaling pathway (Novgorodov et al., 2007). The authors suggest that glutamate treatment of OPC may increase S1P export and S1P extracellular levels to modulate OPC motility and claim the possibility for S1P to be a part of the neuron-oligodendroglial communication network in developing brain, that could have a role also during remyelination processes. These data are also consistent with the physiological effect of S1P₅ in OPCs during differentiation postulating that changes in receptor coupling with heterotrimeric G-proteins may occur, thus leading to the activation of different signaling pathways.

Finally, SphK1 has a protective role against apoptosis in OPC cultures (Saini et al., 2005). In particular, Neurotrophin 3 (NT-3) accounts for SphK1 translocation to plasma membrane and activation to exert its pro-survival effect in these cells. Remarkably, down-regulation of SphK1 negatively affects the capacity of NT-3 to protect oligodendrocyte progenitors from apoptosis. In agreement with these findings, analysis of plaques from MS brains shows reduced levels of S1P and increased sphingosine and C16/18-ceramide (Qin et al., 2010).

In the light of above results, we recently performed a study aimed to merge current knowledge on S1P pathway with A_{2B} R signaling. Namely, by using either or both pharmacological approach and receptor silencing, we demonstrated that an interplay occurs between A_{2B} Rs and SphK/S1P axis in OPCs (Figure 4). Indeed, SphK1 enzyme is activated when OPCs are cultured in the presence of the A_{2B} R selective agonist BAY60-6583, thus rising S1P production, whereas its silencing by small interference RNA (siRNA) increases the expression of S1P lyase, promoting S1P removal (Coppi et al., 2020a). This observation led to hypothesize that the anti-differentiating effect exerted by A_{2B} R activation in OPCs is mediated by an increase in S1P intracellular levels (Figure 4). This hypothesis was confirmed by findings that the SphK inhibitors VPC96047 or VPC96091 markedly increased MAG and MBP expression in OPC cultures, indicating enhanced cell maturation, and also significantly increased I_K currents, necessary to OPC differentiation (Coppi et al., 2020a). Thus, it appears that an increase in S1P production possibly accounts for the anti-differentiating effect of BAY60-6583 in OPCs, whereas A_{2B} R silencing, by promoting S1P removal through the activation of S1P lyase, facilitates OPC maturation. An additional proof that A_{2B} Rs and S1P signaling are interconnected in OPCs resides in the fact that this receptor subtype is upregulated during cell differentiation, an effect that is completely prevented when cells are differentiated in the presence of the pan SphK inhibitor VPC96047 (Coppi et al., 2020a).

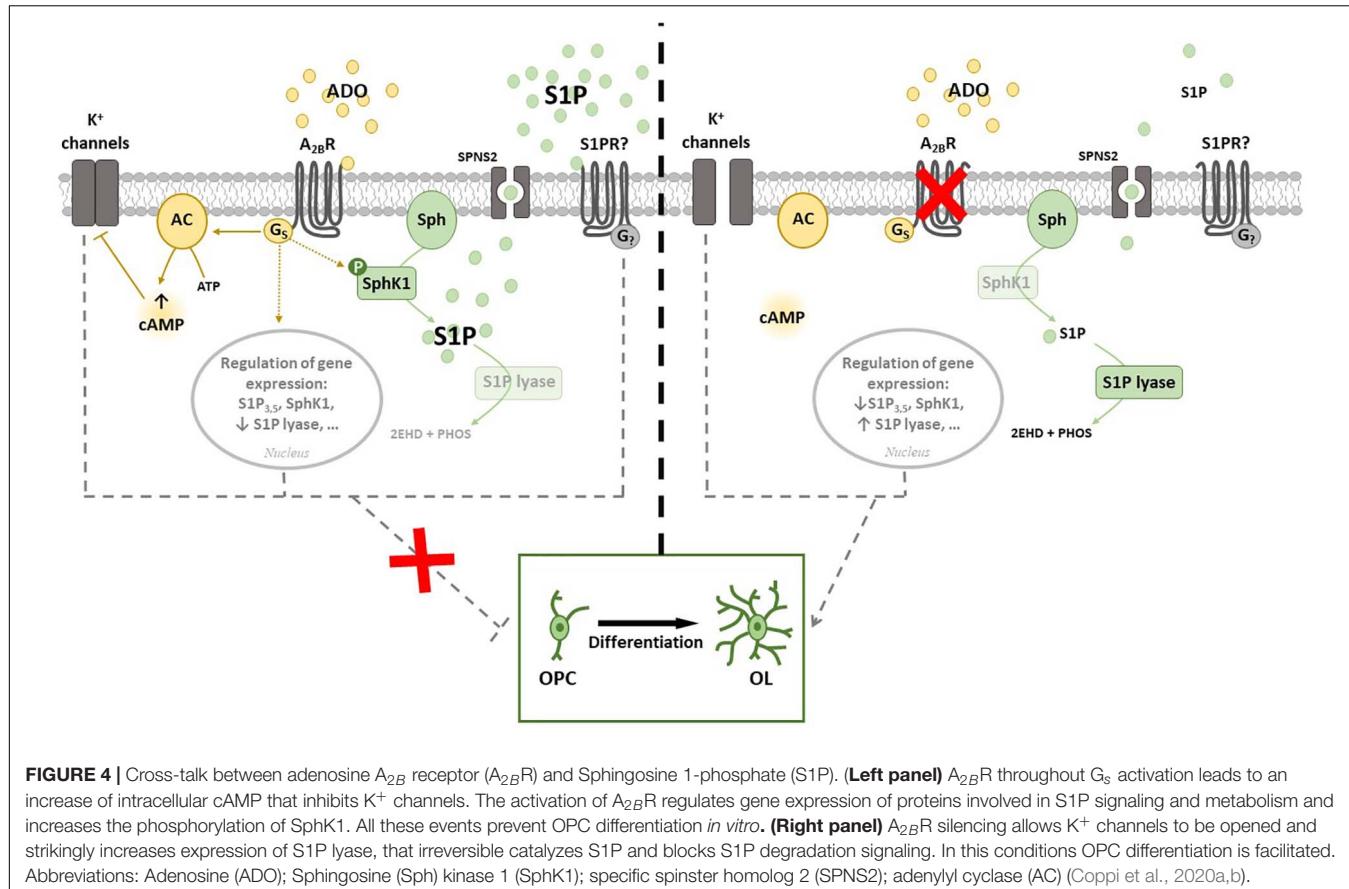


FIGURE 4 | Cross-talk between adenosine A_{2B} receptor ($A_{2B}R$) and Sphingosine 1-phosphate (S1P). **(Left panel)** $A_{2B}R$ throughout G_s activation leads to an increase of intracellular cAMP that inhibits K^+ channels. The activation of $A_{2B}R$ regulates gene expression of proteins involved in S1P signaling and metabolism and increases the phosphorylation of SphK1. All these events prevent OPC differentiation *in vitro*. **(Right panel)** $A_{2B}R$ silencing allows K^+ channels to be opened and strikingly increases expression of S1P lyase, that irreversible catalyzes S1P and blocks S1P degradation signaling. In this conditions OPC differentiation is facilitated. Abbreviations: Adenosine (ADO); Sphingosine (Sph) kinase 1 (SphK1); specific spinster homolog 2 (SPNS2); adenyl cyclase (AC) (Coppi et al., 2020a,b).

We also found that FTY720-P differently affects BAY60-6583-mediated K^+ current inhibition depending upon the concentration applied. When administered at 1 μM , it mimicked and partially occluded the effect of a subsequent BAY60-6583 application on voltage-dependent K^+ currents. This confirms, again, that S1P and $A_{2B}R$ pathways converge. On the other hand, the effect of BAY60-6583 on ramp currents was significantly enhanced in the presence of low (10 nM) FTY720-P (Coppi et al., 2020a). Similarly, 10 nM FTY720-P increased, whereas 1 μM decreased, MAG expression after 7 days of OPC maturation. Contrasting effects of this compound depending on the concentration used have been previously reported by others, the effects of fingolimod depending on both concentration and treatment duration (Jung et al., 2007; Miron et al., 2008c).

Differently from FTY720-P, when S1P is used as a ligand, the effect on K^+ currents was not observed (Coppi et al., 2020a). This apparent discrepancy may be ascribed to the fact that receptor ligation by FTY720-P is restricted to all S1P receptors except S1P₂, which however is activated by S1P. Moreover, the functional outcome induced by S1PR modulators could differ from one ligand to another since it could differently affect receptor fate. Indeed, it has been shown that FTY720-P can induce S1P₁ receptor degradation, whereas S1P affects receptor recycling. Finally the lack of effect exerted by exogenous S1P on I_K is in agreement with previous data in different cellular models, such as skeletal muscle cells, where agonist-induced S1P

intracellular production and activity, called inside-out signaling, has different, even sometimes opposite, actions compared to exogenous S1P (Donati et al., 2004; Cencetti et al., 2010; Cencetti et al., 2013). This effect can be explained by a localized release of bioactive lipid in membrane microdomain where the availability of certain receptor subtypes is limited. The spatial regulation of S1P biosynthesis within the cell, together with its localized partitioning into plasma membrane domains, determines the subset of engaged S1PRs and thus the biological outcome (Donati et al., 2013).

CONCLUSION

Multiple evidences indicate that adenosine may interact with S1P signaling in orchestrating the processes concurring to OPC maturation and thus to the remodeling of brain development and/or repair after a demyelinating insult.

In particular, adenosine $A_{2B}R$ appears to play a critical role in oligodendroglogenesis since its agonism activates SphK1 and reduces OPC differentiation. These data are in agreement with the fact that, on one hand, SphK inhibition decreases $A_{2B}R$ expression leading to an increase in OPC differentiation, and on the other, specific down-regulation of $A_{2B}R$ reduces SphK1 and potently induces S1P lyase expression thus pushing S1P toward catabolism, and thereby facilitating OPC

maturation. On the whole, the available results suggest that A_{2B}R antagonism represents a possible co-adjuvant strategy to improve remyelination promoted by the prodrug sphingosine analog, FTY720 (Fingolimod) for the treatment of MS, as confirmed by previous findings in the literature reporting a protective role of A_{2B}R block in EAE models (Wei et al., 2013; Liu et al., 2020).

AUTHOR CONTRIBUTIONS

EC, FP, PB, AP, and FCe: conceptualization. FP and PB validation. EC, AP, PB, FP, FCe, and CD: resources. EC, FCe, FCh, and

AP: writing—original draft preparation. MV, FCh, FCe, and CD writing—review and editing. All authors contributed to the article and approved the submitted version.

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

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