

**The sphingosine 1-phosphate signaling pathway in epilepsy: a possible role for the immunomodulator drug fingolimod in epilepsy treatment**

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## **Abstract**

It is currently known that erythrocytes are the major source of sphingosine 1-phosphate (S1P) in the body. S1P acts both extracellularly as a cellular mediator and intracellularly as an important second messenger molecule. Its effects are mediated by interaction with five specific types of G protein-coupled S1P receptor. Fingolimod, is a recognized modulator of S1P receptors, and is the first orally active disease-modifying therapy that has been approved for the treatment of multiple sclerosis. Magnetic resonance imaging data suggest that fingolimod may be effective in multiple sclerosis by preventing blood-brain barrier disruption and brain atrophy. Fingolimod might also possess S1P receptor-independent effects and exerts both anti-inflammatory and neuroprotective effects.

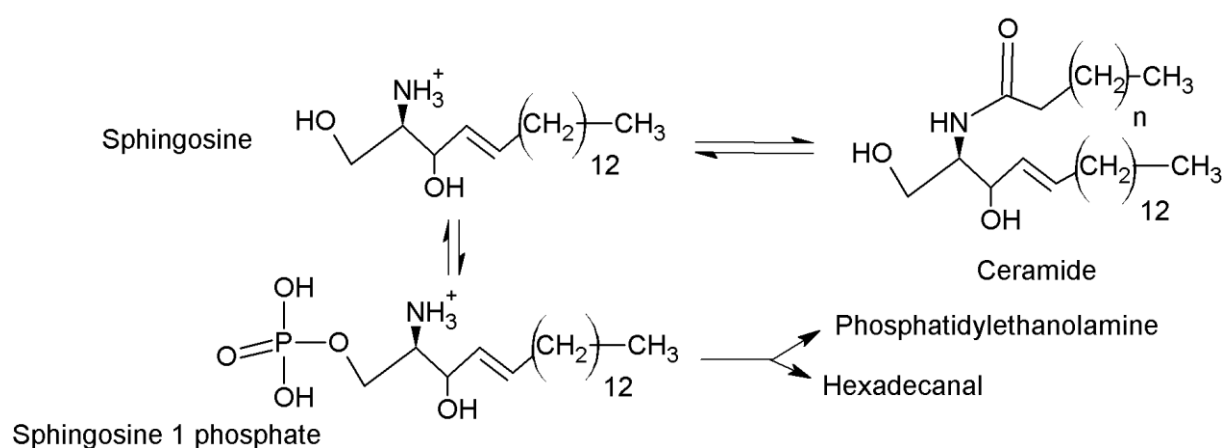
In the therapeutic management of epilepsy, there are a great number of antiepileptic drugs, but there is still a need for others that are more effective and safer. S1P and its receptors might represent a suitable novel target also in light of their involvement in neuroinflammation, a well-known process underlying seizures and epileptogenesis. The objective of this manuscript is to review the biological role of S1P and its receptors, focusing on their expression, effects and possible involvement in epilepsy; furthermore, we summarize the possible anti-seizure properties of fingolimod and discuss its possible usefulness in epilepsy treatment. We conclude that fingolimod, being already commercially available, might be easily tested for its possible therapeutic effectiveness in epileptic patients, both after a more comprehensive evaluation of the real potential of this drug and following a clear evaluation of the potential role of its main targets, including the S1P signaling pathway in epilepsy.

**Running title:** Sphingosine 1-phosphate pathway in epilepsy

**Keywords:** Sphingosine 1-phosphate (S1P) signaling; Fingolimod; Neuroinflammation; Neurodegeneration; Central nervous system diseases; Seizures; Epilepsy; Epileptogenesis.

## Introduction

The German physician Ludwig Wilhem Thudichum was the first to describe sphingolipids as an important family of lipid signaling molecules derived from membrane sphingomyelin, that are abundant in the brain [brik1]. There are both complex sphingolipids (gangliosides, cerebroside), and more simple sphingolipids *e.g.* ceramide, sphingosine and the derivative sphingosine-1-phosphate (S1P), that function as key lipid signaling molecules in response to many extracellular stimuli [2]. Among sphingolipid metabolites (Figure 1), S1P has a fundamental second messenger role in regulating several important cellular processes.

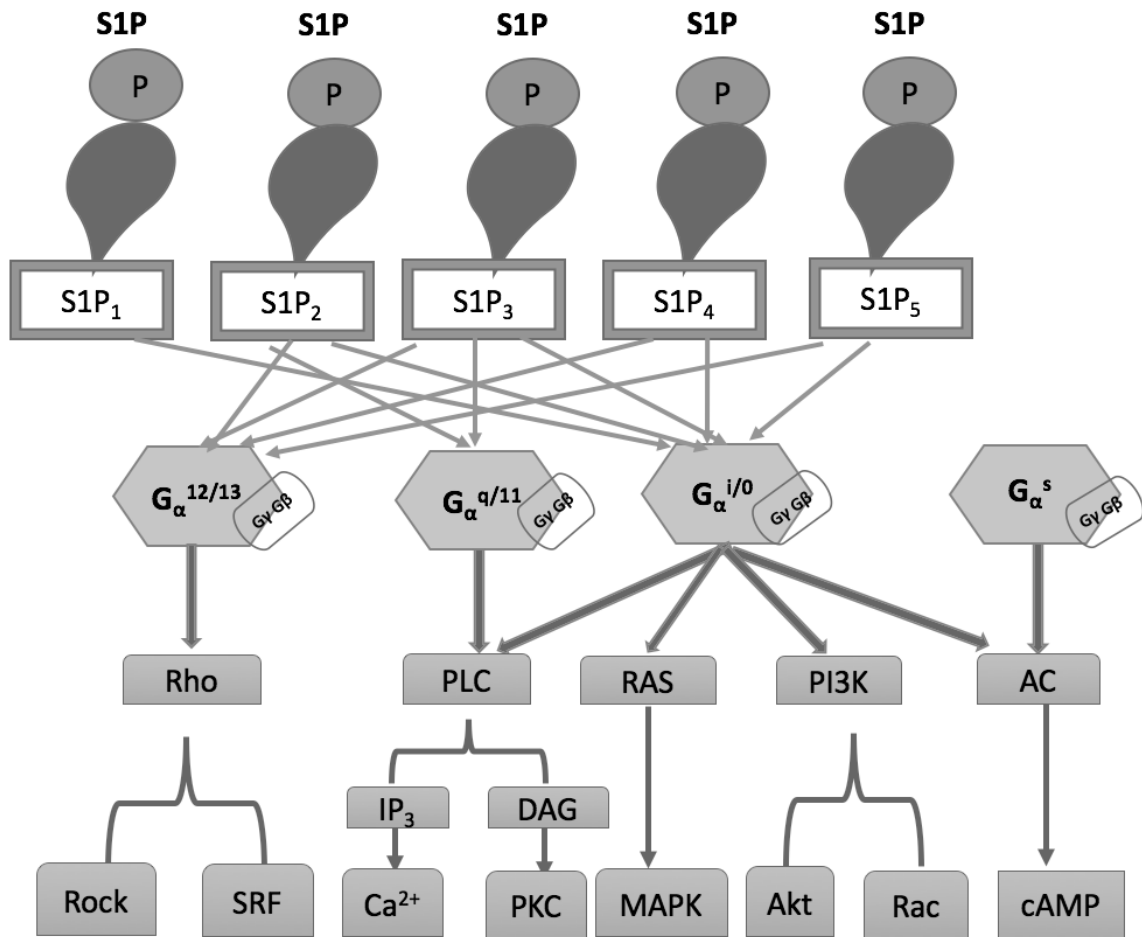


**Figure 1.** Chemical structures of sphingolipid metabolites.

Chemically, S1P is a zwitterionic lysophospholipid that is phosphorylated in the long-chain base of sphingosine [3]. S1P, a pleiotropic signaling molecule, can either act intracellularly as a messenger or extracellularly as a ligand for its membrane receptors [4]. In particular, S1P specifically interacts with high-affinity with five types of G protein-coupled receptor (GPCR) and can affect multiple signalling pathways (Figure 2) [3], whereas its intracellular signaling mechanisms have only recently been discovered and partly described [5]. In particular, fingolimod, a recognized modulator of S1P receptors, and the first orally-active disease-modifying drug that has been approved for the treatment of multiple sclerosis (MS), (see

below), could have S1P receptor-independent effects by acting on other intracellular targets of S1P such as histone deacetylases (HDACs) or by influencing both the metabolism and signaling of several other lipids [1, 6].

S1P is responsible for regulating important events in biology *e.g.* normal development of the arterial system, regulation of blood vessel caliber, control of heart rate, maintaining endothelial integrity, fetal implantation, recirculation of lymphocytes, wound healing and hair-cell survival [4]. The stimulation of numerous signaling pathways by S1P results in intracellular  $\text{Ca}^{2+}$  mobilization, polymerization of actin, chemotaxis/migration, and escape from apoptosis [5]. S1P, during embryogenesis, is important for normal cardiovascular and central nervous system (CNS) development, while in the adult, a S1P concentration gradient is present between plasma and interstitial fluids [7]. Furthermore, when there is cell activation by pro-inflammatory cytokines *e.g.* interleukin-1 beta ( $\text{IL-1}\beta$ ) and tumor necrosis factor-alpha ( $\text{TNF-}\alpha$ ) at inflammatory sites, the production of S1P is increased [8].



**Figure 2.** Signaling pathways linked to sphingosine 1-phosphate receptor (S1PR) activation. S1P interacts with five types of G protein-coupled receptor (S1P1-5), each coupled to several different G proteins to elicit different downstream events. Abbreviations: AC, adenylyl cyclase; PI3K, phosphatidylinositide 3-kinase; PLC, phospholipase C; cAMP, Cyclic adenosine monophosphate; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; Rock, Rho-associated protein kinase; SRF, Serum Response Factor; PKC, Protein kinase C; Mitogen-Activated Protein Kinase, MAPK; Akt, protein kinase B or PKB.

The therapeutic importance of S1P, revealed by modulation of S1P signaling, is highly significant in immune-mediated diseases, particularly, multiple sclerosis (MS). With regard to the cellular functions of S1P, it is not a surprise that the S1P signaling pathway has also been indicated as a potential therapeutic target for inflammation, cancer, Alzheimer's disease,

atherosclerosis and diabetes [5]. Moreover, in the last few years, the potential role of the S1P pathway in epilepsy has been underlined; in fact, in S1P2 receptor-deficient (S1P2<sup>-/-</sup>) mice, spontaneous, sporadic, and occasionally lethal seizures have been reported [9]. Further evidence on the possible involvement of the S1P pathway in epilepsy comes from a study by Gao et al. (2012) in which the S1P receptor modulator fingolimod (Gilenya<sup>TM</sup>, Novartis Pharma AG, Basel, Switzerland) was found to exert significant antiepileptogenic effects in the rat lithium-pilocarpine model of *status epilepticus* (SE) [10] and a subsequent study in the pentylenetetrazole (PTZ)-induced kindling mouse model [11].

One important remaining challenge in epilepsy therapy and research is to develop novel drugs that can influence disease development. Neuroinflammation is not only present in the chronically epileptic brain, but brain inflammation appears to be a characteristic feature of abnormal hyperexcitable brain tissue from which spontaneous, recurrent seizure activity can originate [12]. Since clinical trials have failed to demonstrate that conventional anti-seizure drugs have such disease-modifying activity, other promising therapeutic strategies are necessary [13]. Some drugs clinically used for other pathologies might have also potential indications for inhibiting inflammation, and might be investigated for antiepileptogenic activity as well as the modulators of S1P signaling such as fingolimod [14]. Here in, we review the biological role of the S1P signaling pathway, focusing our attention on its possible relevance in the pathophysiology of epilepsy and the probability of developing new drugs that target the S1P pathway as novel antiepileptic/antiepileptogenic agents. Moreover, we also review the preclinical results obtained to date for fingolimod in an epilepsy animal model in order to better clarify whether it could be a potential “*disease-modifying drug*” for this neurological disease.

## **Sphingosine 1-phosphate and its receptors**

### *S1P metabolism*

Sphingolipids, a class of lipids, are fundamental structural components of biological membranes. Sphingomyelin (SM), is a major membrane sphingolipid, and is the precursor of several important bioactive products; similarly, by enzymatic metabolism, several bioactive molecules that play crucial roles in the regulation of essential biological processes *e.g.* cell differentiation, cellular senescence, apoptosis and proliferation are physiologically obtained [15].

Enzymatic cleavage of SM by sphingomyelinases produces ceramide (Figure 1), formed by sphingosine linked to fatty acid, which can have different lengths by virtue of which several ceramides can be synthesized. Ceramides, also synthesized *de novo*, are degraded by ceramidases to sphingosine. Phosphorylation of sphingosine by sphingosine kinase enzymes, SphK1, SphK2, produces S1P (Figure 1), which is transported outside the cell by chaperone-bound S1P and is compartmentalized in the circulatory system. Several enzymes mediate the catabolism of S1P; S1P lyase is responsible for the breakdown of S1P, whereas two distinct specific S1P phosphatases give rise to sphingosine, which can be used to various aims, for instance, to synthesize ceramides [16].

Several cell stimuli and extracellular agents can regulate the enzymes involved in this lipid metabolism [16]. SphK1 and SphK2 that contribute to S1P formation are expressed ubiquitously and show a high sequence homology. However, SphK2 differs from SphK1 by an additional sequence at the NH<sub>2</sub> terminus and the middle of the sequence. Moreover, these kinases present also a subcellular localization; the differences would explain why SphK1 and SphK2 have different biological functions in some cell types as well as overlapping effects [17]. S1P can be produced by cells of the innate immune system, endothelial cells (EC), as well as neurons and astrocytes in the CNS. Recently, by using SphK 1/2 double deficient mice, it has been confirmed that erythrocytes rather than platelets are the major source of plasma S1P [18]. However, it has also been demonstrated that S1P is released from platelets during activation

and thrombotic processes. In agreement with these studies, S1P has been shown to be present at high concentrations in the circulatory system compared with other organs [8].

### *Sphingosine-1-Phosphate Receptors*

The main molecular effects of S1P are mediated both by binding with the five types of GPCRs expressed and localized on the membrane surface of many cell types, and also by its intracellular action as a second messenger (Figure 2). In fact, it has been proposed that S1P functions as an intracellular mediator in agreement with the increase in intracellular S1P levels upon cell stimulation by cytokines, growth factors and hormones, which activate sphingosine kinase activity [5, 19]. With regard to the intracellular action of S1P, some targets have only recently been identified. These targets are involved in several CNS disorders including epilepsy. Therefore, the actions of S1P are much more intricate than previously considered [5]. In 1992, Sadahira et al. [20] proposed that S1P could control cell motility via putative extracellular receptors. Particularly, S1P was thought to bind with high affinity to five related GPCRs, previously described as Endothelial Differentiation Genes (EDGs) [21] and renamed by the IUPHAR to S1P<sub>1-5</sub> [19]. All S1P receptors couple with Gi, whereas S1P<sub>2-5</sub> also signal via G<sub>12/13</sub> and S1P<sub>2-3</sub> additionally through Gq8. The activation of these G-proteins regulates diverse downstream signaling pathways including phospholipases C and D, adenylate cyclase, phosphoinositide 3-kinase, c-Jun N-terminal kinase, ERK, p38 mitogen-activated protein kinase, Rac, Rho and non-receptor protein-tyrosine kinases and tyrosine phosphatases [4]. S1P<sub>2-4-5</sub> gene receptors were found to be located on chromosome 19, with S1P<sub>2</sub> and S1P<sub>5</sub> in proximity of 19p13.2. Conversely, S1P<sub>1</sub> was identified on 1p21, whereas S1P<sub>3</sub> is on 9q22.2 [3, 22].

S1P receptors are formed by an NH<sub>2</sub>-terminus, with potential N-linked glycosylation sites, seven hydrophobic transmembrane  $\alpha$ -helices (TM1-TM7) forming a polar internal tunnel, a C terminus, containing phosphorylation sites for serine/threonine protein kinases and by three hydrophilic extracellular and three intracellular loops. Since these receptors belong to the



superfamily of GPCRs, they show related structural elements with other receptors coupled with G-protein. In fact, about 20% of the amino acid sequence in S1P receptors is shared with cannabinoid receptors and ~30% with lysophosphatidic acid receptors.

In all the GPCRs that are activated by cationic ligands, the Asp residue located close to the end of TM3 is conserved and seems to be important for binding of charged amines. The C terminus is conserved among most of the GPCRs and functions fundamentally as a palmitoylation site. In addition, the intracellular loops and C terminus have various sites, which can be phosphorylated by serine/threonine protein kinases [23]. Parrill et al. [24] demonstrated that the basic amino acids Arg<sup>(120)</sup> and Arg<sup>(292)</sup> are important for binding with the S1P phosphate group, whereas the acidic Glu<sup>(121)</sup> residue binds the ammonium moiety of S1P. Expression of S1P receptors varies with cell-type; generally, S1P receptors are present in the immune system, vasculature and CNS [25]. However, there are no specific antibodies currently available that can differentiate between the different S1P receptor isoforms. S1P<sub>1</sub> gene expression is widely reported in the brain, heart, liver, lung, kidney, spleen, thymus, adipose tissues, and skeletal muscles of mice [26]. Furthermore, S1P<sub>1</sub> protein is present in brain, heart, lung, kidney, spleen, and vasculature in adult mice [26]. In humans, S1P<sub>1</sub> is the dominant receptor on lymphocytes/leukocytes [26].

In the last few years, it has been observed that different pathophysiologically important processes are regulated by S1P<sub>1</sub> receptors. Knockout studies in S1P<sub>1</sub> receptor-deficient mice indicate embryonic lethality, with defective vasculogenesis and neural tube formation, supporting an important role of S1P<sub>1</sub> receptors in developmental processes, but not for mice deficient in S1P<sub>1-4</sub> receptors [1]. Preclinical studies have shown that S1P<sub>2</sub> is expressed abundantly in the heart and lung and less so in the brain of adult mice and rats [27]; nevertheless, it is highly expressed during embryonic development [28].

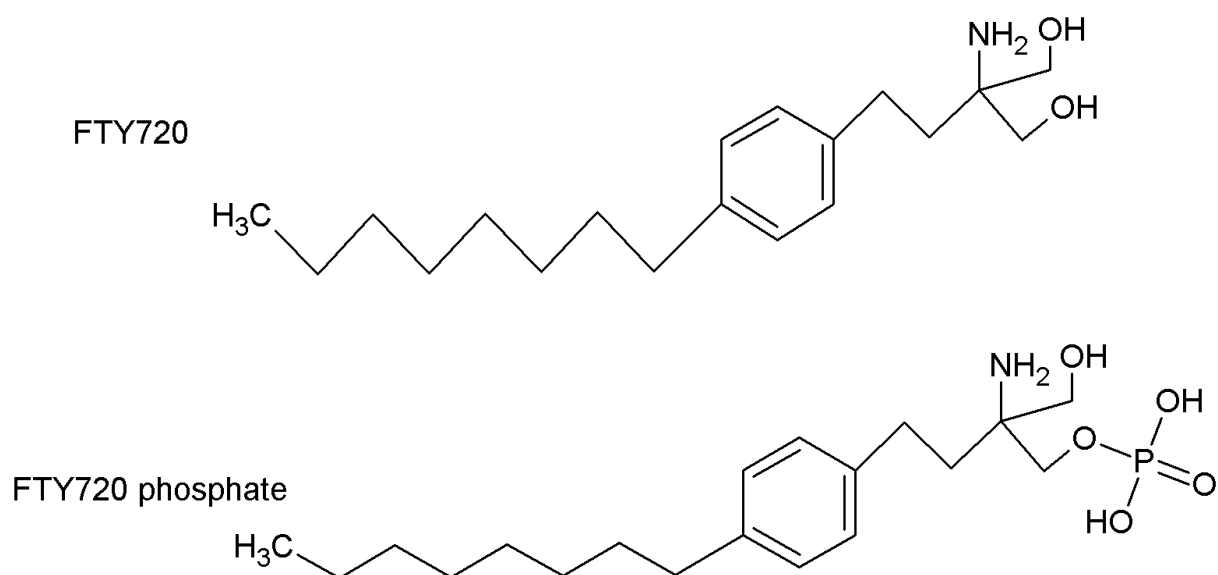
A preclinical study in 2007 showed that S1P<sub>2</sub> expression is important for correct functioning of the auditory and vestibular systems [29]. Other studies *in vivo* have demonstrated that S1P<sub>2</sub> is

involved in regulating portal vein pressure [30], in forming atherosclerotic plaques [31], inflammation [32] and retinal angiogenesis [33]. It is also implicated in regulating vascular integrity through phosphorylation of vascular endothelial-cadherin, thereby preventing its translocation to cell-cell contact sites [19]. Recently, S1P<sub>2</sub> activity was associated with autoimmune risk and epileptogenesis [4]. S1P<sub>3</sub> expression was found to be very high in heart, lung, kidney, spleen, diaphragm, intestine and certain cartilaginous regions [22]. S1P<sub>4</sub> is expressed in lymphoid tissues and peripheral leukocytes and also in smooth muscle cells of the human lung [34, 35]. S1P<sub>5</sub> is found in the spleen and in CNS predominantly in the white matter with high levels of mRNA in oligodendrocytes [36]. When S1P binds, S1P<sub>5</sub> induces phosphatase-dependent inhibition of ERK1/2, resulting in an anti-proliferative phenotype [37]. Recent data demonstrated that S1P<sub>5</sub> is present in natural killer (NK) lymphocyte cells [38].

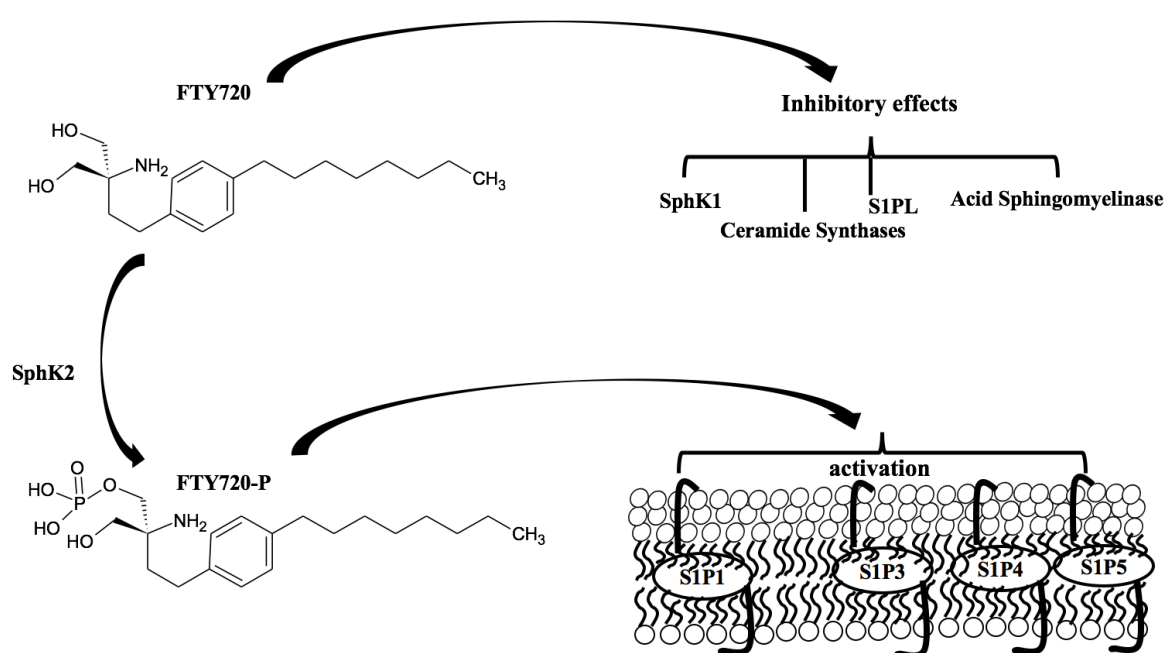
## **Fingolimod and S1P signaling**

### *Chemistry*

Fingolimod (FTY720), is structurally similar to the sphingolipid sphingosine, and is derived from the fungal metabolite myriocin isolated from cultures of *Isaria sinclairii* [39]. Fingolimod is a pro-drug which *in vivo*, is phosphorylated by SphK-2 to the active metabolite fingolimod phosphate (pFTY720) (Figure 3) which can then activate intracellular targets via S1P [1] (Figure 4). Fingolimod can interact with sphingolipid metabolizing enzymes, and has inhibitory actions on SphK1 [40], S1P lyase [41], ceramide synthases [42], cytoplasmic phospholipase A2 (cPLA2) [43] and the acid sphingomyelinase [44] (Figure 4). The direct inhibitory effects of fingolimod occur at micromolar concentrations, while agonism at 4 of the 5 receptors occurs at low nanomolar concentrations [1]; therefore the pharmacological relevance of these direct effects is doubtful.



**Figure 3.** Chemical structures of fingolimod (FTY720) and fingolimod phosphate (FTY720-P).



**Figure 4.** Fingolimod (FTY720) and fingolimod phosphate (FTY720-P) interact with sphingolipid metabolizing enzymes and activate S1P receptors respectively. FTY720 phosphorylation is exclusively mediated by SphK2. FTY720-P binds with high nanomolar affinity to all S1P receptors, but not S1P2. In addition, fingolimod targets SphK1, ceramide synthases, S1PL, sphingosine kinase 1, sphingosine 1-phosphate lyase; cPLA2, cytoplasmic phospholipase A2, and acid sphingomyelinase.

FTY720 includes a prochiral quaternary carbon that bears two hydroxymethyl groups (CH<sub>2</sub>–OH). Replacement of one of the hydroxymethyl groups yields an pFTY720 analogue as a mixture of racemates: the pro-(S) hydroxymethyl group of FTY720 is much more important for biological activity than other groups, while the (R)-hydroxymethyl group (biologically less important) may be chemically and physico-chemically significant in simplifying the molecule and improving the solubility [45].

### *Pharmacokinetics*

Results from studies in healthy volunteers, MS patients and renal transplant recipients have proved that fingolimod can be efficiently absorbed with an oral bioavailability >90% and its absorption is not influenced by food intake [46]. The half-life of fingolimod and pFTY720 is 6-9 days, and steady-state blood levels are attained after 1-2 months of daily dosing [47]. Due to its long half-life and sluggish absorption, fingolimod shows a regular concentration profile over time and it can be administered in once-daily dosing [48].

A 2006 study showed that fingolimod is extensively distributed into various organs including the brain. Distribution and clearance appeared to be linear in the dose range of 0.3 to 4 mg/kg. The fingolimod blood clearances in dogs, monkeys and humans are 0.0617, 0.113, and 0.0433 ml/h/kg, respectively [49].

Fingolimod is metabolized by three pathways: *i*) reversible phosphorylation to pFTY720 [50]; *ii*) hydroxylation and oxidation, giving a series of inactive carboxylic acid metabolites and *iii*) formation of non-polar ceramides [6]. Fingolimod is cleared via metabolism by cytochrome P450 (CYP) 4F2 with minor contributions from CYP2D6, 2E1, 3A4, and 4F12 to give inactive carboxylic acid metabolites, which are then excreted in the urine [46, 48]. *In vitro*, fingolimod and pFTY720 have little or no ability to inhibit and no ability to induce other drug-metabolizing CYP enzymes [46]. Furthermore, CYP3A inducers and CYP3A inhibitors have little or no

effect on the pharmacokinetics of FTY720 and pFTY720 while, ketoconazole, which inhibits CYP4F2, increases the blood concentrations of fingolimod by up to 70% [46, 48]. No clinically significant effects of age, sex or ethnicity on the pharmacokinetic clearance of fingolimod have been reported [51]. Fingolimod blood clearance is  $6.3 \pm 2.3$  L/h (NDA 02257-FDA, 2010). Following oral administration, ~ 81% of a fingolimod dose is excreted slowly in the urine as inactive metabolites [47]. Fingolimod and pFTY720 are not excreted intact in urine, but are found in the feces in amounts < 2.5% of each dose. After 34 days, 89% of the administered dose is recovered (NDA 02257-FDA, 2010).

#### *Mechanism of action, pharmacological effects and side effects*

As mentioned above, fingolimod is phosphorylated *in vivo* by SphK2 to yield the activate form pFTY720, which binds specifically to four of the five G protein-coupled S1P receptors. Once phosphorylated, fingolimod acts as a S1P receptor modulator [52]. Despite the ability of fingolimod to bind to these receptors, to date, its exact mechanism of beneficial action in MS is not yet understood. However, it has been recognized that fingolimod's effects in managing symptoms of MS could be related with its ability to inhibit the egress of lymphocytes from lymphoid tissue, thereby reducing the extent of infiltration of auto-aggressive lymphocytes into the CNS, to initiate inflammation and tissue degeneration [48, 53]. *In vitro* experiments in animals also indicate that fingolimod may exert beneficial effects in MS by interacting with neuronal S1P receptors [52, 54].

Magnetic resonance imaging (MRI) outcomes show that fingolimod reduces gadolinium-enhanced lesions and the morphological correlates of clinical relapses in patients with relapsing-remitting MS, suggesting that fingolimod may indeed exert additional direct effects on brain cells [55]. Following oral administration, fingolimod may then directly impact neuropathological processes *e.g.* gliosis, neurodegeneration and endogenous repair mechanisms [56]. Direct fingolimod effects within the CNS may also occur by binding

intracellular targets of S1P [1]. In particular, a preclinical study by Foster and collaborators indicated that fingolimod could prevent the disruption of BBB permeability through S1P signaling [57]. Particularly, fingolimod, based on its lipophilic nature, entered quickly into the CNS where the drug and its active form were found in the brain parenchyma [57]. In addition, fingolimod administration protected mice from vascular endothelial growth factor (VEGF)-induced vascular leakage [58] and bacterial lipopolysaccharide (LPS)-induced pulmonary edema [59]. Fingolimod also exerts anti-inflammatory effects in animal models of CNS injuries, such as brain ischemia [60] and spinal cord injury [61] as well as in an experimental model of epilepsy [10].

In clinical trials, fingolimod was found to be generally safe and well-tolerated by patients [62, 63]. Despite this, non-fatal herpes virus infection, neoplasms and influenza infections are some adverse effects of special interest that have been reported in subjects treated with fingolimod. Likewise, macular oedema is usually common in fingolimod treated subjects, but was reversible after drug withdrawal [63, 64]. To date, two cases of fatal hemophagocytic syndrome associated with infections as well as several cases of progressive multifocal leukoencephalopathy (PML) have been observed after fingolimod treatment. However, the risk of PML during fingolimod treatment seems significantly low in comparison to natalizumab, the monoclonal antibody also commonly used in the treatment of MS. Particularly, the majority of PML cases were related to a “carry-over” effect, when patients received natalizumab before fingolimod administration [65]. A rare case of posterior reversible encephalopathy syndrome (PRES) (severe headache, nausea, visual defects) has also been reported [66]. Many of the observed adverse effects can be linked to S1PRs action. For others, the mechanism is uncertain [48].

Overall, the main adverse effects that have been associated with the clinical use of fingolimod include: headache, fatigue, flu-like symptoms (nasopharyngitis), back pain, cough, diarrhea, lower respiratory tract infection, transient bradycardia, slowed atrioventricular conduction,

dyspnea, elevation of liver enzymes, and hypertension. Bradycardia and slowed atrioventricular conduction are adverse effects reported at treatment initiation [62, 64].

### **S1P in the brain**

The maximum brain concentration of S1P is around 4-40 nmol/g wet weight, corresponding to ~4-40 $\mu$ M [67], and during brain injury or stroke, the local concentration could further increase or be freed from platelets in blood clots [68]. Interestingly, on rat pituitary GH4C1 cells, S1P receptors regulate intracellular  $\text{Ca}^{2+}$  levels by directly inhibiting voltage-operated  $\text{Ca}^{2+}$  channels [69]. It is likely that a similar mechanism could also operate in central neurons. In this context, SphK1 may play a major role considering that it is the primary isoform found in mouse brain [70] and is highly abundant within the dendrites and dendritic spines of cerebellar Purkinje cells [71]. The expression of SphK1 in GH4C1 rat pituitary cells via sphingosine may reduce inhibition of the voltage-operated  $\text{Ca}^{2+}$  channels (VOCCs)[70]; moreover, in hippocampal neurons, SphK1 is activated by delta-catenin/NPR (neural plakophilin-related armadillo repeat protein) [72].

S1P is involved in the growth and survival of oligodendrocytes [73]; the survival by neurotrophin-3 (NT-3) of oligodendrocytes depends on SphK1. In fact, there is a functional link between NT-3 and S1P signaling. Furthermore, in these cells, S1P itself could stimulate cAMP response element-binding protein (CREB) phosphorylation, an important NT-3 survival signaling pathway [74]. S1P is also important in stimulating the motility of glioblastoma cells and is critical for the invasion process [75]; S1P<sub>1-3</sub> stimulate glioma cell proliferation and amplify glioma migration and invasion by S1P<sub>1</sub> and S1P<sub>3</sub> activation [76]. It has been proposed that S1P is implicated in the regulation of neurotransmitter release. In fact, S1P can increase excitability in cultured rat dorsal root ganglion neurons via S1P receptors, while S1P generated intracellularly from ceramide can increase neuronal excitability by mechanisms that are unclear [77]. Recently, S1P was shown to affect glutamate secretion in primary hippocampal neurons

acting as a secretagogue to trigger glutamate secretion or potentiating depolarization-evoked glutamate secretion [78]. Furthermore, in sensory neurons, NGF-induced production of S1P caused an increase in neuronal excitability [77].

The exact mechanism that underlines the involvement of S1P in neuronal excitability is unknown; however, the role of S1P in modulating neuronal function is confirmed, as above mentioned, by the observation that S1P2-null mice exhibit spontaneous seizures and significant increases in the amplitude of excitatory postsynaptic currents [79]. Zhang and collaborators showed that intracellular S1P could increase sensory neuron excitability by acting as an internal second messenger to suppress outward  $K^+$  currents [77]. In particular, the direct exposure of isolated capsaicin-sensitive sensory neurons to S1P altered their capacity to fire action potentials by inhibiting outward  $K^+$  currents, while externally applied S1P produced a time-dependent suppression of  $K^+$  current ( $I_K$ ). In the presence of 30 mM tetraethylammonium (TEA), a well-established blocker of some subtypes of delayed rectifier  $K^+$  channels, treatment with S1P did not produce a significant inhibition of  $I_K$ . These results suggested that S1P might act on the same population of  $K^+$  channels as TEA [77]. Furthermore, S1P in hippocampal neurons was suggested to be involved in glutamate secretion by an autocrine/paracrine mechanism [78].

#### *S1P receptors in the CNS and Fingolimod*

S1P<sub>1</sub>, S1P<sub>2</sub>, S1P<sub>3</sub>, and S1P<sub>5</sub> are expressed in the CNS [26]. Particularly, S1P<sub>5</sub> is expressed at high levels while, S1P<sub>1-3</sub> are mainly expressed in the developing brain [80]. All these four S1PRs are present in neurons, astrocytes, microglia/macrophages, and oligodendrocytes, where they are expressed in different patterns [1].

The level of S1P<sub>5</sub> normally expressed in astrocytes is very low, but upon exposure to growth factors, its expression is upregulated [81]. Following activation by pathological stimuli, S1P<sub>1</sub> and S1P<sub>3</sub> levels in activated astrocytes are upregulated [82], implying an important role for these



receptor subtypes in pathogenesis. The expression level for S1P receptors in microglia is also dynamically changed upon activation: S1P<sub>1</sub> and S1P<sub>3</sub> are downregulated, but S1P<sub>2</sub> is upregulated [83].

Activation of S1P<sub>1</sub> leads to Rac (GTPase) activation and neurite extension, while, when nerve growth factor (NGF) downregulates S1P<sub>2</sub> and S1P<sub>3</sub>, there is retraction neurites by activation of Rho [84]. S1P<sub>1</sub> activation following S1P formation (dependent on SphK1) produces depolarization-induced glutamate release in hippocampal neurons [78]. S1P<sub>1</sub> is involved in neural stem cell migration towards sites of spinal cord injury [85]. It mediates stimulation of ERK/Egr-1/FGF-2 in C6 glioma and ERK activation in astrocytes [86, 87] also promoting astrocyte motility [88]. S1P<sub>1</sub> is involved in platelet-derived growth factor-induced proliferation of oligodendrocyte precursors [89].

Regarding the expression/function of S1P<sub>2</sub> at endothelial barriers in the CNS normally, or during induction of CNS autoimmunity, no data are available. In addition, the S1P<sub>2</sub> gene is little expressed in the brain of adult mice and rats [27], while it is highly expressed in the brain during embryonic development [28].

S1P<sub>2</sub> is essential for neuronal development/excitability in zebrafish [79] and there are observations concerning neuroexcitability of S1P<sub>2</sub> knockout mice. Although these mice do not display gross anatomical defects within the CNS and appear normal, electrophysiological defects and seizure development were observed in the S1P<sub>2</sub>-null mutants of the C57Bl/6J background; this was suggested to be due to impairment of Ca<sup>2+</sup>-regulated signal transduction [79]. However, the S1P<sub>2</sub> (H218) receptor gene plays an important role in the development and mediation of neuronal excitability; in fact H218<sup>-/-</sup> mice were found to exhibit spontaneous seizures that were accompanied by ictal-like EEG discharges and hyperexcitability of neocortical pyramidal neurons [79]. In fact, S1P<sub>2</sub><sup>-/-</sup> receptor knockout mice develop, between 3 and 7 weeks of age, spontaneous, sporadic and rarely, lethal seizures associated with anxiety and memory impairment [79]. S1P<sub>2</sub> activation inhibits neurite extension in neurons [90] and

glioblastoma motility [91], while it increases glioma invasion [76]; it also induces changes in the morphology of C6 glioma cells [92] and PLC/Ca<sup>2+</sup> in C6 glioma cells.

S1P<sub>3</sub> could inhibit the extension of neurites in pheochromocytoma-derived PC12 cells [90]. S1P<sub>5</sub>, as mentioned above, is present in oligodendrocytes, where it shows different functions [36]. It facilitates oligodendrocyte precursor retraction and stimulates mature oligodendrocyte survival [36]; also, activation of S1P<sub>5</sub> inhibits migration of oligodendrocyte progenitors [93]. Furthermore, S1P<sub>5</sub> stimulates phospholipase C (PLC)-Ca<sup>2+</sup> and phospholipase D (PLD) in C6 glioma [86].

In astrocytes, S1P<sub>5</sub> expression is not detectable under basal conditions, but can be upregulated by astrocytes grown in culture [94]. Considering the possibility of novel drug development, the fact that S1P receptors are differentially expressed in different organs, that receptor expression is modulated in different diseases, as well as the differential coupling to G proteins may make S1P receptors potentially useful therapeutic targets.

The biological activity of fingolimod/pFTY720 is exerted via S1P receptors at different potencies and affinities towards the S1P receptor isoforms (Table 1), and may promote distinct responses in target cells [95]. Specifically, (S)-configured FTY720-P acts as a full agonist at S1P<sub>1</sub>, S1P<sub>4</sub> and S1P<sub>5</sub> and with lower potency at S1P<sub>3</sub> without activity at S1P<sub>2</sub> [50]. Furthermore, the drug can function as an antagonist of S1P<sub>1</sub>, slowing lymphocyte egress by down-modulating/internalizing lymphocyte S1P<sub>1</sub> receptors [25]. Considering the resident cells of the CNS, it is possible for fingolimod to exert direct effects on brain cells implicated in regulating immune reactivity, on cells that are targeted by disease processes and on cells that are involved in CNS repair such as oligodendrocyte progenitor cells [54].

<b>Table 1.</b> Potency (EC <sub>50</sub> ) value of fingolimod-phosphate (FTY720-P) at human S1P receptors.	
<b>Receptor</b>	<b>FTY720-P</b> Potency (EC <sub>50</sub> ) value
S1P <sub>1</sub>	0.3 nM
S1P <sub>2</sub>	>10 000 nM
S1P <sub>3</sub>	3.1 nM
S1P <sub>4</sub>	0.6 nM

S1P <sub>5</sub>	0.3 nM
EC <sub>50</sub> = molar concentration of compound resulting in 50% of maximal GTPγS binding. Particularly, a membrane-based [ $\gamma$ -35S] GTPγS binding assay was used to determine the relative potency and efficacy of the compound at isolated receptors.	

### *Fingolimod and neurological diseases*

Being a lipophilic compound, fingolimod can readily penetrate into the CNS where it accumulates in the brain and spinal cord [57]. Considering this as well as the abundant neural expression of S1P receptors, it is plausible that fingolimod could affect brain cells directly [1] as evidenced by previous studies [98, 99]. Accordingly, as reported by many studies, fingolimod could be used as a new potential therapeutic application for managing several CNS diseases including epilepsy (Table 2) [1, 100, 101]. Furthermore, many of these CNS disorders including stroke, are often associated with an increased risk of seizure development [102].

Concerning the possible cellular target(s) of fingolimod in the CNS, there is well-known evidence for a role of astrocytes in MS pathophysiology [103]. Particularly, Choi et al. [104] showed that deficiency of S1P<sub>1</sub> on astrocytes led to an attenuation of experimental autoimmune encephalitis and that the protective effect of fingolimod against the latter was lost in mice with astrocytic, but not neuronal S1P<sub>1</sub> deletion. Furthermore, a study present in the literature supports an anti-inflammatory effect of fingolimod on astrocytes *in vitro*, regulating specific neuroinflammatory responses by a process of desensitization [105]: in this study, astrocytes derived from human fetal CNS specimens were subjected to initial single fingolimod exposure, then daily exposures up to five days. The single use of drug inhibited S1PR ligand-induced pERK1/2 signaling for >24 h and the effect was maintained even during the next treatment days.

Regarding neurodegenerative diseases, different reports show a putative therapeutic effect of fingolimod against Alzheimer's disease progression in a rat model of intrahippocampal injection of beta-amyloid peptide Ab<sub>1-42</sub>; fingolimod treatment ameliorated spatial learning and

memory deficits in the model and reduced the observed neuronal damage and caspase-3 activation in the hippocampus [106]. Fingolimod injections in a R6/2 mouse model of Huntington disease (HD), beginning in the subtle phase of HD symptoms, significantly improved motor function and prevented the usual weight loss and brain atrophy that is associated with the disease [107]. Likewise, more recently, chronic administration of fingolimod from pre-symptomatic stages, (8 weeks of age up to 20 weeks) was found to enhance hippocampal synaptic plasticity and improve memory performance in an R6/1 mouse model of HD by regulating brain-derived neurotrophic factor (BDNF) signaling and preventing astrogliosis [108]. Recent evidence shows that fingolimod has neuroprotective effects in both mouse and rat models of focal brain ischemia, by decreasing the anti-inflammatory mechanism without a direct effect on neurons [60].

Furthermore, Potenza et al. [109] have demonstrated that fingolimod has disease-modifying properties in a mouse model (mSOD1<sup>G93A</sup>mice) of amyotrophic lateral sclerosis. Particularly, these positive effects, accompanied by a modulation of microglial activation and innate immunity, endorse fingolimod as a potential new therapeutic treatment for amyotrophic lateral sclerosis. Likewise, fingolimod was also able to reduce synucleinopathy and improve gut motility in A53T transgenic mice, a mouse model of Parkinson's disease [110].

<b>Table 2.</b> Sphingosine-1-phosphate/Fingolimod as a potential new therapeutic approach to CNS disorders.		
<b>Disease</b>	<b>Models</b>	<b>Refs</b>
Alzheimer's Disease	Human cells and experimental models	[1, 106, 111]
Amyotrophic Lateral Sclerosis	mSOD1 <sup>G93A</sup> mice	[109]
Anxiety	Mouse model of chronic unpredictable stress	[112]

Brain Tumors	Human cells and experimental models	[1, 113]
Cerebral Malaria	Human and experimental cerebral malaria	[114]
Depression	Mouse model of chronic unpredictable stress	[112]
Epilepsy	Lithium-pilocarpine model, PTZ model and WAG/Rij rat model	[10, 11, 115]
Huntington Disease	R6/2 mouse model	[107]
Intracerebral Hemorrhage	Several experimental models	[1]
Multiple Sclerosis	Approved, in 2010, for the treatment of relapsing-remitting multiple sclerosis	[1, 50]
Neuroblastoma	Xenograft model	[116]
Parkinson's Disease	A53T transgenic (Tg) mice	[110]
Rett Syndrome	MeCP2 -deficient mice	[117]
Stroke	Several experimental models and a clinical proof-of concept study	[1, 118]

### *S1P, Fingolimod and epilepsy*

To date, there are numerous antiepileptic drugs (old and new generation) that are commercially available; they all represent symptomatic therapies suppressing seizures but without any demonstrated antiepileptogenic or disease-modifying properties [13, 119]. Therefore, one of the principal unmet needs in the field of epilepsy therapeutics is the discovery of new disease-modifying drugs that may totally prevent epilepsy (antiepileptogenic drugs) or slow its progression as well as its related comorbidities [120, 121]. In particular, a rational antiepileptogenic strategy could be to target cell-signaling pathways that primarily trigger the downstream mechanisms causing epileptogenesis [119, 122].

Sphingolipids such as S1P exert crucial functions in the CNS [4]; therefore, it is currently suggested that drugs acting on the S1P signaling pathway might be a valid novel therapeutic target for the treatment of some neurological disorders such as epilepsy. Fingolimod, the first drug developed that is able to modulate the sphingolipid signaling pathway, approved for the

treatment of MS, might therefore represent an already available treatment option (see above) [1, 14].

Since fingolimod has potent anti-inflammatory effects in MS and in other CNS injuries, it may well be effective on inflammation during epileptogenesis [60, 61]; in fact, besides inhibiting lymphocyte egress, it acts on a number of neuronal and non-neuronal cells exerting neuroprotective and anti-inflammatory effects in the brain [123]. Human brain endothelial cells (ECs) are one of the CNS cell types that can phosphorylate fingolimod to its activated form. Fingolimod promotes assembly of adherens junctions and decreases the permeability of the BBB via S1P<sub>1</sub> [25, 54].

To date, the molecular mechanisms underlying the reported antiepileptogenic properties of fingolimod remain unclear. Fingolimod as well as S1P might act through extracellular S1P receptors as well as binding to intracellular S1P targets [14]. Particularly, after fingolimod is phosphorylated by SphK2, it mimics the intracellular actions of S1P and inhibits class I histone deacetylases (HDACs) to regulate histone acetylation, gene expression and brain functions [6]. As previously mentioned, Gao et al. [10] found that fingolimod possessed anti-inflammatory and antiepileptogenic effects in the rat lithium-pilocarpine model of temporal lobe epilepsy. In this study, the chronic treatment (14 days) with fingolimod (1 mg/Kg), beginning 24 hours after SE induction, significantly decreased the occurrence, frequency, duration and severity of spontaneous recurrent seizures; after SE, it also counterbalanced several hallmarks of epileptogenesis such as microglial cell activation, and abnormally elevated IL-1 $\beta$  and TNF- $\alpha$  expression as well as pathological sprouting of mossy fibers in the hippocampus during the latent phase. Furthermore, it reduced neuronal loss; in fact, in the study by Gao and colleagues, fingolimod-treated rats showed more neuronal nuclear antigen (NeuN)-positive cells and less Fluoro-Jade B (FJB)-positive cells in the hippocampus in contrast with the control group, indicating that fingolimod had a neuroprotective effect against SE-induced neuronal loss [10]. Very recently, it has also been demonstrated that fingolimod exerts neuroprotective effects in a

PTZ-kindling mouse model. Its effects have been tested by two different approaches: pre and post-treatment. In the pre-treatment study, the administration of fingolimod, 1h before PTZ-administration, was able to reduce seizure onset. Moreover, such treatment also decreased microglial activation and neuronal death in hippocampal areas CA1 and CA3. Likewise, fingolimod post-treatment reduced seizures as well as increased endogenous remyelination in kindled mice. These data could support fingolimod as a new therapeutic approach for demyelination linked to epilepsy [11]. Furthermore, a preliminary study, presented at the 12<sup>th</sup> European Congress on Epileptology held in Prague in September 2016, reported that fingolimod could also have antiepileptogenic effects in WAG/Rij rats, which represents a well-established genetic model of absence epilepsy, epileptogenesis, and neuropsychiatric comorbidity [124]. These antiepileptogenic effects were accompanied by positive effects on learning, memory and depressive-like behaviour, which represent the most crippling comorbidities of epilepsy [115, 125]. Since comorbidity of epilepsy greatly diminishes the quality of life, their treatment is a pressing need [126, 127]. Likewise, another poster presentation at the 12<sup>th</sup> European Congress on Epileptology, studied both the mRNA expression levels of fingolimod target molecules S1PRs in hippocampal regions of mice after SE and the antiseizure effects of fingolimod in the pilocarpine as well as in the kainate model of temporal lobe epilepsy. Increased expression levels and expression changes of fingolimod targets in the hippocampus of mice as well as antiepileptogenic effects of fingolimod *in vivo* were observed. Accordingly, fingolimod could also be a promising therapy option in focal epilepsies [128].

Regarding presumed non-immunomodulatory effects of fingolimod in epilepsy, subsequent studies to those of MacLennan and colleagues (mentioned above) indicated that S1P<sub>2</sub> plays an crucial and functionally important role in controlling neuronal excitability; in particular, it was demonstrated that S1P2<sup>-/-</sup> mice appear normal and seizure-free [129, 130]. Du and colleagues reported that S1P2<sup>-/-</sup> mice exhibit lethal seizures around weaning age [131]. More recently, the seizures expressed in juvenile S1P2<sup>-/-</sup> mice were extensively characterized in the study of

Akahoshi et al. [9], where they underlined that early CNS insults remain in the surviving adults, resulting in enhanced hippocampal/neocortical gliosis, impaired spatial learning memory, increased anxiety, and, possibly, impaired maternal nurturing. Therefore, the selective blockage of S1P<sub>2</sub>-dependent signaling may induce such CNS dysfunction in addition to auditory/vestibular defects [28]. Such controversial phenotypic alteration might be linked to the genetic background differences of investigated S1P<sub>2</sub><sup>-/-</sup> mice.

The involvement of inflammation in epilepsy and epileptogenesis has been widely studied, while actually the role of mossy fiber sprouting remains controversial [12, 132]. Regarding neuroinflammation, occurring in several brain disorders such as Alzheimer's disease, Parkinson's disease, stroke, and epilepsy, among others, leads to a perturbation of BBB permeability [133]. In particular, proinflammatory mediators influencing endothelial tight junctions and the basal membrane may provoke and sustain BBB breakdown [134]. The early identification by neuroimaging studies of such BBB perturbations could be used as a biomarker to prevent or modify the epileptogenic processes related with the development of epilepsy [135, 136].

It is now well recognized that the S1P signaling pathway is involved, through S1P receptors, in the modulation of BBB permeability. In fact, the increased BBB integrity promoted by S1P pathway activation leads to a reduced infiltration of several pro-inflammatory factors. Among the S1P receptors modulating the BBB permeability, S1P<sub>2</sub> has been associated with the epileptogenic process [79]. Furthermore, the role of the SphK/S1P signaling pathway in neuronal excitability has been previously demonstrated by NGF studies. In particular, NGF, via sphingomyelin metabolism, induces the formation of S1P, which enhances the sensory neuronal excitability acting as an intracellular messenger to suppress outward K<sup>+</sup> currents. However, the precise molecular mechanism whereby S1P suppresses these currents is not well understood [137]. Recently, Li et al. [138] have also demonstrated that the increased neuronal excitability induced by S1P is mediated by S1P<sub>1</sub> and/or S1P<sub>3</sub> receptors. The role of S1P in controlling



neuronal excitability and epilepsy has also been suggested by the observation that the SphK1/S1P pathway protects mouse hippocampal neurons after kainic acid (KA) neurotoxin treatment. Particularly, in the study of Lee et al., the temporal dynamics of hippocampal SphK1 and S1P1 expression during KA-induced excitotoxicity were characterized and they found that they correlated with elevated levels of glial fibrillary acidic protein (GFAP) expression. GFAP (primarily expressed in astrocytes) has multiple CNS functions, including maintenance of BBB integrity, and is a well-recognized marker of CNS injury [139]. The SphK1/S1P neuroprotective effect seems to be the result of its ability to control astrocyte responses in damaged regions, common in several brain disorders such as epilepsy [140]. To date, further studies are now clearly required in order to better clarify the mechanism(s) whereby S1P induces neuronal hyperexcitability and neurotransmitter release. These studies could also simplify the understanding of pathologies correlated with increased excitability such as epilepsy, and thus lead to novel therapeutic approaches [141].

SPHK-1 and S1P are also able to regulate neurotransmitter release via a  $\text{Ca}^{2+}$ -dependent mechanism. In particular, it has been proposed that S1P acts by enhancing depolarization-induced  $[\text{Ca}^{2+}]_i$  increase in the cells, through voltage-operated calcium channels (VOCCs) [142, 143]. Subsequently, it has been demonstrated that SphK regulates the function of VOCCs in rat pituitary GH4C1 cells by removing its substrate sphingosine (which normally inhibits depolarization-induced  $\text{Ca}^{2+}$  influx) and not through its catalytic product (S1P) [70]. Moreover, S1P, acting as an intracellular second messenger, could increase the  $\text{Ca}^{2+}$  influx in the cells by enhancing large-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  (BKCa) channel activity as demonstrated in human ECs [144]. Of note,  $\text{Ca}^{2+}$  homeostasis is widely involved in several CNS disorders such as epilepsy, whereas it is now argued whether BKCa channels might be a suitable target for the development of potential AEDs [145]. Furthermore, Kajimoto et al. [78] have reported that S1P pathway activation leads to glutamate secretion from primary hippocampal neurons, both acting as a secretagogue to facilitate glutamate release and through the enhancement of high  $\text{K}^+$

depolarization-induced glutamate efflux. According to Kanno and Nishizaki [146], the glutamate secretion occurs in restricted hippocampal areas via S1P<sub>3</sub> receptors.

At the level of the BBB, P-glycoprotein (P-gp) is widely expressed and protects the brain by extruding harmful xenobiotics. This pump, when overexpressed, represents an obstacle for drug delivery in the CNS. Therefore, it has been suggested as a possible underlying cause of drug resistance in patients affected by epilepsy [147]. Recently, in epileptic rat brain, it has been reported that P-gp overexpression could be regulated by inflammation and nuclear factor-kappa B (NF-κB) activation [148]. Moreover, studies have also demonstrated that S1P as well as fingolimod, through S1PR<sub>1</sub>, are able to rapidly and reversibly reduce P-gp pump activity and thus might also ameliorate drug delivery into the CNS. In particular, it was observed that after S1P and fingolimod administration, there was an increase in the uptake of some P-gp substrates such as verapamil, whereas blocking S1PR<sub>1</sub> abolished this effect. Therefore, this action of fingolimod on P-gp pump activity could be used to ameliorate the clinical ineffectiveness of AEDs in patients with intractable epilepsy [149].

During epileptogenesis, the hippocampus is subjected to several morphological changes, such as mossy fiber sprouting, neurite extension, retraction and neurogenesis. To date, the role of these structural changes in the formation of an epileptogenic network is not well understood; however, it has been hypothesized that they could be used as biomarkers for epileptogenesis [150]. Moreover, it has also been observed that neuronal migration disorders are related to malformations of cortical development, which are characterized by severe epileptic syndromes. These alterations in neuronal migration have been linked to abnormal cellular morphology and circuit formation [151].

Several reports describe an involvement of SphK/S1P signaling in neurogenesis, cell migration, survival and differentiation [152]. Of note, it was observed that S1P could, through S1P receptors, induce mammalian target of rapamycin (mTOR) pathway activation. Both these pathways are also involved, in several cells type, in the modulation of cell growth,

differentiation, and autophagy [153]. Particularly, it has been reported that the S1P pathway, by activating the mTOR signaling pathway, is able to inhibit the development and function of regulatory T cells [154]. Furthermore, it has been demonstrated that sphingolipids such as ceramide and S1P, through the activation of mTOR, are able to inhibit autophagy and related cell death of human leukemia HL-60 cells [155]. The involvement of mTOR in epileptogenesis and epilepsy is currently under evaluation and is widely supported by several studies; however, the exact molecular mechanisms underlying its role are still not well understood. As a result of the mTOR involvement in epilepsy, the mTOR inhibitor everolimus is currently under evaluation in patients with tuberous sclerosis complex (TSC), in which epilepsy is the most common neurological symptom [156-158]. Hence, the relationship between S1P and mTOR signaling deserves further studies, since it could lead to new and potentially useful information in epilepsy research.

Regarding novel therapeutic approaches for epilepsy treatment, particular attention is reserved for cannabinoids, even though their underlying mechanisms of action are only partly established [159-161]. Sim-Selley et al. [162] reported that central administration of S1P induced some cannabinoid-like effects, including antinociception. These effects appeared to be CB<sub>1</sub> receptor-independent. Recently, regarding pain modulation, the same research group suggested probable points of interaction between the cannabinoids and endogenous sphingolipid systems [163]. Furthermore, a previous study also demonstrated that fingolimod as well as S1P could be potential antagonists of CB<sub>1</sub> receptors, as demonstrated both *in vitro* and *in vivo*. A further probable point of interaction between the two systems might be the peroxisome proliferator-activated receptors (PPARs). In fact, several cannabinoid effects, including antiseizure effects are also mediated by PPAR receptors [164-166]; likewise, several S1P functions also seem to be mediated via PPAR receptors [167]; PPAR $\gamma$  is an intracellular target for S1P and the S1P:PPAR $\gamma$ :PGC1 $\beta$  complex might be a suitable target for regulation of neoangiogenesis [77].

According to this evidence, the probable interaction between these two systems deserves particular attention, considering that they might be relevant for antiepileptogenic properties.

A further link between the S1P signaling pathway and epilepsy could be related to histone deacetylases (HDACs). The HDACs are important enzymes that influence the acetylation status of histones and several fundamental cellular proteins. HDAC inhibitors would seem to be a valid therapeutic approach to treat several brain disorders such as epilepsy. In fact, the antiepileptic drug valproic acid also seems to act through its effects as a HDAC inhibitor [168]. Likewise, S1P as well as fingolimod, after their phosphorylation by sphingosine kinases (especially SphK2), can inhibit the enzymatic activity of HDACs, avoiding the elimination of acetyl groups present on lysine residues inside histone tails [6]. Overall, sphingolipids or their derivatives would seem to be suitable targets both to understand the mechanisms underlying the etiopathology of several brain disorders such as epilepsy and also to develop novel therapeutic approaches to manage them [50].

## **Conclusions**

During the last decades, several reviews have appeared that focus on the intricate regulation of the sphingolipid pathway both in physiological and pathological conditions; indeed, sphingolipids may represent a source of potential targets for therapeutic intervention in various settings. Currently, it is known that sphingolipids, such as S1P, regulate many physiological and pathological processes in the CNS [4]; S1P signaling modulates the development of the embryonic nervous system, the migration of neuronal progenitors, proliferation of astrocytes and trophic factor release, activation of microglia and oligodendrocyte proliferation and survival [54, 85]. Furthermore, in the adult brain, it is also involved in the regulation of neurotransmitter release and participates in the inflammatory response by acting on glial cells further than being synthesized as a response to neuroinflammation by neurons, astrocytes, oligodendrocytes and microglial cells [60, 82, 108]. Despite the demonstration of this clear

involvement in CNS functions and responses/participation to pathological processes, our comprehension of the S1P signaling pathway is still in need of clarification. On the other hand, the identification of novel therapeutic targets is a need for several CNS pathologies such as Alzheimer's disease, Parkinson's disease, and schizophrenia among others. Similarly, epilepsy, despite the great number of currently available antiepileptic drugs (AEDs), represents a therapeutic area where undoubtedly new and more effective/safer drugs are necessary [120].

Currently available AEDs are not efficacious in about 30% of epilepsy patients; furthermore, patients also suffer from AED-dependent adverse events and epilepsy-related comorbidities (*e.g.* depression and cognitive impairment). Finally, there is no evidence of efficacy of current AEDs against *epileptogenesis*. Recently, the international league against epilepsy (ILAE) has identified several unmet needs in epilepsy research, including more efficacious and safer drugs further than the need for disease-modifying drugs able to prevent seizure development, inhibit disease progression and/or prevent the development of comorbidities [120, 121, 169].

The possibility of modulating the S1P pathway could thus represent a suitable target for epileptogenesis; this possibility is highly supported by the known role of neuroinflammation in this process and the recognized antiinflammatory effects exhibited by fingolimod. This drug has already demonstrated efficacy in an experimental model of epileptogenesis [10]. Fingolimod is a brain-permeant sphingosine analogue, which can activate different S1P receptor subtypes to perform its biological activity. Besides its primary action to reduce the egress of T lymphocytes from secondary lymphoid organs (thus restraining neuroinflammation and autoimmunity, important for MS treatment), recent evidence suggests that fingolimod action involves S1PRs expressed by CNS-resident cells, including neurons [52]. This was confirmed in the study by Gao et al. [10] where anti-inflammatory and antiepileptogenic effects in the rat lithium-pilocarpine model of temporal lobe epilepsy were demonstrated; furthermore, other studies suggest other non-immunomodulatory effects involved in the control of neuronal excitability and synaptic transmission by this drug [79]. Indeed, further studies are needed to

confirm this antiepileptogenic effect and to well determine the exact mechanism of action involved.

Finally, S1P signaling target has the great advantage of already having a commercially available drug, which would highly simplify the possibility to test this hypothesis in epilepsy patients. Obviously, before any trial would start, much more evidence needs to be accumulated to support its possible efficacy. In this light, it might be useful to study the effects of fingolimod on patients affected by MS *and* epilepsy; in fact, it is known that there is a percentage of patients that also have seizures as a consequence of MS pathology.

## **List of abbreviations**

AEDs = Antiepileptic Drugs

BBB = Blood Brain Barrier

cAMP = Cyclic Adenosine Monophosphate

CNS = Central Nervous System

cPLA2 = cytoplasmic Phospholipase A2

FTY720 = Fingolimod

GPCR = G Protein-Coupled Receptor

HD = Huntington Disease

HDACs = Histone Deacetylases

MS = Multiple Sclerosis

mTOR = mammalian Target Of Rapamycin

NGF = Nerve Growth Factor

NT-3 = Neurotrophin-3

P-gp = P-glycoprotein

PLC = Phospholipase C

PML = Progressive Multifocal Leukoencephalopathy

PTZ = Pentylentetrazole

S1P = Sphingosine 1-Phosphate

S1PRs = Sphingosine 1-Phosphate Receptors

SphK 1/2 = Sphingosine Kinase 1/2

VOCCs = Voltage-Operated Calcium Channels

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## Figure Legends

**Figure 1.** Chemical structures of sphingolipid metabolites.

**Figure 2.** Signaling pathways linked to sphingosine 1-phosphate receptor (S1PR) activation. S1P interacts with five types of G protein-coupled receptor (S1P1-5), each coupled to several different G proteins to elicit different downstream events. Abbreviations: AC, adenylyl cyclase; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; cAMP, Cyclic adenosine monophosphate; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; Rock, Rho-associated protein kinase; SRF, Serum Response Factor; PKC, Protein kinase C; Mitogen-Activated Protein Kinase, MAPK; Akt, protein kinase B or PKB.

**Figure 3.** Chemical structures of fingolimod (FTY720) and fingolimod phosphate (FTY720-P).

**Figure 4.** Fingolimod (FTY720) and fingolimod phosphate (FTY720-P) interact with sphingolipid metabolizing enzymes and activate S1P receptors respectively. FTY720 phosphorylation is exclusively mediated by SphK2. FTY720-P binds with high nanomolar affinity to all S1P receptors, but not S1P2. In addition, fingolimod targets SphK1, ceramide synthases, S1PL, sphingosine kinase 1, sphingosine 1-phosphate lyase; cPLA2, cytoplasmic phospholipase A2, and acid sphingomyelinase.