Università di Genova

Maastricht University

DEPARTMENT OF EXPERIMENTAL MEDICINE

PhD PROGRAM IN EXPERIMENTAL MEDICINE

INTERNATIONAL CURRICULUM OF PHARMACOLOGY AND TOXICOLOGY

DOUBLE DOCTORATE - UNIVERSITY OF GENOA AND MAASTRICHT UNIVERSITY

ROLE OF SPHINGOSINE-1-PHOSPHATE RECEPTOR MODULATORS IN MOUSE CENTRAL NERVOUS SYSTEM: RELEVANCE TO AUTOIMMUNE DEMYELINATING DISEASES

Candidate
Alessandra Roggeri

Promotors
Prof. Dr. Anna Pittaluga
Prof. Dr. Jos Prickaerts

Co-Promotor
Dr. Tim Vanmierlo

PhD Program Coordinator
Prof. Ernesto Fedele

Academic Year 2021-2022

XXXIV Cycle

Introduction

Sphingolipids (SLs) were first discovered by J.L.W. Thudichum in 1876 by fractional crystallization of ethanolic brain extracts [1]. Due to the enigmatic nature of the discovered molecules, the scientist attributed to them the root term “sphingo” reminding the Greek mythical creature, the Sphinx. According to their name, the first groups of SLs (e.g., sphingomyelin, cerebrosides) were discovered in neuronal tissues. However, in the second part of the 20th an increasing number of studies unveiled that SLs can be found in all eukaryotic cells as the main component of plasma membranes [2–4].

Despite the large number of evidence that attributes to SLs the to function of maintenance the structure of the plasma membrane, more recently some of these molecules have been recognized as bioactive. Indeed, a large number of studies showed that SLs can play a key role in many physiological and pathological conditions. For this reason, they are attracting more and more the interest of the scientists as they can be taken in consideration as new targets for the treatment of several pathological conditions.

1. Structure and classification of sphingolipids

From a structural point of view, all the SLs possess three main components: a sphingoid backbone also known as long-chain bases, an acyl chain, and a polar head group. Modifications to the basis spingoid backbone generate a plethora of different molecules with different biological functions. The backbones are non-transient amino alcohol precursors of ceramide and complex sphingolipids, comprising different types of 2-amino-1,3-dihydroxyalkanes or -enes.

In yeast, there are two types of backbone, namely dihydrosphingosine ((2R,3S)-2-aminooctadecane-1,3-diol; DHS) commonly known as sphinganine and phytosphingosine ((2S,3S,4R)-2-aminooctadecane-1,3,4-triol; PHS), which have a carbon chain length of 18-20 [5]. PHS is a bioactive lipid and represents the major backbone of glycosphingolipids in fungi, yeast, and plants. However, in vertebrates it has been detected only in few specific tissues such as the epidermis, small intestine, kidney, and epithelial tissues [5].

In mammals, most common backbones include DHS and sphingosine (2-amino-4-trans-octadecene-1,3-diol) [5]. The two sphingoid bases differ structurally by a trans-double bond located at C4, which is present in sphingosine but not sphinganine [6]. The sphingoid backbone can be converted in derivatives following small modifications such as phosphorylation and acetylation. The base can be N-acylated by amine bond with different kind of long fatty- acid chains and/ or esterified at the primary hydroxyl generating a polar head group. The combination of different backbones subtypes, acyl chains, and head groups gives rise to hundreds of SLs. The nature of the polar head group identifies different SLs subtypes starting from the simplest hydrogen (i.e., for sphingosine and ceramide) to more complex molecules such as phosphocholine or different types of glycans.

1.1 Ceramides

In mammals, most SLs are N-acylated with a long/ very long fatty acids-chain to form the subfamily called “ceramides”. Several ceramide structures can be found and may vary for the length of the fatty acid chain ranging from C16 to C26 carbons atoms and for the degree of unsaturation. The most common are palmitic (C16:0) and stearic (C18:0). Ceramide acts as the backbone of more complex sphingolipids such as sphingomyelin (SM) cerebrosides, and gangliosides, and as such is an important resident of the plasma membrane of eukaryotic cells.

1.2 Phosphosphingolipids

SLs are typified by a higher level of complexity, and they can be formed by addiction of different moieties to the primary alcoholic function at C1 of ceramide (Cer). The ester bond of one or more phosphate groups to the 1-hydroxy group of a ceramide characterizes the subfamily of phosphosphingolipids. The simplest molecules in this group are sphingosine-1-phosphate (S1P) and Cer-1-phosphate (C1P) but the most prevalent phosphosphingolipid in mammalian tissues is SM which has a phosphocholine polar head group. Other forms of phosposphigolipids can be found in consistent quantities only in other type of mammals, fungi and plants.

1.3 Glycosphingolipids

Another important group is represented by glycosphingolipids (GSLs) which have a sugar head group linked with a β-glycosidic bond to the 1-hydroxyl of Cer [7]. In plants, the sugar residue is usually a simple sugar such as glucose, but in mammals the headgroup ranges from a simple sugar to a complex oligosaccharide chain (up to 15-20 sugars). Based on the charge of the headgroup, GSLs can be classified into neutral or acidic GSLs.

When the sugars are uncharged, the molecules are referred to as neutral glycosphingolipids. They are composed by three major lipids: glucosylceramide (GlcCer), galactosylceramide (GalCer), and lactosylceramide (LacCer), which mainly serve as simple biosynthetic precursors for more complex derivatives [8]. GlcCer and GalCer, also known as cerebrosides, are the

simplest GSL structures and consist of a monosaccharide head group attached at the 1-OH position of ceramide. The addition of galactose to GlcCer through a β-1,4-glycosidic bond produces LacCer, the basis for most of the complex GSLs.

Acidic GSLs are complex SLs characterized by a negatively charged head group. Based on the moiety involved, four categories of acidic GSLs can be identify: i) gangliosides, which possess one or more sialic acid groups; ii) glucuronoglycosphingolipids, which possess a glucuronic acid moiety; iii) sulfatoglycosphingolipids, which possess a sulfate group; and iv) phosphoglycosphingolipids, which possess a phosphate group [6,9]. Gangliosides are mainly present at the outer leaflet of the plasma membrane of neuronal cells. Their hydrophobic backbone is anchored into the plasma membrane, whereas the oligosaccharide head group rises above the cytosol for molecular interaction. The variability in the carbohydrate-based head group, the acyl chain length and the degree of saturation of ceramide gives rise to over 200 ganglioside structures [10].

Glucuronoglycosphingolipids are negatively charged because of the presence of a glucuronic acid, a sugar acid derived from glucose with C6 oxidized to a carboxylic acid. Until now, less is known regarding this GSLs subtypes since they are less expressed in humans but more prevalent in insects and plants.

Sulfatoglycosphingolipids are ubiquitously expressed in human tissues but with a higher concentrated in the nervous system. A common form of sulfatoglycosphingolipids is the sulfatide, which constitutes up to 4–6% of myelin lipids [11]. However, sulfatide has also been found in the kidney and small intestine [11]. Like other GSLs, sulfatide structure can vary according to acyl chain length and differences in ceramide structure.

2. Metabolism of sphingolipids

Despite the large number of components and structural differences, all SLs are originated and degraded by the same metabolic pathway converging in the production of Cer. The molecule can have two different origins: a) *de novo* pathway in which SLs give rise from non-sphingolipid precursors and b) the hydrolysis pathway arising from the degradation of complex SLs and in particular SM. The production of *de novo* SLs occurs in the endoplasmic reticulum (ER) with the generation of the sphingoid backbone. In this compartment the pyridoxal phosphate-dependent enzyme serine palmitoyltransferase catalyses the condensation between serine and palmitoyl-CoA to yield 3-ketosphinganine. SPT uses preferentially palmitoyl-CoA (C16:0-CoA) as substrate, which accounts for the prevalence of C18 long-chain base in the sphingolipids of plants, yeast, and mammals. However, small amount of sphingoid base consisting of 12-26 carbons atoms have been reported [12]. Suddenly 3-ketosphinganine is reduced by NADPH to form DHS in a reaction that is catalysed by 3-ketodihydrosphingosine reductase [13]. Depending on the organism, the molecule can then undergo further modifications such as hydroxylation, N-acylation, phosphorylation, and desaturation. In mammals, the backbone is usually N-acylated in the ER by ceramide synthase to produce dihydroceramide [14,15]. Finally, the dihydroceramide desaturase adds a C4–C5 *trans* double bond leading to the generation of Cer, a key node in sphingolipid metabolism [16–18]. Cer is transported into the Golgi apparatus where it undergoes different modifications to generate different types of SLs. While *de novo* synthesis occurs in the ER, further metabolism to generate SL derivatives occurs in the Golgi apparatus [19]. Once formed, Cer is transported from ER to the *trans*-Golgi region by the ceramide transfer protein (CERT) which mediates the non-vesicular trafficking of Cer for the synthesis of SM [20].

On the other side, the hydrolytic pathway arises from the degradation of more complex sphingolipids and in particular from SM, a phospholipid highly expressed in the external layer of the plasma membranes [21]. Following physiological membrane turnover, SM is split in

ceramide and phosphorylcholine by the activity of sphingomyelinases (SMase). SMases are enzymes classified in three major categories: acid, neural, and alkaline based on the optimal pH values of their activity [22]. In accordance with the pH optimum, acid SMase is present only in lysosomes, while neutral SMase is a cell membrane-associated enzyme largely expressed in almost all the cellular compartments [23,24]. While acid and neural SMases are widely distributed in all the mammalian cells, alkaline SMases are exclusively expressed in the intestinal mucosa and human bile [25,26].

Once formed, Cer can achieve enzyme modifications to generate different types of bioactive SLs. It can be metabolized in ceramide-1-P (C1P) by the ceramide kinase (CERK) which specifically phosphorylates Cer derived from SM degradation but not from *de novo* synthesis [27,28], or it can undergo the activity of the ceramidase leading to the formation of sphingosine (Sph). Again, Sph can be phosphorylated by Sph kinases (SKs) to S1P.

3. Sphingosine-1-phosphate

Together with phospholipids, sterols, and glycolipids, SLs form the plasma membranes. For long time, they were object of study for their main role in regulating the structure and activity of plasma membranes [21,29–31]. However, in the past three decades, some members of the SL family members have been recognized as bioactive compounds, playing a key role in both physiological and pathological conditions. An increasing number of evidence has shown that Cer, Sphingosine (Sph), S1P and C1P are involved in a large number of cellular functions [32–34]. In this context, S1P represents the main theme of the thesis and therefore will be further deeply described in the next chapter.

3.1 Sphingosine-1-phosphate metabolism

3.1.1 Synthesis

As mentioned above, S1P is produced from ceramide, by the ATP-dependent phosphorylation of sphingosine, catalysed by sphingosine kinases (SphKs). These enzymes play a key role in the metabolism of SLs and specifically in the regulation of S1P expression. Two isoforms of the enzyme have been cloned and characterized in mammalian cells, namely sphingosine kinase-1 (SphK1) and sphingosine kinase-2 (SphK2). Although SphKs activity has been detected in almost all the tissues [35], differences in tissue distribution and subcellular localization have been observed. Despite some small pools of SphK1 associated with the cytoskeleton and reticulum have been found, SphK1 is predominantly localized in the cytoplasm [36]. Following cell stimulation [i.e., activation of protein kinase C (PKC) or tumor necrosis factor-α (TNF-α) activation] SphK1 translocates to the plasma membrane in an extracellular signal-regulated kinase (ERK)-dependent pathway activation, which increases

enzymatic activity [37]. Otherwise, SphK2 localization appears to be more complex. SphK2 is expressed in the cytoplasm, where it is associated with ER but also with mitochondria [38].

However, the isoform 2 possesses both nuclear localization (NLS) and export (NES) signals [39] which enable it to shuttle in and out of the nucleus. Here the enzyme is able to modify the gene expression by directly inhibiting DNA transcription [40] or even by affecting the activity of histone deacetylase [41]. The importance of SphK1/SphK2 has been demonstrated using knockout (KO) mice. In 2005, Mizugishi and colleagues demonstrated that in mice, the simultaneous genetic ablation of SphK1 and SphK2 results in lower production of S1P which significantly affects both angiogenesis and neurogenesis resulting lethal at the embryonic stage [42]. However, knocking-down SphK1 or SphK2 solely did not show any pathological phenotype, suggesting that, despite the differences, there is redundancy between the two enzymes [42].

SphKs link S1P to the metabolism of SLs and therefore they are crucial to maintain the physiological ration between Cer and S1P. Changes in the intracellular amount of S1P may depend on the availability of Cer and Sph deriving from both *de novo* pathway and from the degradation of more complex SLs. For this reason, the intracellular amount of S1P is tightly tuned by the continuous degradation of the excess of S1P. Recent studies have revealed that Cer can contribute to the regulation of apoptosis, oxidative stress, insulin resistance, senescence, and growth arrest while S1P is more involved in pro-survival signals such as migration, proliferation, and also differentiation [43–46]. This metabolic equilibrium is known as “sphingolipid rheostat” and must be maintained for the phisiological cell homeostasis.

3.1.2 Catabolism

S1P can be degraded by two different pathways. The first, called the “salvage pathway”, leads to the regeneration of SLs. S1P can be dephosphorylated by lipid phosphatases (LPPs) or by

phosphatases1/2 (SPP1/2) producing Sph which can be converted back into Cer by the Cer synthase [47]. While LPPs are generic lipid phosphohydrolases, SPP1/2, discovered at the beginning of the century [48,49], are specific for SLs and lead to S1P inactivation. In human, silencing SPP1 results in intracellular accumulation of S1P and an its increased secretion in the extracellular space [50].

The second catabolic pathway involves the activity of S1P lyase (SPL) which catalyses the proteolytic cleavage between C2-C3 of the acyl chain. The reaction is irreversible and results in the formation of hexadecenal and ethanolamine [51].

3.1.3 Production

Under physiological conditions the highest amount of S1P is present in the blood. In this compartment S1P binds mainly to high-density lipoprotein and its concentration can reach approximately 200 nM in humans and 700 nM in mice [52–55]. Otherwise, only low-nanomolar amounts of S1P can be detected in tissue interstitial fluid [56]. Therefore, in mammals, a S1P gradient is constitutively maintained between vascular and extravascular compartments, thus playing a key role in controlling the trafficking of T-cells from lymphoid organs to the lymph.

For a correct immune response, new lymphocytes produced in the bone marrow and the thymus need to enter the lymphoid organ (spleen and lymph nodes), where the immune responses are generated. In inflammatory conditions, T-cells are induced to leave the lymph nodes by a mechanism that depends by the interaction of S1P with one of its receptors. In particular, in lymph nodes, S1P is secreted from lymphatic endothelial cells via the transporter spinster-like protein 2 (SPNS2) and guide the T-cell egression in a gradient-dependent [57].

The presence of a S1P gradient *in vivo* raises several questions about the cellular sources and the mechanisms involved in the homeostasis of the S1P gradient. For long time, the source of S1P, as well as the transport mechanism involved in its secretion, remained unclear. However,

growing number of studies have revealed that different cell types can secrete S1P with mechanisms that differ between various cell types.

3.1.4 Source

In the last decade, several blood cells subtypes have been proposed as preferential sources of circulating S1P, including erythrocytes, platelets, endothelial cells, mast cells, and macrophages.

In 2007, Ito and colleagued identified erythrocytes and platelets as the main sources of S1P producing about half of its total blood level [58]. The authors detected high SphK activity in platelets but no SPL one, which correlates with the high amount of stored-S1P found in these cells. Interestingly, they also demonstrated that erythrocytes lack both SPL and S1P phosphohydrolase. The absence of any degradation pathway may explain the ability of these cell to store S1P efficiently [58]. However, *nuclear factor erythroid 2 (Nfe2)*–deficient mice, despite having no circulating platelets, have normal plasma levels of S1P [59] suggesting that platelets may not be the main source of S1P under physiological conditions.

In the same years, the role of red blood cells (RBCs) as source of S1P in the blood was investigated using KO mice. Venkataraman and co-workers used the SphK triple allele KO mice [$SphK1^{-/-}SphK2^{+/-}$; SphK(3N)] to confirm whether S1P in plasma was derived from hematopoietic cells. Transplantation of wild-type (WT) bone marrow to KO mice was able to reconstitute plasma S1P levels, suggesting that WT hematopoietic cells are capable of releasing S1P [60]. Surprisingly, reconstitution of $Sphk1^{-/-}Sphk2^{+/-}$ bone marrow cells in WT hosts failed to reduce plasma S1P, proposing the existence of an additional non-hematopoietic source of plasma S1P. Based on this observation it was found that injection of adenoviral of *Sphk1* into the liver of $Sphk1^{-/-}$ mice was sufficient to restore plasma S1P levels to WT condition [60]. Analysis of the transgene expression unveiled positivity in both hepatocytes and endothelial

cells. However, only the latter has been found to release S1P *in vitro* [60]. The data suggest that even the endothelium may also be an important non-hematopoietic source of plasma S1P.

3.1.5 Transport

S1P can be transported to the outer side of the cells to elicit paracrine or autocrine effects. To this aim, the S1P produced intracellularly is exported with active or passive transport mechanisms. The high hydrophilicity due to the presence of the phosphate group impedes its diffusion through the plasma membrane. How S1P is exported is poorly understood, but several hypotheses are emerging. Both ATP-binding cassette (ABC) transporters and SPNS2 have been proposed as suitable transporters for S1P movements across the cell membrane.

ABC transporters are a group of membrane-associated proteins that mediate the ATP-dependent extrusion of various substrates including cytokines, lipids, and other hydrophobic and amphiphilic compounds from cells [61]. In human, most (approximately 50%) of the 48 ACB transporters mediate the transport of lipids or lipidic compounds, including phospholipids, cholesterol, and SLs [62]. The transporter-mediated S1P export has been described for the first time in rat platelets. The authors unveiled that in these cells S1P is not released by exocytosis, but through two possible mechanisms: one ATP-dependent carrier-mediated and one Ca^{2+} -dependent. The first depends on PKC-dependent pathway(s) that are activated by thrombin, and it is inhibited by a PKC inhibitor (staurosporine) and a ABCA1 inhibitor (glyburide). The second one is induced by Ca^{2+} influx and it is insensitive to either staurosporine or glyburide [63]. A few years later, the same authors further characterized this mechanism by revealing that S1P transport activity is an ATP-dependent event that does not require ATP hydrolysis [64]. Consistently, no significant changes in S1P levels were detected in *Abca1*, *Abca7* and *Abcc1*/*Mrp1* null mice, potentially suggesting the existence of compensatory mechanisms of transport mediated by other carrier [65].

Based on these new insights many works have suggested that SPNS2 may also be involved in S1P trafficking. SPNS2 belongs to the solute carrier family 22 (SLC22), a family of ion transporter within the major facilitator superfamily. The involvement of SPNS2 in S1P transport was first observed in experiments conducted in zebrafish [66]. Mutation of *SPNS2* gene has been found to induce a split heart phenotype (cardia bifida) resembling the phenotype observed in animals with a mutation in the sphingosine-1-phosphate receptor 2 (S1P2R) homologue [66]. In these mice, the injection of S1P in blastula-stage embryos was able to restore the cardia bifida, therefore confirming the correlation between SPNS2 and S1P. To further investigate this crosstalk, additional *in vitro* experiments were performed on Chinese hamster ovary (CHO) cells to examine whether the expression of zSpns2-EGFP could induce S1P export. The experiments were successful, showing a time-dependent export of S1P in treated cells [66]. Consistently, mass spectrometry analysis of blood and plasma of SPNS2-deficient mice showed lower levels of both S1P and dihydro-S1P compared to WT mice [67]. However, knocking out SPNS2 in mice is not sufficient to block the release of S1P from erythrocytes and platelets [68] suggesting that SPNS2 is involved in the release of S1P from endothelial cells but not from red blood cells (RBCs), where the characterization of S1P transport still remain ambiguous. More recently, in 2018, two separate research groups discovered the major facilitator superfamily transporter 2b (Mfsd2b) and proposed it as new candidate for S1P transport from erythrocytes and platelets. Mfsd2b is an orphan transporter that belongs to MFS transporter family and possesses 12 transmembrane domains. The Mfsd2b protein is found in bone marrow, spleen, and blood, where it is mainly present in platelets and RBCs [69]. Kobayashi and colleagues characterized the Mfsd2b as novel S1P transporter in nucleated erythroid cell line MEDEP-E14 [70]. In their experiments they observe a similar mechanism of S1P release by comparing cells expressing Mfsd2b or SPNS2. Since both proteins belong to the MFS transporters family, the authors proposed Mfsd2b as a new S1P transporter in erythroid cells [70]. Interestingly, in the same years, a lipidomic analysis of

Mfsd2- KO RBCs and platelets showed an intracellular accumulation of S1P, suggesting that Mfsd2b is an exporter, rather than an importer, of S1P. Accordingly, overexpression of Mfsd2b in humans and mice results in an increased S1P export from RBCs and platelets [69]. A few years later the same research group performed S1P transport assays on erythrocytes isolated from *Mfsd2b*-KO and *SPNS2*-KO mice. The results revealed that the erythrocytes isolated from *SPNS2*-KO mice showed normal S1P transport activity while *Mfsd2b*-KO erythrocytes displayed a significant reduction in S1P transport [71]. Consistently, the plasma level analysis of S1P from erythrocyte-specific KO of Mfsd2b was reduced by approximately 50% compared to WT mice [71]. Taken together the results support the hypothesis that Mfsd2b, rather than ABC or SPNS2 transporters, are responsible for the release of S1P from erythrocytes.

4. Sphingosine-1-phosphate receptors

Despite some new hypotheses about the intracellular activity of S1P [72–74], it is believed that the molecule is classically transported out of the cells to act by means of five subtypes of class A G protein-coupled receptors (GPCRs) located on the cell surface. In 1990, during the isolation of endothelial differentiation gene (Edg) clones from primary cultures of human umbilical vein endothelial cells, the Edg1 receptor was discovered [75].

The Edg receptors family [also known as lysophospholipid receptor (LPL-R)] consists of eight members and shares structural and functional features with the cannabinoid receptor group [76]. The endogenous receptor ligand remained unknown for nearly a decade leading to the classification of Edgs as orphan receptors [77]. Then, in 1998 independent research groups identified S1P as the high-affinity ligand of Edg1. Afterwards, from 1990 to 2000 other four Edg receptors (Edg3, Edg5, Edg6, and Edg8) were recognized as high-affinity receptors for S1P [78–80] and the orphan receptors were renamed sphingosine-1-phosphate receptor 1 (S1P1R; Edg1), S1P2R (Edg5), S1P3R (Edg3), S1P4R (Edg6), and S1P5R (Edg8) [81]. However, three of the Edg receptors show greater affinity for another lysophospholipid: the lysophosphatidic acid (LPA). Therefore the Edg family is now clustered into two subfamilies: the SIP receptors subfamily, which includes the receptors listed above, and the LPA receptors subfamily, which comprises LPA_1 (Edg22), LPA_2 (Edg4), and LPA_3 (Edg7) [77].

From a structural point of view, S1PRs share all the common characteristics of GPCRs. They possess seven α-helices transmembrane hydrophobic transmembrane domains (TM1–TM7) that pass through the phospholipid bilayer. On the outer side of the membrane, the receptors expose the N-terminal domain together with three extracellular loops (ECL; ECL1– ECL3) while at the inner side the C-terminus domain and other three intracellular loops (ICL; ICL1–ICL3) are present [82].

The receptors are widely distributed in all tissues. Particularly, in mammals, S1P1R and S1P3R have a widespread distribution in most cells, while the subtype four (S1P4R) is preferentially expressed on immune cells and the subtype five (S1P5R) in the skin, the central nervous system (CNS) and in natural killer cells. S1PRs couple different inhibitory and/or excitatory G proteins resulting in the activation of different intracellular signalling pathways. Taking into consideration that a single receptor subtype can couple G proteins that mediate opposite intracellular signals, the possibility exists that activation of a specific receptor may generate different/opposite effects, based on the G protein involved in the transduction mechanism.

4.1 Sphingosine-1-phosphate receptor 1 (S1P1R)

In 1990, in a study aimed at identifying immediate early genes that regulate the differentiation of endothelial cells, S1P1R was the first receptor recognized as target of S1P [75]. In mammals, S1P1R is expressed in nearly all tissues, including lungs, brain, and immune organs, where it controls many physiological and pathological functions. Differently from the other S1PR subtypes which are known to couple different types of G protein, S1P1R univocally links G_i protein [83].

S1P1R has been found to play a key role in vascular development in embryonic stages in which specific deletion of *S1P1R* on endothelial cells impairs vascular maturation [84,85], despite the expression of genes involved in vasculo- and angiogenesis (i.e., VEGF, Flk-1, Flt-1, Ang-1, and Tie-2) are not altered compared to WT animals [84]. In mutant mice at embryonic day 12.5 (E12.5) the aorta was not enveloped by vascular smooth muscle cells (VSMCs) as observed in control mice. Transverse sections of aorta from control embryos showed two to three layers of VSMCs surrounding the vessel. Differently in *S1P1R*$^{-/-}$ *mice* the aorta was poorly covered by mural cells [84]. A few years later, another research group demonstrated that in the aortas isolated at the embryonic stage of EC-specific S1P1R KO mice lack of VSMC coverage

compared to WT mice at the same developmental stage [85]. The authors also found that the S1P-mediated activation of Rac, which occurs in WT mice, was absent in S1P mutant mice. Since Rac is a key regulator of actin cytoskeleton and cell motility, they speculated that the lack of S1P1R inhibits the migratory capacity of EC.

S1P1R is also widely present in the immune system where they regulate the T-cells egression from the secondary lymph nodes [86–88]. The specific deletion of *S1P1R* in these cells (TCS1P1KO mice) increases the number of mature thymocytes specific for thymic emigration [87]. Furthermore, the results from *in vivo* migration assay with intra-thymic injection of FITC unveiled a reduction (-60%) of the number of thymic egression in TCS1P1KO mice compared to WT [87]. To investigate the signaling pathway involved in the process of T lymphocytes egression, Lo and colleagues (2004) demonstrated, using in a *in vivo* assay, that the lymphocyte egression from the lymph nodes depends on a processes involving a Pertussis toxin (PTX) - sensitive Gi protein [88]. Notably, once activated by S1P ligands, S1P1R interacts with Gi leading to the activation of several downstream molecules such as phospholipase C (PLC), phosphoinositide 3-kinase (PI3K), Ras guanosine triphosphatase (GTPase) and inhibits adenylyl cyclase (AC), that in turn reverberates on secondary signalling pathway including Rac GTPase, mitogen-activated protein kinase (MAPK), protein kinase B (Akt), and mammalian target of rapamycin (mTOR). Collectively, these observations support the involvement of S1PRs in controlling of T-cell egression[88].

4.2 Sphingosine-1-phosphate receptor 2 (S1P2R)

S1P receptor subtype 2 (S1P2R) was discovered few years after S1P1R, in 1993 [89]. Researchers first isolated a new cDNA clone from rat aortic smooth muscle, which encoded for a novel GPCR characterized by a high sequence homology to S1P1R. Similarly, S1P2Rs are widely expressed in the organism (muscle, ECc, CNS, immune system) and mediate various

physiological functions including cell proliferation and migration. Unlike S1P1Rs, S1P2R couples different types of G protein, namely G_i, $G_{12/13}$ and G_q [83]. S1P2R plays an important role in regulating the immune response. Knocking down S1P2R in mouse bone marrow-derived macrophages significantly reduces the release of proinflammatory cytokines [i.e., interleukin -1β (IL-1β), IL-6, and TNF-α] induced by the *A. actinomycetemcomitans*, a Gram-negative *bacterium* [90]. Interestingly, while S1P mediates the lymphocyte egression through activation of S1P1R, the ligand inhibits macrophage chemotaxis at the site of inflammation by acting at S1P2Rs [91]. The authors also investigated the signaling pathway involved in this process, suggesting that it could involve $G_{12/13}$ proteins, leading to the production of cyclic-AMP (cAMP) that in turn activates protein kinase A (PKA) and inhibits macrophages migration [91]. The inhibitory activity of S1P2R also occurs in VSMCs [92], where this receptor subtype predominates compared to the subtypes 1 and 3. The S1P2Rs reduces cell migration through a G_q and $G_{12/13}$ -mediated inhibition of Rac-induced signaling [92]. The exact role of S1P2R in the regulation of cell proliferation and migration is still matter of investigation, particularly for its possible implication in cancer. Indeed, implantation of Lewis lung carcinoma or B16 melanoma in S1P2R-KO mice causes an accelerated tumor growth and angiogenesis with enhanced association of VSMCs and pericytes. These *in vitro* results confirm that ECs isolated from S1P2R-KO exhibited enhanced Rac activity, Akt phosphorylation, cell migration and proliferation, suggesting S1P2R as a possible target for new anticancer treatment [93]. More recently (2020) a study involving colorectal cancer patients demonstrated a reduced expression of S1P2R in the mucosa but no difference in the expression of S1P1R and S1P3R subtypes [94].

4.3 Sphingosine-1-phosphate receptor 3 (S1P3R)

In 1996, a novel GPCR gene, typified by high homology with that of the gene encoding for the human S1P1R and rat S1P2R, was cloned. The gene encodes a protein named S1P3 receptor

(S1P3R) [78]. S1P3R shares some common features with S1P2R. Indeed, the receptor couples different G proteins, including the G_i, G_q and $G_{12/13}$ but not Gs [83]. S1P3R is expressed in almost all tissues, with higher presence in the heart, lung, kidneys, and spleen [95]. Like the other family member, S1P3R plays an active role in cardiovascular and neurological functions as well as immune responses. S1P3R promotes the mobilization of both monocytes and macrophages in mice suffering from atherosclerosis [96] and controls leukocytes rolling (crucial step of inflammatory cell recruitment) [97]. Of note, the leukocyte rolling ultimately dependent on P-selectin (a transmembrane protein that acts as a cell adhesion molecule) as blocking P-selectin with an antibody causes complete abrogation of leukocytes rolling in both KO and WT mice. Analyses carried out on human umbilical endothelial cells (HUVECs) revealed that *in vitro* treatment with 1 μM S1P resulted in rapid (5 min) insertion of P-selectin on the cell surface, while the application of a selective S1P1R agonist (SEW2871) was ineffective [97]. Overall, *in vitro,* and *in vivo* results identified S1P and its interaction with S1P3Rs as an essential step of immune cell recruitment. The cascade of events triggered by S1P3Rs was studied using ECs-deficient mice lacking the G_q on a global G_{11}-deficient background. These mice showed a significant reduction of leukocyte rolling efficiency, suggesting that the receptor mainly acts primarily via G_q-dependent signalling pathway [97].

4.4 Sphingosine-1-phosphate receptor 4 (S1P4R)

In 1998, the S1P4 receptor (also known as Edg-6) was first isolated from *in vitro* differentiated dendritic cells [79]. The S1P4R mRNA expression pattern is conserved in human and murine tissues. Unlike the other receptors, S1P4R mRNA is detectable in lymphatic and hematopoietic vessels but not in other compartments [79]. S1P4R can couple G_i and $G_{12/13}$ proteins but not G_s as suggested by the finding that intracellular cAMP production is not observed following receptor activation [98]. Intracellular signaling elicited by S1P4Rs was investigated in CHO

cells. Study i*n vitro* showed the activation of a PTX-sensitive PLC-mediated pathway following stimulation with 1 μM S1P, confirming the involvement of Gi-mediated signaling pathway [98]. Furthermore, the authors observed that S1P4R activation can induce cytoskeletal rearrangements, which would involve $G_{12/13}$ proteins since low nanomolar concentration of S1P induces the activation of the small GTPase Rho.

In contrast to other receptors, the specific expression of S1P4R by immune cells has revealed a main role of the receptor in the trafficking and differentiation of these immune cells [99].

4.5 Sphingosine-1-phosphate receptor 5 (S1P5R)

The sphingosine -1-phosphate receptor 5 (S1P5R) (Edg8 receptor) was the last S1P receptor to be described in the literature. In 2000, Im and colleagues uniquely identified this receptor as the fifth high-affinity S1P receptor [80]. While human S1P5R expression is elevated in the cardiovascular system, in rats its expression is limited to the brain, the spleen and the skin, suggesting a possible species-specific dependence of the physiological role of the receptor [100]. As far as its distribution in the CNS, S1P5R was initially proposed to be present in the white matter. However, more recently data have been provided showing its expression in almost all organs/tissues [101]. Interestingly, it has been reported that the receptor has the highest expression compared to other S1PRs in both benign and malignant tissues [101]. S1P5R couples either G_i or $G_{12/13.}$ In *in vitro* experiments on rat oligodendrocytes precursor cells (OPCs), activation of S1P5R has been shown to inhibit cell migration through the engagement of $G_{12/13}$ and the consequent activation of small GTP-ase Rho- Rho/Rho-associated protein kinase 1 (ROCK) signalling pathway [102]. Gi coupling has been further confirmed in CHO-K1 cells overexpressing the rat receptors. Here the activation of S1P5R with S1P inhibited the intracellular amount of cAMP in a PTX-sensitive mechanism, confirming the coupling with G_i protein [103].

5. Sphingosine-1-phosphate receptors in the central nervous system

Since their discovery, S1PRs have been extensively studied for their role in vascular development, cells migration and differentiation. However, their implication in CNS functions is so far poorly understood. Growing interest in this field began in 2010, when a pro-drug (fingolimod; FTY720) targeting S1PRs was approved as the first oral treatment for multiple sclerosis (MS). With the exception of S1P4R, all the S1PRs have been proposed as potential targets of the drug. Despite the differences in the degree of expression, all the receptors can be found in neurons, astocytes, oligodendrocytes, and microglia where their presence is largely cell-specific. Importantly, the expession level can vary depending on several factors, such as age, tissue specialization, differentiation stage, and external stimuli. Central S1PRs are involved in many different phisiological functions including chemical transmission, cell migration, differentiation and develepment.

In 2009, Meng and Lee analysed the presence of S1PRs in CNS mouse embryos, using *in situ* hybridization and reported that their expression level changes during development. S1P1R is most represented in the neuroepithelium of the brain and in the neuronal tube while S1P2R is preferentially detected in migrating neural cells crest. Interestingly low amount of S1P4R was detected while the S1P5R was expressed in branchial arches, and its expression increased in both peripheral and CNS between E11.5 and E16.5. This observation led the authors to suggest that S1P5Rs may be more involved in the later stages of differentiation in the CNS. In contrast, S1P3R was mostly detected in non-neuronal cells surrounding neural structures in the early stage of development, while in the later stages it was predominantly expressed within the trigeminal and dorsal root ganglia in cells that the authors suggested to be glial precursor cells [104].

5.1 Neurons

In the CNS, S1P plays a key role in neuron development, neurite extension and neuron survival [42,105,106]. However, over the past decade, a growing number of studies have confirmed that S1P directly participates in modulating membrane excitability, with particular emphasis on their role in controlling the efficiency of neurotransmitter release in different areas of the CNS.

In 2007, Kajimoto and colleagues investigated the involvement of S1P in controlling the release of neurotransmitters from rat hippocampal neurons. Based on the observation that SphK1 localizes at active presynaptic puncta, they demonstrated that glutamate release induced by a high-potassium depolarizing stimulus was significantly suppressed when cells were treated with dimethylsphingosine (DMS), an inhibitor of SphK1 [107]. They also investigated the effect of endogenous S1P on glutamate release, trying to understand which receptor subtype might be involved. Interestingly, silencing both S1P1R and S1P3R by administration of specific siRNAs resulted in an almost complete inhibition of S1P-induced glutamate release, suggesting that secretion is probably mediated by both receptors [107]. Although they did not investigate the mechanism underlying this event, the authors suggested a possible activation of a signaling pathway involving the Gi-Rac protein. The pathway is known to stimulate the fusion of synaptic vesicles to plasma membranes leading to the release of neurotransmitters [107]. Three years later, the same research group further investigates the physiological relevance of SphKs/S1P receptor signalling in rat hippocampal slices. S1P (0.1 μM) significantly increased AMPA-miniature excitatory postsynaptic currents (mEPSCs) in CA3 pyramidal neurons. However, the same concentration did not affect the rate of AMPA-mEPSCs recorded in CA1 pyramidal neurons, suggesting that S1P activity on glutamate release is closely related to the CA3 hippocampal region [108]. The increase in AMPA-mediated mEPSCs was blocked either by VPC23019 (0.3 μM), an antagonist of S1P1R and S1P3R, or by suramin (100 μM), a S1P3R-selective antagonist, suggesting the latter as the receptor primarily involved in this mechanism [108]. In support to the hypothesis, they also observed that AMPA-mediated mEPSCs in CA3

pyramidal neurons were reduced by the PLC inhibitor U73122 or the protein kinase C (PKC) inhibitor GF109203X. Since S1P3R, unlike S1P1R, couples the G_q protein which promotes the activation of the PLC-PKC pathway, the results lead to the conclusion that in the hippocampus S1P stimulates glutamate release through activation of S1P3R [109]. Consistently, in mice, S1P3R ablation reduced excitability in hippocampal neurons isolated from CA3 area, showing higher inter-spike intervals and lower input resistance than WT neurons [110]. However, application of S1P (1 μM) to CA_3-principal neurons results in a significant increase in the amplitude of eEPSC in both *S1P3*$^{-/-}$ and WT mice, with a slightly greater effect in the latter[110]. Therefore, the results suggest that beside S1P3R other S1PRs may contribute to control neuronal excitability and synaptic transmission in the hippocampal region. Interestingly, experiments performed in other regions of the CNS gave different results. In a study of 2009, S1P was shown to mediate inhibition of glutamate release from cortical neurons by activating S1P1R [111] suggesting a possible region- and receptor-dependent S1P activity. More recently, FTY720, a broad-spectrum S1PRs agonist, has been shown to inhibit, in a concentration-dependent manner, the 4-aminopyridine (4-AP)-induced glutamate release in isolated rat cortical nerve endings (synaptosomes [112]). The inhibition was PTX-dependent, suggesting that the effect involves S1P1R coupled to the PTX-sensitive Gi protein. The study also unveiled that the release activity in these particles was completely blocked by gallein, a Gβγ inhibitor, which was predictive of the involvement of Gβγ -PLC signaling pathway rather than PKA-cAMP way in the releasing activity [112].

Despite there is no clear evidence describing the mechanism by which S1PRs ligands modulate glutamate release, some papers have suggested the involvement of N-methyl-D-aspartate (NMDA) receptors [113,114]. Western blot analysis showed that in rat hippocampal slices, prolonged (1 to 4 h) exposure to FTY720-phosphate (FTY720P; 10 μM) increased GluN2B phosphorylation at Tyr1472 [114]. To investigate whether the increased level of phosphorylation was associated with an accumulation of NMDA receptor subunits in plasma

membrane, the authors performed a cell-surface biotinylation in hippocampal slices that were pre-treated for 3 hours in absence or presence of FTY720P (10 μM). While the amount of GluN2B in whole homogenates remained unchanged, the density of GluN2B and GluN1 subunits significantly increased in the surface fraction [114] suggesting that FTY720P modulates the membrane insertion of NMDA receptor subunits. In 2012, Rossi *et al.* demonstrated that *in vivo* treatment with FTY720 (0.3 mg/kg) modulates glutamate transmission in corticostriatal coronal slices of mice affected by experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Oral therapeutic administration of FTY720 resulted in reversal of clinical symptoms in EAE mice and restoration of impaired duration of sEPSC. Na^{+}/Ca^{++}exchangers and NMDA receptors presynaptically located were proposed as possible factors involved in controlling glutamate release efficiency and sEPSC frequency in EAE. Interestingly while presynaptic NMDA receptor-mediated activity was restored after *in vivo* administration of FTY720P, the impaired functioning of axonal Na^{+}/Ca^{++}exchanger did not recover, hinting a synapse-specific protective mechanism [115]. Therefore, the role of S1PRs as modulator of glutamate transmission in the CNS remains unclear. Differences in the outcomes elicited by S1P may depend on the area and on the neuronal population under study, as well as on the relative proportions of the S1PR subtypes. Furthermore, the response to S1P is highly dependent on the coupled G protein. When the Gi protein signaling pathway is involved, hyperpolarization of neurons and inhibition of neurotransmitter release are expected to occur. Conversely, activation of $G_{q/11}$ increases the intracellular Ca^{2+} concentration which in turn would fit better with an excitatory effect

5.2 Astrocytes

The relative expression of S1PRs in astrocytes is S1P3R > S1P1R > S1P2R > S1P5R [116]. The effect of S1PRs in these cells has been studied with regard to inflammation. Accordingly, in rat astrocytes, pre-exposure to FTY720P (1 μM for 1 h) prior to incubation with

lipopolysaccharide (LPS; 100 ng/mL) resulted in a reduction of the C-X-C motif chemokine 5 (CXCL5) release. Comparable results were obtained pre-treating the cells with a selective S1P1R agonist (SEW2871, 1 μM for 1 h) suggesting the involvement of this receptor subtype in astrocyte protection [117]. Other experiments were conducted on induced human astrocytes (iAstrocytes) to investigate the effect of siponimod (BAF312), a selective agonist of S1P1/5 receptors on nuclear factor-κB (NF-κB), a family of transcriptional factor that regulate the transcription of sevaral genes involved in the amplification of inflammatory responses preferentially in astrocytes. *In vitro* treatment with BAF312 (100 nM) inhibited the nuclear translocation of NF-κB induced by interleukin-17 (IL-17) and interleukin-1β (IL-1β; both 10 ng/ml), consistent with an anti-inflammatory property of the compound in iAstrocytes [119].

By the end of the last century a growing number of studies unveiled that astrocyte play a direct and active role in controlling the efficiency of neuronal network and in particular the efficiency of synaptic transmission. Starting from these observations, the term ‘tripartite synapse’ was coined to indicate the existence of a bidirectional communication between neuron and astrocytes [118–120].

In neurodegenerative diseases, neuroinflammation leads to impairment of glutamate transmission, favouring the release of aminoacid and concomitantly unbalancing the mechanisms of glutamate transport and reuptake. Normally, the amount of glutamate in the synaptic and extra-synaptic space is finely tuned by neuronal and astrocytic transporters. These events are particularly relevant as glutamate is the primary excitatory neurotransmitter in the CNS and its accumulation in the synaptic space leads to hyperexcitation and subsequent neuronal death. Altogether, the process is called “glutamate excitotoxicity” which is one of the hallmarks of many neurodegenerative and neuroinflammatory diseases. The mechanism of glutamate scavenger is therefore particularly relevant to prevent these pathological events. Two major glutamate transporters, namely the L-glutamate/L-aspartate transporter (GLAST) and the glutamate transporter-1 (GLT-1) are expressed on astrocytes and neurons and are implicated in

the reuptake mechanism. Notably, beside neurones, also astrocytes participate to hyper-glutamatergicity by releasing gliotransmitters which in turn modulate the neuronal component. Glutamate exocytosis in these cells is a Ca^{2+}-dependent event and involves molecular events comparable to those in neurons. In the early 1900s by using fluorescence imaging techniques the Ca^{2+} levels in astrocytes were monitored and the study revealed that astrocytes excitability strictly depends on changes in intracellular Ca^{2+} concentrations [120]. The role of S1P and its receptors in astrocytes has been partially investigated, but it is known that during inflammation, the expression of GLAST and GLT-1 in astrocytes is modified. *In vitro* studies conducted on iAstrocytes unveiled that both BAF312 and FTY720 prevent the cytokines-induced downregulation of glutamate transporters, indirectly hinting that S1P signaling has a role to recover pathological glutamate to physiological homeostasis in astrocytes [121]. Although there is no information on the effect of the S1P/S1PRs interaction in modulating glutamate release from astrocytes, some authors have highlighted the ability of S1PRs modulators to alter the intracellular amount of Ca^{2+} in these cells, which indirectly would support the thesis that S1PRs activation may be implicated in this event.

Specific activation of S1P1R (but not of S1P2R and S1P3R) on non-neuronal cell lines (HEK-293, CHO and RH7777) results in an inhibition of PTX-sensitive Ca^{2+} mobilization. The authors observed that prolonged activation of S1P1R inhibits the ATP-evoked Ca^{2+} release by inducing the activation of PKCs which are negative regulators of PLC [122]. Based on these observations, in 2013 the role of FTY720 in mediating Ca^{2+} mobilization in astrocytes was investigated [123]. Human foetal astrocytes were pre-treated with FTY720 (100 nM) and then stimulated with the proinflammatory cytokine IL-1β to induce the release of Ca^{2+} ions from intracellular stores. The S1PRs modulator inhibited the of Ca^{2+} mobilization with an effect that persisted also when the compound was applied daily for 5 days, confirming that also in astrocytes the S1PRs signalling is involved in the control Ca^{2+} ions mobilization. On primary rat astrocytes, *in vitro* treatment with S1PRs agonists generates a dose-dependent increase of

Ca^{2+} levels [124]. Interestingly, application of S1P induced an increased amount of Ca^{2+} through a cellular event that was PTX-independent [124].

5.3 Oligodendrocytes

Oligodendrocytes are myelinating cells of the CNS that undergo a specific program of proliferation, differentiation, and myelination to produce the insulating sheath of axons [125]. Therefore, they play a key role in controlling the conduction rate of the action potential, promoting saltatory conduction through the axons. In MS, an autoimmune demyelinating disease, the pathological process produces dysfunction and apoptosis of oligodendrocytes leading to demyelination and neurodegeneration. FTY720, a broad-spectrum S1PRs agonist, was reported to favour remyelination which is supposed to rely on events depending on S1P and S1PRs ligands acting at oligodendrocytes.

Oligodendrocytes develop from OPCs going through several differentiation stages and the expression pattern of S1PRs in these cells changes along the different stages of maturation. OPC displays preferential expression of S1P1R which increases during development [102,126–132] and lower levels of S1P2R, S1P3R, and S1P5R [102,127]. Changes in S1PR density highlight the potential to exploit S1PRs signaling to modulate distinct OPC and oligodendrocyte-specific processes. *In vitro* stimulation of S1PRs in both rodent and human oligodendrocytes promote cell survival [126,128,129], taking however into consideration that the protective activity of S1PRs- mediated signalling in oligodendrocytes is absent in OPCs. Downstream analyses demonstrated that S1PR signaling activates and phosphorylates extracellular signal-regulated ERK1/2 and Akt specifically in oligodendrocytes but not OPCs, protecting them from stress-induced apoptosis [126,128,129]. Interestingly, in mature human oligodendrocytes the cytoprotective role of S1PRs activation was mimicked by specific agonist of S1P5R, raising the possibility of attributing distinct characteristics of S1PR agonists to

subtype-specific activation [129]. Furthermore, silencing *S1P1R* attenuated the PDGF-induced proliferation of OPCs, showing that S1P1R and S1P5R exert different functions during oligodendroglial development [126]. *In vitro* studies have shown that S1PR activation triggered by low nanomolar concentrations of S1P and FTY720 results in enhanced OPC differentiation into both pre-oligodendrocytes and mature oligodendrocytes [126,133,134]. Treatment with FTY720 mediates the phosphorylation of ERK1/2, CREB, and p38 mitogen-activated protein kinase (p38MAPK) in bipolar $O4^+$ cells [135]. However, in more differentiated $O4^+$ cells, only p38MAPK was phosphorylated following treatment with the agonist [135]. Pre-treatment with mitogen-activated protein kinase (MEK) and p38MAPK inhibitors reduces agonist-mediated increase in $O4^+$ cells, but only inhibition of p38MAPK results in reduced differentiation of $O4^+$ cells in mature stage [135]. Overall, the results demonstrated that the ERK1/2 pathway regulates the OPCs maturation in pre-oligodendrocytes, but not the transition to mature oligodendrocytes, mainly mediated by p38MAPK activation. Interestingly, other studies have shown that S1P5R activation inhibits OPC differentiation, while activation of S1P1R improves the differentiation process [131]. Consistently, mice lacking the S1P5R subtype develop a regular pattern of myelination, while S1P1R KO mice exhibit reduced levels of myelin proteins, thinner myelin sheaths, and greater vulnerability to chemically-induced demyelination, highlighting more prominent involvement of subtype 1 in the context of myelination [130,136]. Similarly, deletion of S1P1R in the oligodendroglial lineage of mice leads to a delay in OPC differentiation into mature oligodendrocytes during early myelination [137]. It appears plausible that S1P1R, mainly expressed in OPCs, can play a pivotal role in early differentiation, while S1P5R, the most representative subtype in oligodendrocyte, may be involved in late myelination processes.

Although S1P5R subtype is expressed at lower levels in OPCs than oligodendrocytes, and no survival-promoting characteristics have been observed during following stimulating of the subtype in OPCs, activation of S1P5R by S1P blocks OPC migration in a transwell migration assay [102]. The reduced migratory capacity upon S1P5R activation is thought to be explained

by the commitment of Gα12/13 protein. Activation of these protein is coupled to the ROCK signaling pathway, which negatively affects the cellular migratory responses [102,138]. Consequently, the S1P- induced changed OPC migration was prevented when knocking down solely S1P5R [102]. The data are also in line with the physiological effect of S1P5Rs activation in mouse OPCs. *In vitro*, S1P induces a dose-dependent retraction of the cellular processes in pre-oligodendrocytes but it is devoid of activity in mature cells. The retraction is transient and it is mediated by activation of S1P5R and Rho kinase-mediated phosphorylation of collapsin response mediator protein [130]. Interestingly, S1P-mediated activation of S1P5R in mature oligodendrocytes, on the other hand, exhibits a pro-survival activity. Curiously, in this case, the effect is mediated by the activation of S1P5R but through a PTX-sensitive Gα/i-protein, which correlates with the activation of Akt signaling [130]. The results underline that the different functions of S1P5R activation during differentiation may be related to changes in coupling of the receptor with heterotrimeric G proteins, thus leading to the activation of different intracellular signaling pathways.

Although the main role of oligodendrocytes is to correct transmission of the electrical impulse, these cells have been observed to express glutamate transporters and may also participate in the control of glutamate homeostasis and neuron survival. In primary cell cultures of rat oligodendrocytes, the expression of metabotropic glutamate receptors (mGluRs) at different stages of differentiation was investigated. Western blot and immunohistochemical analyses confirmed that mGluR density is developmentally regulated and in particular they are highly expressed in day 0 and day 2 cultures, but are significantly downregulated at day 6 and 10 cultures [139]. Furthermore, in OPCs, activation of group 1 mGluRs, but not other groups, mitigates excitotoxic injury induced by kainate exposure, through the activation of PKCα [139]. Oligodendrocytes are also recognized as an important source of several neurotrophic factors such as glial cell line-derived neurotrophic factor, insulin-like growth factor-1 and transforming growth factor-b. The release of these molecules exerts a beneficial effect on the surrounding

cells in both physiological and pathological conditions. *In vitro*, oligodendrocytes-derived brain-derived neurotrophic factor (BDNF) mediates an increase in vesicular glutamate transporter (VGLUT)-positive neurons, mechanism which is counteracted by anti-BDNF antibody [140]. Reciprocally, glutamate induces the release of BDNF through metabotropic (but not ionotropic) receptors, activating the intracellular PCL signaling pathway [140]. Additionally, oligodendrocytes participate in the maintenance of glutamate homeostasis by mediating glutamate reuptake. The two major glutamate transporters GLAST and GLT-1 have been observed to be strongly expressed in cultured human foetal oligodendrocytes [141]. *In vitro*, blockade of GLT-1 with an inhibitor, results in a substantial block of glutamate reuptake capacity, suggesting that this mechanism is preferentially facilitated by GLT-1 [141]. Treatment with the proinflammatory TNFα leads to the reduction of GLT-1 receptors on oligodendrocytes and a parallel inhibition of glutamate uptake [141], suggesting that in inflammatory condition, impairment of oligodendrocyte function may also participate to glutamate excitotoxicity.

This latter observation in especially important in the context of MS where the damage to oligodendrocytes and myelin sheaths impairs synaptic transmission. Therefore, target oligodendrocytes and OPCs could be beneficial not only for myelination and remyelination processes but could also be of interest as indirect target to regulate glutamate amount with is largely increased in the disease.

5.4 Microglia

Microglia are macrophage-like immune cells resident in the CNS, but despite some functional and structural similarities, they do not express cellular markers typical of peripheral macrophages and unlike them, they are relatively inactive under physiological conditions. As a result of injury or alteration of CNS homeostasis, microglia can rapidly turn into an active state. This condition is characterized by cell migration, differentiation and changes in cell morphology, as well upregulation and overproduction of cytokines and inflammatory mediators

[142]. In the inactive state, microglia possess a branched morphology characterized by cytoplasmic processes and protrusion. Then, as a result of inflammatory stimuli or brain injury, the shape of microglia changes from ramified to amoeboid, rapidly transforming into M1-like polarized cells. Beyond their immunological role, microglia cells also participate in brain development, controlling neurons survival, proliferation and modulating the function and number of synapses, playing an active role in synapses pruning [143,144]. Interestingly, microglia play an important role in the control of neurotransmission hence the concept of "Quad-partite" synapse. *In vitro* experiments unveiled that microglia express receptors specific for different transmitters such as acetylcholine, GABA, and glutamate so that the release of neurotransmitters can affect microglia by modifying the membrane potential, the release of cytokines and also the intracellular level of Ca^{2+} [145]. Activated microglia has been observed to produce glutamate from extracellular glutamine and it is able to release it through hemichannel of gap junction [146]. This is suggested by the finding that carbenoxolone (CBX), a gap junction blocker, significantly attenuates the release of glutamate from microglia and ablates neuronal cell death in a dose-dependent manner [147]. *In vitro,* activation of LPS-induced microglia releases a large amount of glutamate which could participate to excitotoxicity [147]. The role of microglia and astrocytes in controlling the amount of extracellular glutamate at the early stages of inflammatory diseases was studied *in vitro* using a mixed culture consisting of astrocytes, neurons, and microglia. L-glutamate (L-Glu) uptake was significantly reduced after 72 hours of treatment with a low concentration of LPS (10 ng/mL), which is sufficient to activate microglia but no other cells. The authors used a pharmacological approach to determine the role of L-Glu transporter in controlling glutamate reuptake [148]. Inhibition of GLAST (with UCPH-101) but not GLT-1 increased the L-Glu amount in a concentration-dependent manner, confirming that GLAST and not GLT-1 was mainly involved [148]. The results also unveiled no change in GLT-1 density but a significant reduction of GLAST in astrocytes [148].

Furthermore, application of CBX suppressed L-Glu release from activated microglia and reversed the LPS-induced downregulation of GLAST [148]. This is consistent with the view that the increased extracellular L-Glu level was sustained by activated microglia which releases L-Glu following direct LPS-stimulation [148]. The authors proposed that microglia play a direct role in controlling the amount of glutamate in pathological condition, releasing glutamate thought the hemichannel of gap junction and consequently inducing the downregulation of glutamate transporters on astrocytes.

Recently, FTY720 has been proven to be effective in human patients for the treatment of cerebral ischemia. Since the pathology is closely linked to microglia activation, a growing interest in S1P-S1PR crosstalk in microglia has emerged in recent years. In rat microglia cell cultures, S1P1R and S1P3R mRNA expression is relatively high, and it is significantly reduced after stimulation with LPS (0.1 μg / mL) or S1P (20 μM). Conversely, low levels of S1P2R mRNA were detected in both control and LPS-activated cells while only a very low amount of S1P5R mRNA was detected in acutely isolated cells, further reduced in the activated state [149]. *In vitro* experiments in BV2 microglia cell line showed that, following LPS stimulation, S1P increases the expression of inflammatory mediators (i.e., TNF-α, IL-1β, and iNOS) [150]. Similar results were obtained *in vivo.* Microinjection of S1P (1 nmol/0.5 μL) into the brains of adult mice activates microglial cells, resulting in in an increase in the number of Iba1-immunopositive cells compared to vehicle-injected controls [151]. Interestingly, pre-treatment with FTY720 (3 mg/kg) reduced the neuroinflammatory outcomes [151]. A study aimed at evaluating the S1PR subtype involved in the inflammatory mechanism revealed that both S1P1Rs and S1P3Rs have a pathogenic role in the activation of microglia [152,153]. Specifically, S1P3R is associated with microglia activation, proliferation and it supports the shifting into the amoeboid shape. Indeed, S1P3R regulates microglia polarization to the "M1-like" phenotype, the proinflammatory and toxic form of microglia. In mouse model of transient middle cerebral artery occlusion and reperfusion (tMCAO), *in vivo* administration of

CAY10444, a selective S1P3R antagonist, reduces the number of M1-polarized cells and inhibits microglia proliferation. The results were further supported by *in vitro* experiments in which suppression of S1P3R activity by S1P3R-specific shRNA lentivirus (or by a pharmacological antagonist) decreases the mRNA expression level of M1-soluble markers (i.e., TNF-α, IL-6, and IL-1β) [152]. In this animal model, therapeutic oral administration of a selective S1P1R antagonist (AUY954) as well as knocking down the S1P1R with its specific shRNA lentivirus abolished brain damage after tMCAO, confirming the pathogenic role of S1P1R in activated microglia [153]. More recently, S1P2R has been identified to mediate ischemic brain injury [154]. Deletion of S1P2R drastically reduces the number and proliferation of activated microglia and reverse the morphology of these cells to the inactive, branched shape. The molecular mechanisms underlying these events were further investigated *in vitro* on BV2 microglia cell line. LPS-induced M1 polarization is characterized by increased phosphorylation of ERK1/2, p38 MAPK, and JNK MAPK. Intriguingly, pharmacological inhibition of S1P2R using a selective antagonist (JTE013) attenuates the phosphorylation of ERK1/2 and JNK without affecting p38 MAPK [154]. Administration of the same antagonist *in vivo* leads to comparable outcomes, arguing that S1P2R influenced M1 polarization through ERK1/2 and JNK pathways in post-ischemic brain. Overall, the results suggest that S1P and S1PRs are directly involved in microglia activation and therefore that this signaling pathway can be considered as an indirect pharmacological target in all the neurodegenerative disease in which microglia activation is involved.

6. Multiple Sclerosis

6.1 General overview

MS is chronic inflammatory autoimmune disease characterized by the presence of demyelinated plaques in the CNS that cause axonal pathology and progressive neurological deterioration. The onset of the disease peaks between the ages of 20 and 40, and women are affected two to three times more often than men (sex ratio 2,5:1).

The multifocal zone of inflammation caused by T-cell and macrophage infiltration leads to the disruption of the myelin sheath and death of the oligodendrocytes. This mechanism results in the formation of plaques composed of inflammatory cells and demyelinated axons in both white and grey matter that alter the transmission of nerve impulses and lead to neuronal dysfunction [155]. Inflammatory plaques, determined histologically or by magnetic resonance imaging (MRI), are the main pathological hallmark of MS. The disease engages progressive and unpredictable episodes of axonal demyelination resulting in lesions along myelinated axons of nerve fibres in brain, brainstem, spinal cord, and optic nerves [156]. The loss of myelin sheaths causes alterations in the conduction of electrical potentials along neuronal processes in the CNS. In particular, in demyelinated areas electrical conduction is characterized by reduced speed, temporal dispersion of impulses, and conduction failure [157].

6.1.1 Classification

The previous classification of MS was based on the old concept that two distinct MS phenotypes exist, one characterized by high inflammatory levels (relapsing-remitting) and the other typified by neurodegeneration (progressive). However, it is now widely recognized that axonal and neuronal loss occur starting from the early stages of the disease, leading to an early cognitive impairment. Likewise, patients with progressive forms of MS may have evidence of inflammatory activity through new MRI lesions [158]. Since the distinction of MS subtypes is

essential in determining the therapeutic approach, the classification has been revised considering these new insights.

6.1.1.1 Clinically isolated syndrome

The term clinically isolated syndrome (CIS) is commonly referred to a first episode of neurological disturbance (exacerbation or attack) caused by inflammation or demyelination in the CNS [159]. CIS can be monofocal or multifocal based on the number of lesions that characterize the attack. CIS, by definition, is generally isolated in time and space (monofocal) and patients experience a single neurologic sign caused by a single lesion in the optic nerve (a common feature reported in many CIS cases), in the spinal cord, in the brainstem or in the cerebellum. However, in some CIS cases, the clinical hallmarks are disseminated in space (multifocal) i.e., optic neuritis with an extensor plantar response or simultaneous optic neuritis and internuclear ophthalmoplegia [159]. A patient is classified as having CIS when there is clinical evidence of a single relapse, and the MRI does not fully meet RRMS criteria. While in some cases clinical symptoms remain an isolated episode in CIS patients, in others CIS converts to MS. In approximately 85% of patients who develop MS, the disease is preceded by acute neurological symptoms owing to a single demyelinating lesion [160].

6.1.1.2 Relapsing-remitting MS

Relapsing-remitting MS (RRMS) is the most common form of MS (about 85% of patients with MS) and it is characterized by attacks to the myelin by the immune system. The relapsing-remitting course is defined by acute relapse (or attack) followed by a partial/complete recovery (referred to as remission) with periods of relative clinical stability in between. The attack is defined as a neurologic damage associated with an acute inflammatory demyelinating event that lasts at least 24 hours [161]. During the period of remission, symptoms may disappear or become permanent, but no apparent progression of the disease is observed. Based on the

presence or absent of new MRI activity, this pathological form can be further sub-classified as active or inactive respectively. Additionally, RRMS can be classified as worsening or non-worsening if there is an increased disability due to an incomplete recovery following a relapse phase. The diagnosis of RRMS begins with the observation of some typical symptoms (i.e., optic neuritis, sensory and/or motor manifestations of myelitis) along with signs related to demyelinating lesions supplemented by imaging coherent with the presence of diffuse multiple lesions.

6.1.1.3 Primary-progressive MS

Primary-progressive MS (PPMS) relates to patients who suffer from deterioration of neurologic functions from the onset of symptoms without relapses or remission. In general, PPMS may be characterized by a stable period with or without relapse as well as a period in which symptoms worsen with or without new relapses or lesions. Approximately 15% of people with MS are diagnosed with PPMS and the average age of onset is about 10 years later than that of the relapsing form. Patients with PPMS experience more severe symptoms than those with RRMS. Beyond the most common symptoms (i.e., bouts of fatigue, numbness, vision problems, spasticity or stiffness, bowel and bladder problems, and problems with learning, memory or information processing) people with PPMS are more likely to have disabilities in walking and mobility.

6.1.1.4 Secondary-progressive MS

Most patients with RRMS develop secondary progressive MS (SPMS), in which there is a progressive worsening of neurologic functions. Usually, SPMS is diagnosed retrospectively following observation of gradual worsening after an initial relapsing phase and it occurs in up to 40% of patients by 20 years after the initial event. Several studies have revealed that inflammation is less present in SPMS compared to RRMS, resulting in smaller number of lesions and fewer levels of inflammatory infiltrations in the plaques. Furthermore, patients with SPMS display a more lesions in the spinal cord that in the brain compared in the other forms of

MS. To date, there are no clear clinical, imaging, immunological, or pathological criteria to establish when RRMS converts to SPMS as the transition is generally gradual [162]. Therefore, numerous studies are underway to look for potential biomarkers or imaging techniques that distinguish SPMS from RRMS or possibly predict the transition from RRMS to SPMS, although the biomarker currently suggested requires further validation prior to clinical use.

6.1.2 Etiology

So far, the etiology of MS remains unclear although both genetic predisposition and environmental cues appear to have a role. Abnormalities in different cell types have been observed in the immune system, as well as in the CNS of MS patients, and therefore the identification of primary and secondary events remains challenging. MS is mainly recognized as an autoimmune disease as pathogenic central T lymphocytes that are reactive against myelin antigens and myelin-forming cells (oligodendrocytes) have been found in brain lesions of MS patients. Furthermore, peripheral autoreactive T cells cross the blood-brain barrier (BBB) and start to produce inflammatory cytokines facilitating the effects of other inflammatory mediators such as B cells and macrophages, also promoting the production of antibodies against neurons and their myelin sheaths and axons [163]. The pathological process leads to the formation of demyelinating plaques with consequent neuronal loss and neurodegeneration. *In vivo*, both antibodies and complement accumulation were found in MS lesions in early active chronic lesions [164]. For this reason, T lymphocytes and particularly $CD4^+$ T helper have long been considered the primary immune drivers in MS. However, this notion has been reassessed in recent years and the role of innate immunity, B cells and $CD8^+$ T cells has emerged. In this context, recent clinical trials with anti-CD20 antibodies that deplete B-cells have been successful, demonstrating the key role of B-cells in the course of MS. Although the mechanism of action of B-cells remains unknown, it is likely to control autoantibody secretion, immunomodulation, and autoantigen-specific antigen presentation. Activation of B-cells was detected in over than 95% of MS patients. Interestingly, markers of B-cells activation have been

found in the cerebrospinal fluid (CSF) of MS patients and are correlated with the onset of relapse and disability progression [165]. The loss of any clear clinical signs indicating the transition from CIS to MS makes this correlation relevant not only for understanding the pathogenetic mechanism underlying MS, but also in the prospect of seeking a predictive marker. Interestingly, the increase of both mature B-cell and plasma blast in the CSF is related to increased MRI lesions and disease progression and it appears to be faster in oligoclonal bands (the activated form of B-cells)-positive patients compared to negative ones [165].

Recently, the lack of adequate vitamin D has been taken into consideration as a possible factor implicated in the development of MS. The ultraviolet light from the sunlight induces the photosynthesized form of vitamin D from the dermal layer of the skin. Vitamin D preserve the balance of calcium in the body, and it is essential for keeping the proper function of many organs and system, including the immune system. Interestingly, low levels of vitamin D and its metabolites (25-hydroxyvitamin D [25(OH) D] and 1,25- dihydroxyvitamin D [1,25(OH)2D]) are associated with a higher risk to develop MS [166,167]. In 2008, a cross-sectional study was conducted to measure the blood levels of two major metabolites of vitamin D: 25-hydroxyvitamin D [25(OH) D] and 1,25- dihydroxyvitamin D [1,25(OH)2D] [166] in MS patients. Consistent with previous findings, the serum level of both metabolites was significantly lower in the progressive phenotype compared to RRMS patients [166]. However, only low levels of 25(OH)D have been associated with the occurrence of relapses as well as to an increase in the Expanded Disability Status Scale (EDSS) score. Although the association with relapse rate did not reach statistical significance, a higher level of 25(OH)D was found in patients with RRMS without relapses compared to patients with relapses despite comparable EDSS scores [162]. Based on this correlation vitamin D was tested for its potential therapeutic activity in EAE mice. Oral or intraperitoneal administration of vitamin D has been found to relieve the clinical symptoms in EAE. In 2010, Chang and colleagues reported that D3 (1,25(OH)2D3) reduced the differentiation and migration of $CD4^+$ T-helper 17 cells in EAE

mice [168]. Moreover, a significant decrease in the number of mature splenic dendritic cells was observed after administration of 1,25(OH)2D3 [169].

6.2 Multiple sclerosis and glutamate transmission

In the recent years, an increasing number of studies have focused on altered glutamate transmission as a key factor in the etiopathogenesis of MS, particularly for its potential role in promoting neurological symptoms that develop during the course of the disease. Therefore, despite the clear immunological component of the disease, MS has recently been recognized as a synaptopathy [170]. The term is even more used to define disruption of synaptic structure and function as a key factor in many psychiatric and neurodegenerative disorders [171]. Damage to the grey matter occurs early in MS and appears to be partially independent of demyelination. Analyses conducted on *post-mortem* brain of MS patients have shown synaptic damage typified by the loss of both pre- and postsynaptic components [170]. In MS patients, as well as in EAE mice, the most commonly used animal model for MS, both glutamatergic and GABAergic system are compromised [172–174]. This pathological condition generates an accumulation of glutamate in the synaptic cleft resulting from an alteration of many factors including the mechanism(s) of release, metabolism, or uptake. The accumulation of glutamate leads to an increased and excessive activation of glutamate receptors (GluRs) and then to excitotoxicity that collaborates with immune cells to damage neurons and surrounding cells [170].

Both the immune and nervous systems participate in increasing the glutamate concentration in the CNS of MS patients. Beside neurons, activated astrocytes, macrophages and microglia also release glutamate providing a continuous supply of neurotransmitters and contributing in general to excitotoxicity. In 2009, Centonze and colleagues demonstrated that alteration of excitatory transmission occurs early in the striatum of EAE mice [175]. In this area, anomalies in the amount of glutamate downregulate early gene *Arc/Arg3*, which is involved in the control

of the trafficking of AMPA [175]. In conjunction with gene downregulation, the authors observed an increase in the expression, phosphorylation, and activity of AMPA receptors [175]. Early and acute alterations of excitatory neurotransmission also occur in other areas of the CNS in EAE mice at different stages of the disease [176]. Interestingly, early presynaptic deficit (detected as a reduction in glutamate overflow) was detected in the cortex of EAE mice and may be related to early neuropsychiatric manifestations. On the other hand, in the acute stage of the disease, an impairment of glutamate transmission occurs in both the cortex and the spinal cord. However, immunohistochemical results unveiled that only in the latter region the alteration is associated with demyelinating and high inflammatory levels [176]. Of note, unlike the cortex, glutamate exocytosis was amplified and not inhibited in the nerve endings of the spinal cord [176,177].

In this altered context, attention has focused on the glutamate reuptake system, which may play an important protective role during MS. GLT-1 and GLAST are the two receptors primarily involved in glutamate reuptake. However, contrasting results were obtained. In the forebrain and cerebellum of EAE mice the expression level of GLT-1 mRNA increases in both brain regions despite appearing earlier in the forebrain than in the cerebellum [178]. The increase may appear contradictory to the excitotoxicity that characterizes MS. However, this could reflect a compensatory mechanism activated to counteract the increase in glutamate amount that occurs during EAE, aimed at preventing cell damage. Interestingly, the authors found that the increased mRNA expression occurs before the onset of neuropathological symptoms, confirming the hypothesis of early impairment of glutamate transmission in MS. In contrast, at the same stage, the protein density of GLT-1 was found to be slightly increased in the forebrain but significantly decreased at the acute stage. Similarly, in the cerebellum, the density of GLAST and GLT-1 decreases just before the acute phase of the EAE [178]. Intriguingly, in samples derived from *post-mortem* optic nerves from MS patients, transcripts and protein densities of both receptors are increased [179]. The enhanced expression could reflect an

adaptive molecular change to limit excitotoxicity of glutamate and subsequent neurodegeneration. In line with impaired glutamate exocytosis and reuptake, glutamine synthase and glutamate dehydrogenase, involved in glutamate catabolism, are also downregulated in the white matter of both EAE and MS patients [180].

The close relationship between inflammation and neurotransmission plays a fundamental role at all stages of the disease. Until a few years ago, inflammation-driven demyelination was thought to develop mainly during the first stages of MS concomitantly with episodes of reversible neurological deficit (relapses), while the later stages were mainly characterized by inflammation-independent neurodegenerative processes [181]. However, the new disease modified therapies developed in the last decade for treating MS, which target inflammatory demyelination, reduce the frequency of relapses but also improve some of the cognitive impairments and neurobehavioral abnormalities associate with MS. These findings suggest that target inflammation can indirectly restore the impaired neurotransmission even in the early stage of the disease.

6.3 Sphingosine-1-phosphate receptors as modulators for the treatment of multiple sclerosis

In 2010, FTY720 was approved as the first oral treatment for RR form of MS. The compound and all derivatives are structurally analogue of the endogenous S1P and are considered agonists of S1PRs. However, their mechanism is usually referred to as a “functional antagonism,” since binding to S1PRs causes receptor internalization and ubiquitylation following by receptor degradation [182], thereby reducing the receptor-mediated effects. This functional antagonism has been recognized as the main mechanism underlying the immunomodulatory effect of the molecules. In inflammatory conditions, an increase amount of S1P proteins in the blood and enhanced density of S1P1R on T- lymphocytes are observed. Since S1P/S1P1R interaction is

known to induce egression of lymphocytes from the secondary lymph nodes, FTY720-induced internalization of S1P1R generates overall lymphopenia which in turn reduces infiltration of T cells into the CNS [183]. Although functional antagonists are expected to mediate inhibition of S1PRs-mediated signaling, some contradictory results are described in the literature. In 2009 an *in vitro* study on CHO cells demonstrated that FTY720 triggering the S1P1R internalization causes persistent downstream activation of the S1P-dependent intracellular signaling [184]. However, in a study conducted on endothelial cells, it was observed that after internalization, S1P1R translocates to the nuclear compartment to regulate the transcription of various growth factors [185]. Comparable re-localization was observed in activated T lymphocytes, where nuclear translocation of S1P1R determines the transition from a promigratory to a proliferative activity [185]. While these processes are not completely clarifying, they add new knowledge about the potential effects of S1PR modulators.

Interestingly, the ability of FTY720 and its derivatives to cross the BBB has given rise to a growing number of studies focusing on a possible direct central role of S1PR modulators. While the researchers have drawn attention on the effect of these compounds on demyelination, remyelination and OPC, some work also focussed on the effect on neurotransmission.

6.3.1 FTY720 (fingolimod)

To date, four S1PRs modulators have been approved by the US Food and Drug Administration (FDA) for the treatment of different forms of MS. The first compound is FTY720, approved for the treatment of RRMS in 2010. It a pro-drug that in mammals is phosphorylated by SphK2 to form bioactive FTY720P. Due to the similarity of chemical structure to the endogenous S1P, FTY720P binds to four out of five S1PRs, showing high affinity for S1P1,3 and 5 receptors, low affinity for S1P4R, and no activity towards S1P2R [186] (Table 1). In EAE rats, FTY720 rapidly crosses the BBB and is phosphorylated *in loco* by CNS cells. Here the concentration of

FTY720P remains stable, even creasing over time [187]. The ability of FTY720 to reach high concentrations in the CNS makes studying its potential direct effect intriguing. In 2013, Di Menna and colleagues demonstrated that FTY720 protects cortical neurons against excitotoxicity by acting directly on neurons [113]. The neuroprotective effect FTY720 and FTY720P was assessed *in vitro* before, during, or after an excitotoxic pulse (NMDA for 10 min). The two compounds were neuroprotective when applied 18-20 hours prior to the NMDA stimulus. The positive effect was attenuate by PTX and completely abrogated by S1P1R antagonist, suggesting the involvement of this receptor subtype in neuroprotection [113]. Other studies have suggested that FTY720 indirectly provides neuroprotection by stimulating the production of neurotrophic factors, such as BDNF, leukemia inhibitory factor (LIF) and the heparin-binding EGF-like growth factor in neurons and astrocytes [188,189]. *In vivo* experiments on EAE mice have shown that oral administration of FTY720 rescues synapse deficits [112,115,190]. Rossi and colleagues, demonstrated that oral prophylactic administration of FTY720 (0.3mg/kg) reduced the clinical symptoms of EAE mice and prevented the alteration of sEPSC shape that characterized MS [115]. However, FTY720 was unable to modulate the frequency, amplitude, and kinetic properties of sEPSCs recorded by the control neurons, suggesting that only pathological process associated with inflammation was sensitive to the compound. In EAE mice, oral FTY720 was able to restore presynaptic and postsynaptic alterations of glutamatergic transmission and to promote recovery of dendritic spines. Regarding the glutamate exocytosis it has been demonstrated that *in vivo* prophylactic FTY720 efficiently restored the presynaptic impairments in EAE mice [190]. The efficiency of both GABA and glutamate overflow was restored following FTY720 treatment to levels comparable to those observed in control, non-immunized, mice [175].

Name	Receptor affinity		Indication	Approval (year)	References
	High	Low			
FTY720 Fingolimod Gilenya Novartis©	S1P1R (EC_{50} = 0.3 nM) S1P3R (EC_{50} = 0.9 nM) S1P5R (EC_{50} = 0.50 nM)	S1P2R (EC_{50} >10000 nM) S1P4R (EC_{50} =345 nM)	RRMS	2011 (EMA)	[191–193]
BAF312 Siponimod Mayzent Novartis©	S1P1R (EC_{50} = 0.39 nM) S1P5R (EC_{50} = 0.38 nM)	S1P2R (EC_{50} >10000 nM) S1P3R (EC_{50} >1000 nM) S1P4R (EC_{50} >750 nM)	CIS RRMS SPMS	2020 (EMA)	[194–197]
RPC1063 Ozanimod Zeposia Celgene©	S1P1R (EC_{50} = 0.41 nM) S1P5R (EC_{50} = 11 nM)	S1P2R (EC_{50} >10000 nM) S1P3R (EC_{50} >10000 nM) S1P4R (EC_{50} >7 nM)	CIS RRMS SPMS	2020 (EMA)	[193,198]
Ponesimod (ACT-128800) Actelion©	S1P1R (EC_{50} = 5.7 nM) S1P5R (EC_{50} = 11 nM)	S1P2R (EC_{50} >10000 nM) S1P3R (EC_{50} >10000 nM) S1P4R (EC_{50} >7000 nM)	RRMS	2021 (FDA)	[199]

Table 1. Sphingosine-1-phosphate receptor modulators approved for the treatment of multiple sclerosis (MS). Characteristics of sphingosine-1-phosphate receptor (S1PR) modulators: receptor affinity; year of approval by European Medicines Agency (EMA) or the Food and Drug Administration (FDA); indication for MS treatment [clinically isolated syndrome (CIS); relapsing-remitting MS (RRMS)and secondary progressive MS (SPMS).

Although the mechanism underling these events need to be further investigated, the hypothesis is that FTY720 can directly modulate chemical transmission by acting presynaptically. Accordingly, *in vitro*, FTY720 inhibits in a concentration-dependent manner the release of glutamate induced by 4-AP in rat cerebrocortical synaptosomes [112].

6.3.2 BAF312 (siponimod)

Siponimod (BAF312) is a second-generation modulator of S1PRs, approved in 2019 by the FDA for the treatment of CIS, RRMS, and acute SPMS. BAF312 does not require phosphorylation to be activated and, unlike FTY720, it binds with nanomolar affinity only S1P1R and S1P5R and exhibits low affinity towards the other receptors (Table 1) [200]. BAF312 carries out the immunomodulatory effects mainly by reducing the number of

circulating lymphocytes [200,201]. However, some new insights suggest its direct involvement in the control of CNS activities as it has been demonstrated that BAF312 penetrates rodents CNS and protects from the development of EAE [202]. In a modified EAE model with cytokines-mediated induction of cortical lesions (focal autoimmune encephalomyelitis**)** intracerebral administration of BAF312 (3mg/kg) improved cortical network functionality, without inducing lymphopenia suggesting an inflammation-independent mechanism of action.[203]. Similarly, in the EAE mouse model, the continuous intracerebroventricular infusion of BAF312 (0.45 μg/day) exerted a positive effect on the imbalanced synaptic transmission that occurs during the disease. Both *in vivo* and *in vitro*, BAF312 enhanced the inhibitory tone by increasing GABA transmission and by promoting the survival of GABAergic interneurons [204]. Physiologically, glutamate homeostasis at the synaptic cleft is also maintained by the GLAST and GLT-1. Their role is to uptake the glutamate from the synaptic cleft and keep its extracellular concentration below neurotoxic levels. However, a high level of inflammatory mediators leads to decreased receptors' expression that would result in an increased bioavailability of glutamate. In this context, it has been recently observed that, in human iPSC-induced astrocytes, the treatment with high concentrations of BAF312 (100 nM) maintains the expression of GLAST and GLT-1 at the physiological level [121], contributing to restore the unbalanced transmission of glutamate.

6.3.3 RPC1063 (ozanimod)

Ozanimod (RPC1063) was recently approved (March 2020) by the FDA for the treatment of CIS, RRMS, and active SPMS [205]. It is an active agonist of S1P1R and S1P5R while it shows significantly lower affinity for the other S1PRs (Table 1) [193]. Despite the similarity to BAF312, the safer profile of this therapeutic agent made it suitable of further investigations [206]. However, little information is available on the impact of this compound on the CNS.

Oral therapeutic administration of RPC1063 has been shown to ameliorate, in a dose-dependent manner, the EAE clinical score [193]. Since the results appear only partially depend on the reduction of autoreactive lymphocyte trafficking, the authors suggested a RPC1063 direct effect of the molecule in the CNS [193]. Interestingly, the treatment with RPC1063 reduced plasma levels of the neurofilament light chain, a human biomarker of neurodegeneration, which positively correlates with inflammation and demyelination in the spinal cord of EAE and cuprizone mice [207]. *In vivo*, treatment with RPC1063 showed reduced axonal breaks in the corpus callosum, and enhanced functional capacities in cuprizone-induced demyelination [208]. Furthermore, RPC1063 directly modulates activity of astrocytes *in vitro* through the activation of the Akt and ERK pathways, thus inhibiting the release of lipopolysaccharides (LPS)-induced pro-inflammatory cytokines such as IL-1β, TNFα and IL-6 [192]. Recently, Musella *et al*., investigated the anti-inflammatory/neuroprotective effects of RPC1063, by performing *ex vivo* studies in EAE brain [209]. The authors found that RPC1063 (1000 nM) reduced the duration of glutamate-mediated spontaneous currents in EAE corticostriatal slices, reaching values comparable to those of healthy mice [209].

6.3.4 ACT-128800 (ponesimod)

Ponesimod (ACT-128800) is a potent S1P1R agonist and exhibits 650 fold higher selectivity than the endogenous ligand S1P [210]. It has been approved (March 2021) by the FDA for the treatment of adults with CIS, RRMS and active SPMS (Table 1). In preclinical studies, the effect of ACT-128800 (30 mg/Kg) in EAE mice was assessed by prophylactic (after 1-day post-immunization (d.p.i)) and therapeutic (after 15 d.p.i) oral administration. It is interesting to note that both preventive and therapeutic administration leads to a significant reduction of the clinical score even at the chronic phase of the disease [211].

AIM OF THE THESIS

The aim of my PhD thesis was to investigate the role of S1PRs in the CNS of both healthy and EAE animals, paying attention at the impact of the therapeutic oral administration of FTY720 on the expression and the functions of these receptors. The project originated from previous findings published by the group of Anna Pittaluga in 2017 [212] showing that oral administration of FTY720 (0.3 mg/Kg/day for 14 days starting or from the pre-symptomatic stage of the disease, i.e. 12±1 d.p.i. or at the acute stage, i.e. 25±1 d.p.i) recovered efficiently the unbalance of glutamate transmission in nerve endings (we refer to as synaptosomes) isolated from the spinal cord, the cortex and the hippocampus of EAE mice at different stages of the disease [212]. Until now, inhibition of T-lymphocytes migration from the periphery to the CNS was proposed to be the main mechanism underlying the beneficial effect of FTY720 and derivatives in the treatment of MS. Nowadays, however, an increasing number of studies are also focusing on the beneficial effects that these compounds exert directly on the CNS, opening new scenarios in the drug-induced effects. The study aimed at verifying whether the recovery of glutamate impairments induced by FTY720 may rely on a direct action of the drug at S1PRs in central neurons and astrocytes. To verify the hypothesis, the first part of my PhD project aimed at addressing the following tasks:

I. To investigate the presence and the pharmacological profile of S1PRs in synaptosomes and gliosomes (i.e., astrocytic processes) isolated from the cortex of control healthy mice.

II. To extend the study to EAE mice and to evaluate if, in pathological conditions, the expression of release-regulating S1PRs is modified during the course of the disease.

III. To investigate whether the chronic oral administration of FTY720 could recover the pathological-related adaptations of central S1PRs in EAE mice.

While the first part of the thesis focuses on the role of S1PRs in both neurons and astrocytes, the second part aims at investigating the molecular mechanism underling the possible effect of

S1PRs ligands on oligodendroglia cells, the myelinated cells of the CNS which are primarily involved in MS pathology. OPC are precursor cells that give raise to mature and myelinating oligodendrocytes during the developmental stages. However, in the adulthood, a pool of these undifferentiated cells remains in the CNS and following injuries they are induced to migrate and differentiate to generate new myelinated cells. Alterations in both migration and differentiation mechanisms of these cells are known to be involved in MS ethology and progression.

In this context, this part of the project is based on evidence in literature suggesting that in mature oligodendrocytes S1P5R is highly present and it is involved in the survival of mature oligodendrocytes and in the promotion of myelination [126,213,214]. BAF312, has been demonstrated to cross the BBB, stimulate remyelination and prevent synaptic degeneration that occurs in EAE mice [204,215]. Interestingly, more recent evidence unveiled that the compound is also able to increase BDNF amount in the brain of both control and EAE mice. Taken together, these insights suggest the possibility that BAF312 could directly acts on oligodendroglia possible involving the activation of BDNF-tropomyosin receptor kinase B (TrkB) pathway. To study this hypothesis, we proposed:

I. To investigate the impact of the BAF312 on OPCs differentiation.

II. To highlight the role of TrkB receptors on the BAF312 -induced differentiation of OPC by using selective TrkB antagonists.

III. To confirm the effect of BAF312-induced differentiation of OPCs by investigating the impact of the drugs on cells isolated from both TrkB and BDNF-KO mice.

While the first part of the project was carried out at The University of Genova, in the laboratories of Professor Anna Pittaluga, the second part of the thesis was carried out at the laboratory of Professor Tim Vanmierlo, BIOMED, Faculty of Medicine, Hasselt University (Belgium) during the COVID-19 pandemic, which largely limited the opportunity to perform

confirmatory experiments. Nonetheless, although preliminary, the results discussed in this thesis would add new knowledge on the relationship between S1PRs and oligodendroglial cells. The study gave rise to a review, published at the end of 2020 [216], written in collaboration with the team of the Professor Vanmierlo.

MATERIALS AND METHODS

1. Animals

Mice (female, strain C57BL/6J) were purchased from Charles River (Calco, Italy) and were housed in the animal facility of the Department of Pharmacy, Section of Pharmacology and Toxicology, School of Medical and Pharmaceutical Sciences, University of Genoa (authorization n. 484 of 2004, June 8th). EAE mice were sacrificed at 25 ± 1 days post immunization (d.p.i.) by cervical dislocation and decapitation to collect the cortices. The experimental procedures were approved by the Animal Subjects Review Board of the University of Genoa and by the Italian Ministry of Health (DDL 26/2014 and previous legislation; protocol n. 50/2011-B and 612/2015-PR), and they are in accordance with the European legislation (Directive 2010/63/EU for animal experiments) and the ARRIVE guidelines.

2. EAE induction and clinical score

For EAE induction, mice (female, strain C57BL/6J, 18–20 g, 6–8 weeks) were immunized accordingly to a standard protocol with minor modifications [217]. Briefly, animals were administered with incomplete Freund's adjuvant containing 4 mg/ml Mycobacterium tuberculosis (strain H37Ra) and 200 μg of the myelin oligodendrocyte protein 35-55 (MOG 35–55) peptide. Three aliquots of the suspension were injected subcutaneously at two sites in the flank and one closer to the base of the tail. Immunization with MOG35–55 was followed by intraperitoneal administration of 400 ng of PTX on day 0 and after 48 h. The clinical scores (0 = healthy; 1 = limp tail; 2 = ataxia and/or paresis of hindlimbs; 3 = paralysis of hindlimbs and/or paresis of forelimbs; 4 = tetraparalysis; 5 = moribund or death; intermediate clinical signs were scored by adding 0.5). Score and weight were recorded daily.

3. FTY720 treatment (*in vivo*)

Mice (30) were randomly assigned to the following groups: control, FTY720-untreated EAE, and FTY720-treated EAE mice. FTY720 was administered orally, dissolved in drinking water (0.3 mg/Kg). This route of administration was adopted to minimize the daily handling and stress associated with the other methods of drug delivery (i.e., oral gavage). The drug was administered therapeutically, added to the drinking water starting from the first signs of the disease 11 ± 1 d.p.i. for 14 days, until 25± 1 d.p.i,

4. Synaptosomes and gliosomes preparation

Isolated nerve terminals (we referred to as synaptosomes) are functionally active particles having a diameter of about 1-2 μm that originate from nerve terminals (28). Synaptosomes contain mitochondria, vesicles, cytosolic structures, and part of the ER. They retain all the features (structural and functional) of the nerve endings they originate, remaining able to, uptake, store, and release neurotransmitters. In some few cases, fragments of postsynaptic membrane can remain attached the presynaptic one, due to the presence of specific binding-proteins (i.e., PSD95) which link the pre- to the postsynaptic component of the synaptic cleft. However, the postsynaptic fragment does not re-seal and is completely inactive. Therefore, it cannot influence the presynaptic-induced effects. Gliosomes are pinche-off particles which originated from astrocytic processes, that possess gliotransmitter-loaded vesicles that are competent for gliotransmitter secretion [218]. Mass-spectrometry characterization unveiled that they are enriched for proteins involved in synaptic vesicle-mediated transport as well as other astrocyte proteins, such as vesicle associated membrane protein 3 (VAMP3; involved in astrocyte exocytosis), and Ezrin (perisynaptic astrocyte cytoskeletal protein) [219]. Purified synaptosomes and gliosomes were prepared as previously described [220]. Briefly, after decapitation, the cortices were rapidly removed and homogenized 0.32 M sucrose

solution, buffered to pH 7.4 with Tris-(hydroxymethyl)-amino methane [Tris, final concentration (f.c) 0.01 M] using a glass/Teflon tissue grinder (clearance 0.25 mm) at 900 rpm, 12 strokes. The homogenates were centrifuged at 1000 × *g* for 5 min to remove nuclei and cell debris while the supernatant was stratified on a discontinuous Percoll® gradient (2%, 6%, 10% and 20% v/v in Tris-buffered sucrose) and centrifuged at 33500 × *g* for 6 min. The layer between 10% and 20% Percoll® (synaptosomal fraction) and that between 6% and 2% Percoll® (gliosomal fraction) were collected and washed by centrifugation. The pellets (synaptosomes and gliosomes) were resuspended in different medium according to the intended use. For functional studies (see. "superfusion experiment" in the method section) the pellets were resuspended in a physiological medium with the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; HEPES, 10; glucose, 10; pH 7.2–7.4. For biochemical studies synaptosomes and gliosomes were washed once in phosphate-buffered saline (PBS) and resuspended in modified RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, protease inhibitors).

5. Superfusion experiments

To induce the internalization of [^{3}H]D-aspartate ([^{3}H]D-Asp, f.c.: 50 nM) an analog of glutamate, synaptosomes and gliosomes were incubated for 15 min a 37 °C in a rotary water bath. After the incubation period, identical portions of the synaptosomal/gliosomal suspensions were stratified on microporous filters at the bottom of parallel chambers in a Superfusion System (Ugo Basile, Comerio, Varese, Italy; [221] and maintained at 37 °C. The apparatus consists of 20 identical superfusion chambers at the bottom of which synaptosomes/gliosomes are stratified under moderate vacuum on a microporous filter, located on a filter holder of porous glass. Then, the particles are superfused with standard physiological solution at 0.5 ml/min for a total period of 48 min. Each experimental condition was run in triplicate to

mitigate variability. The effects of different S1PRs agonist (S1P, CS2100 and CYM5541) on [^{3}H]D-aspartate were investigated in both basal or depolarizing condition.

In basal condition stratified synaptosomes/gliosomes were superfused at 0.5 ml/min with the standard physiological solution for 36 min to equilibrate the system. Starting from minute 36 four superfusate fractions were collected, namely: b1, t = 36-39 min; b2, t = 39- 42 min; b3 t = 43-45min, and b4 t = 45-48. At t = 39 min, synaptosomes/gliosomes were transiently (90s) exposed to S1PRs ligands till the end of the superfusion period.

Differently, in depolarizing condition, after 39 minutes of superfusion, synaptosomes and gliosomes were transiently exposed (90s) to a high KCl-containing solution (12 mM KCl for synaptosomes and 15 mM KCl for gliosomes, NaCl substituting for an equimolar concentration of KCl) in presence or absence of the ligands. Different concentrations of KCl solutions are used to have a comparable overflow from synaptosomes and gliosomes [222].

At the end of the experiment, three superfusate fractions [two 3-min fractions (basal release), one before (t = 36–39 min) and one after (t = 45–48 min) a 6-min fraction (t = 39–45 min; evoked release)] were collected. Fractions and superfused synaptosomes were measured for radioactivity. The amount of radioactivity released into each fraction was expressed as percentage of the total radioactivity.

The KCl-induced overflow was estimated by subtracting the neurotransmitter content into the first and the third fractions collected (basal release, b1 and b3) from that in the 6-min fraction collected during and after the depolarization stimulus (induced release, b2). Within the text, the effect of agonists is also expressed as percentage of the KCl-induced overflow of tritium observed in the absence of receptor ligand (percent of control).

6. Confocal microscopy

Mouse cortical synaptosomes and gliosomes (40 μg of protein) were fixed with 2% paraformaldehyde, permeabilized with 0.05% Triton X-100 PBS and incubated overnight at

4°C with the following primary antibodies diluted in 3% bovine serum albumin (BSA) in PBS: rabbit polyclonal anti-S1P1R (1:600, ab 11424 Abcam), rabbit polyclonal anti-S1P3R (1:100; ab 108370 Abcam) goat polyclonal anti-syntaxin 1A (1:3000, AF7237, R&D systems, Minneapolis USA), guinea pig anti-vesicular glutamate transporter type 1 (VGLUT1; 11:1000, AB5905, Millipore, Darmstadt, Germany), and mouse polyclonal anti-GFAP (1:300, G3893, Sigma) . The following day the particles were washed once in PBS and incubated for 1h at room temperature with the respective secondary antibodies: anti-rabbit AlexaFluor-555 (1:900, Molecular Probes, Eugene, Oregon USA), anti-guinea pig AlexaFluor-488 (1:, Molecular Probes, Eugene, Oregon USA), anti-goat AlexaFluor-647 (1:900, Molecular Probes, Eugene, Oregon USA only for synaptosomes) or anti-mouse AlexaFluor-647 (1:900, Molecular Probes, Eugene, Oregon USA, only for gliosomes). Synaptosomes and gliosomes were then applied onto coverslips. Fluorescence imaging (512 × 512 × 8 bit) acquisition was carried out by Leica TCS SP5 laser-scanning confocal microscope, equipped with 458, 476, 488, 514, 543, and 633 nm excitation lines, through a plan-apochromatic oil immersion objective 63X/1.4NA. Sequential channel acquisition was performed to avoid spectral bleed-through artifacts. For images acquisition, storage, and visualisation Leica 'LAS AF' software package was used. The quantitative estimation of co-localized proteins was performed by calculating the "co-localization coefficients" [223]. The coefficients are based on the Pearson's correlation coefficient, a standard procedure for matching one image with another in pattern recognition and express the fraction of colocalizing molecular species in each component of a dual-color image. The calculation was performed by macro routines integrated as plugins (WCIF Co-localization Plugins, Wright Cell Imaging Facility, Toronto Western Research Institute, Canada) using the + Image J 1.51w software (Wayne Rasband, NIH, USA).

7. Western blot analysis

Western blot analyses were conducted to investigate changes in the cortical synaptosomal and gliosomal densities of S1PRs in control (CTR), FTY720-untreated and FTY720-treated EAE mice. The cortices of mice from different experimental groups (CTR, FTY720-untreated EAE, FTY720-treated EAE) were homogenate in 5 mL of sucrose solution. 1/5 of the homogenate was keep and directly lysed in modified RIPA buffer while the remaining part was used to obtain synaptosomes and gliosomes then lysed in modified RIPA buffer. The protein content was determined using the Pierce BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). Samples were resuspended in SDS-PAGE loading buffer, boiled at 95°C for 5 min and then separated by SDS-7.5% PAGE (20µg/lane) and transferred onto PVDF membranes (100V for 1h, at 4°C). Membranes were incubated for 1 h at room temperature in Tris-buffered saline-Tween (t-TBS: 0.02 M Tris, 0.15 M NaCl, and 0.05% Tween 20) containing 5% (w/v) non-fat dried milk and then probed with rabbit anti-S1P1R (1:1000, Abcam), rabbit anti- S1P3R (1:300, Abcam), mouse anti-β tubulin (1:500, T8660, Sigma, St. Louis, MO, USA) antibodies overnight at 4°C. After three washes in t-TBS (0,05%), membranes were incubated for 1h at room temperature with appropriate horseradish peroxidase-linked secondary antibodies (1:10000, Sigma). Images were acquired using the Alliance LD6 images capture system (Uvitec, Cambridge, UK) and analysed with UVI-1D software (Uvitec, Cambridge, UK).

A similar protocol of Western blot analysis was followed at Hasselt university, where the immunoblots have been conducted on Olineu cells. The protocol applied was the same described previously and PVDF membranes were incubated with the following antibodies mouse anti-p-TrkB (1:500) rabbit anti- p-CREB (1:600) and mouse anti- pro-BNDF (1:250).

8. Biotinylation studies

Changes in the cortical synaptosomal and gliosomal level of S1P1R and S1P3R proteins were investigated by performing biotinylation studies followed by immunoblot analysis. Briefly, mouse cortical synaptosomes and gliosomes (from CTR and FTY720-untreated EAE mice) were obtained following the purification protocol on a discontinuous Percoll® gradient (see section before). The preparations were divided in two aliquotes. One aliquot was lysed in modified RIPA buffer to analyse the S1P1/3R content in the total synaptosomal lysate (L). Parallel, synaptosomes and gliosomes obtained from the other aliquot were labelled with sulfo-NHS-SS-biotin (2 mg/ml; Invitrogen) for 1 h at 4 °C in PBS/Ca^{2+}-Mg^{2+} with the following composition (mM): 138 NaCl, 2.7 KCl, 1.8 KH_2PO_4, 10 Na_2HPO_4, 1.5 $MgCl_2$, 0.2 $CaCl_2$, pH 7.4. The biotinylation reaction was then stopped by incubating synaptosomes with PBS/Ca^{2+}-Mg^{2+} containing 100 mM glycine for 20 min at 4°C. After two washes in PBS/Ca^{2+}-Mg^{2+} synaptosomes were lysed in modified RIPA buffer. The biotinylated samples (100 ug) were incubated under shaking with Dynabeads MyOne Streptavidin T1 beads (Invitrogen) for 30 minutes at room temperature to pulldown the biotinylated proteins. Similarly, the beads were added also to non-biotinylated synaptosomes, to check the specificity of streptavidin (negative control, NTC). After three washes in PBS-tween (0.01%) the samples were resuspended in SDS-PAGE loading buffer and boiled for 5 min at 95 °C to separate biotinylated proteins from the beads. The beads were discarded, and the supernatant loaded on a polyacrylamide gel and analysed through immunoblot assay. The immunoreactivity of S1P1/3Rs was detected by using rabbit anti-S1P1 (1:1000) and rabbit anti-S1P3 (1:300) antibodies in the total cortical lysate (L), in streptavidin pulldown of the non-biotinylated synaptosomal lysate (NTC) and in the biotinylated synaptosomes samples (B). Mouse anti-β-tubulin (1:500) and rabbit anti-GluA2 (1:2000, ab 206293 Abcam, Cambridge, United Kingdom) were used as negative and positive control of the biotinylation protocol, respectively.

9. Primary oligodendrocyte precursor cell (OPC) cultures

Primary murine OPCs were obtained from mixed glial cultures, using the standard shake-off method. Briefly, cortices, isolate from postnatal day 0 were dissociated using papain (3 U/ml, diluted in Dulbecco's Modified Eagle Medium (DMEM) containing 1 mM L-cystein; Sigma-Aldrich) though an incubation of 20 min. Ones dissociated, the mixed glial cells were maintained in DMEM on poly-L-lysine-coated (5 µg/ml, Sigma-Aldrich) culture flasks. The medium was supplemented with 50U/ml penicillin and 50 mg/ml streptomycin (P/S; Invitrogen) and inactivated foetal calf serum (FCS). Cells were kept at 37 °C in a humidified atmosphere of 8.5% CO_2. To stimulate the OPC formation, after 7 days the medium was supplemented with bovine insulin (5 µg/ml). On day 14, the cells were shaken using an orbital shaker at 75 rpm and 37 °C for 45 min to detach the microglial layer. A second shake-off was performed for 16 h at 250 rpm, after which the OPC-enriched supernatant was collected, and centrifuged on 300×*g* for 5 minutes. OPCs were seeded onto 24/6 well plates (based on the experiments) and maintained in DMEM medium (+ 10% FCS and 1% P/S) or differentiation medium (DMEM medium, supplemented with 0.5% P/S, 2% horse serum, 0.3 mM transferrin, 0.1 mM putrescin, 0.02 mM progesterone, 0.2 µM sodium selenite, 0.5 µM triiodothyronin, 0.8 mM bovine insulin, 0.5 mM L-thyroxine, 2% B27 supplement) depending on the experiment.

10. BAF312 treatment (*in vitro*)

For pharmacological *in vitro* treatment, 5 ng/µl of platelet-derived growth factor α (PDGFα) was added to the DMEM culture medium to maintain OPC or Olineu in a proliferation state. The cells were treated of 48h or 6 days (based on experimental procedure) with three different concentrations (10nM, 100 nM, and 1 µM) of BAF312 (Tocris) or DMSO (Sigma-Aldrich)

used as a vehicle control. After the treatment OPC were either lysed for RNA isolation and qPCR analyses or fixed on coverslips to perform immunofluorescence studies. After treatment Olineu cells were lysed in RIPA and used for immunoblotting analyses. During the treatment a partial medium change was performed every two days to maintain the proper drug concentration.

11. Immunostaining

At the end of the treatment, primary OPCs were fixed in 4% PFA for 30 min at room temperature. After a wash in PBS, the a-specific bindings were blocked for 30 min with 1% BSA in PBS-tween (0.1%), followed by incubation with primary antibodies (rat anti-MBP 1:500; IgM anti-O_4 1:1000) four hours at room temperature. After three washes on a shaker with PBS, cells were incubated with anti-rat AlexaFluor-488 (1:1000) or anti-IgM AlexaFluor-555 (1:1000)- conjugated secondary antibody for one hour at room temperature. After three washing the nuclei were statically stained with 4′6-diamidino-2-phenylindole (DAPI) for 10 minutes at room temperature. Coverslips were mounted with Dako mounting medium (Dako, Carpinteria, USA) and analysed using a fluorescence microscope (Leica DM2000 LED).

12. RNA extraction and qPCR

Total RNA was isolated from cells, using the RNeasy mini kit (Qiagen) following to the manufacturer's instructions. RNA concentration and quality were analysed with a Nanodrop spectrophotometer (Isogen Life Science). RNA was reverse transcribed using the qScript cDNA Supermix kit. The qPCR was performed to analyse gene expression, using the Applied Biosystems QuantStudio 3 Real-Time PCR System (Life Technologies). The reaction mixture consisted of SYBR Green master mix (Life Technologies), 10 µM forward and reverse primers (Integrated DNA Technologies), nuclease-free water and cDNA template (12.5 ng), up to a total reaction volume of 10 µl. The primer pairs used for amplification are the following**:**

Target	Forward	Reverse
S1P1R	5′-ACTTTGCGAGTGAGCTG-3	5′-AGTGAGCCTTCAGTTACAGC-3′
S1P2R	5′-TTCTGGAGGGTAACACAGTGGT-3′	5′-ACACCCTTTGTATCAAGTGGCA-3′
S1P3R	5’-TGGTGTGCGGCTGTCTAGTCAA-3’	5’- CACAGCAAGCAGACCTCCAGA -3’
S1P5R	5’-CTTGCTATTACTGGATGTCGC-3’	5’-GTTGGAGGAGTCTTGGTTGC-3’
TrkB	5’-TGAGGAGGACACAGGATGTTGA-3’	5’-TTCCAGTGCAAGCCAGTATCTG-3’

Results were analysed by the comparative Ct method and were normalized to housekeeping genes *Pgk-1* and *Cypa.*

13. Reagents and chemicals

Aspartic acid, D-[2,3-3H] were from Perkin Elmer (NET581001MC, Boston, MA, USA). Pertussis toxin and the Freund’s incomplete adjuvant, Solvent Blue 38, Periodic Acid were acquired from Sigma-Aldrich (Saint Louis, MO, USA). Pierce™ BCA Protein Assay Kit was from Thermo Fisher Scientific 168 Third Avenue Waltham, MA USA 02451. Myelin oligodendrocyte glycoprotein (MOG) was purchased from Espikem (Florence, Italy). Mycobacterium tuberculosis (H37Ra) was obtained from DIFCO BACTO Microbiology (Lawrence, KA, USA). FTY720 was supplied by Novartis Pharma AG (Basel, Switzerland). S1P (1370), CS-2100 (EC50 = 4.0 nM, 4543) and CYM-5541 (EC50 = 72-132 nM, 4897) were all purchased from Tocris Bioscience (Bristol, UK)

14. Statistics and calculations

SigmaPlot 10.0 data analysis and graphing software package were used for data handling/statistics and for graph drawing. Analysis of variance was performed by one way ANOVA followed by Dunnett’s or Tukey’s multiple-comparisons test as appropriate. Direct comparison was performed with the student’s t-test. Data were considered significant for $p < 0.05$ at least.

RESULTS

Isolated nerve terminals and glial particles isolated from mice cortex express S1P1R and S1P3R proteins.

The first aim of the project was to investigate the density of S1P1R and S1P3R proteins in both cortical synaptosomes and gliosomes isolated from the cortex of adult (3 months old) healthy mice. To this purpose, Western blot analyses were carried on cortical synaptosomal and gliosomal lysates. To confirm the presence of the receptors, different amounts (10 µg and 20 µg) of cortical synaptosomes and gliosomes were loaded and incubated with the antibodies recognizing one the C-terminus domain of S1P1R and the other one the N-terminus domain of S1P3R. The analysis unveiled the presence of immunostaining consistent with the molecular weight of S1P1R and S1P3R proteins (S1P1R: 44 kDa; S1P3R: 42 kDa). The immunostaining densities were directly related to the amount of the loaded amount of the proteins (Fig. 1), either for synaptosomal lysate (Fig. 1A and B respectively) and glial particles (Fig. 1C and D respectively).

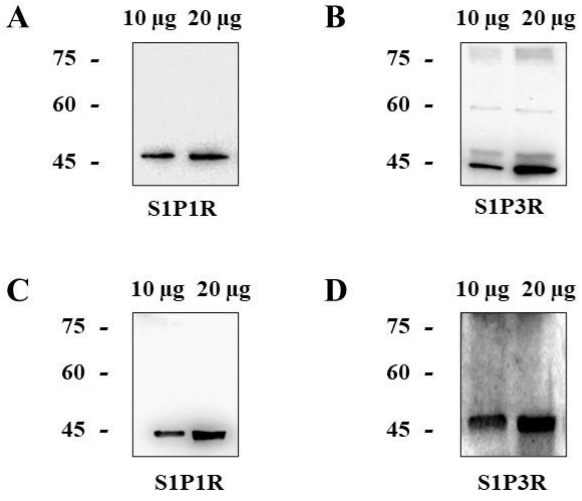
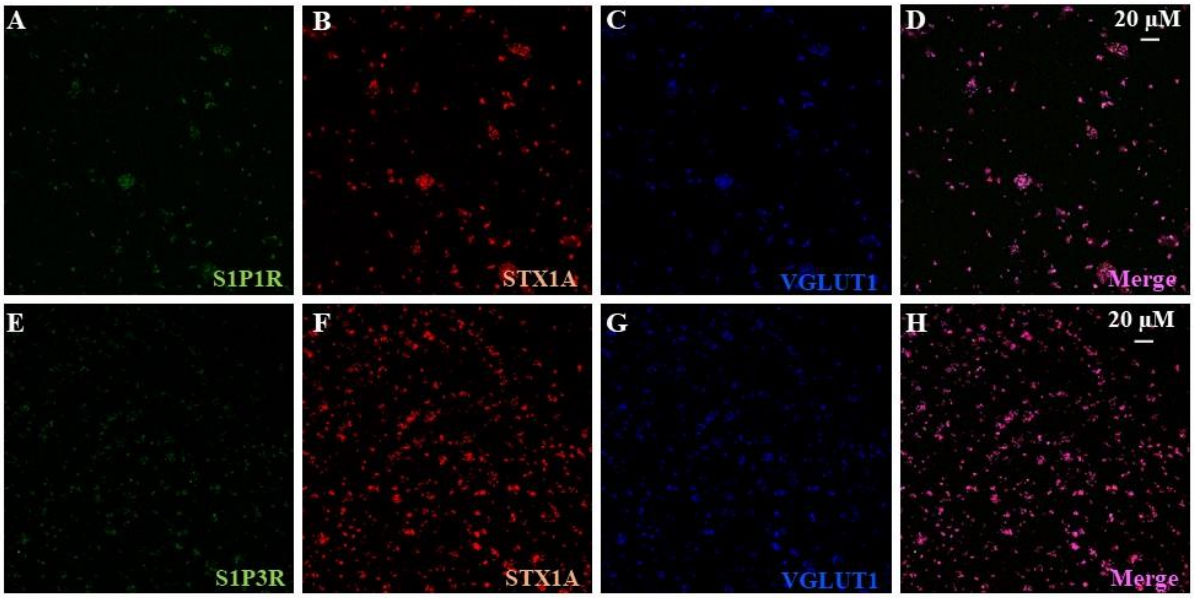
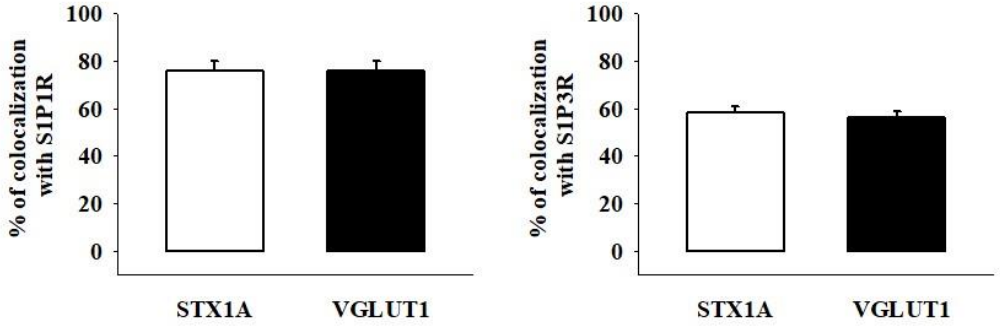


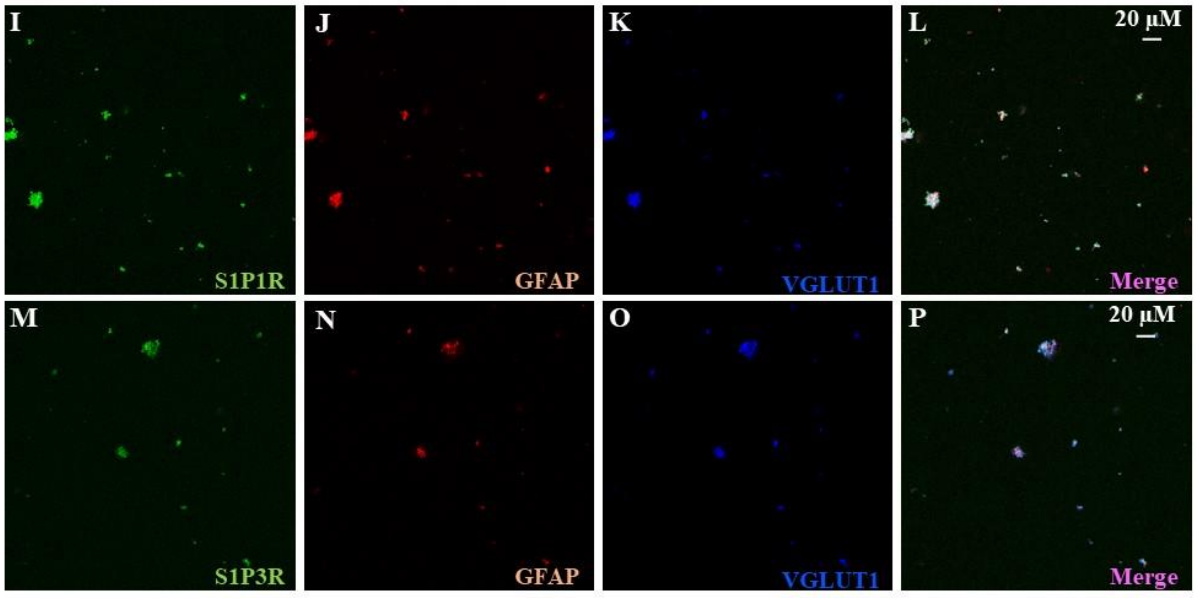
Figure 1. Mice cortical synaptosomes and gliosomes lysates are immunopositive for S1P1R and S1P3R proteins. Different amounts (10 ug and 20 ug) of cortical synaptosomes (**A** and **B**) and cortical gliosomes (**C** and **D**). Western blot analyses were carried out to evaluate the density of S1P1R (**A** and **C**) and S1P3R (**B** and **D**). The images are representative of three analyses run in different days.

The findings from the Western blot analyses confirmed the presence of both S1P1R and S1P3R proteins in synaptosomes and astrocytic lysates. We then asked whether the particles endowed with the two receptors are glutamatergic in nature. To address the question, we performed confocal analyses studying the immunopositivity of S1P1R (Fig. 2A and E) and the S1P3R (Fig. 2I and M) in mouse cortical synaptosomes that express the vesicular glutamate 1 transporter (VGLUT1), here used as a marker of glutamatergic particles (Fig. 2C, G, H, and O). At the same time, synaptosomes were also stained with anti-syntaxin1A (STX1A, Fig. 2B and F) antibody, a marker of presynaptic component, to highlight the presence of the receptor protein in presynaptic structures. Similarly, cortical gliosomes were stained with anti-glial fibrillary acidic protein (GFAP, Fig.2 J and N), a specific marker of astrocytes.


A
S1P1R
B
STX1A
C
VGLUT1
D
20 µM
Merge
E
S1P3R
F
STX1A
G
VGLUT1
H
20 µM
Merge


% of colocalization with S1P1R
100
80
60
40
20
0
STX1A
VGLUT1
% of colocalization with S1P3R
100
80
60
40
20
0
STX1A
VGLUT1

Gliosomes


I
S1P1R
J
GFAP
K
VGLUT1
L
20 µM
Merge
M
S1P3R
N
GFAP
O
VGLUT1
P
20 µM
Merge

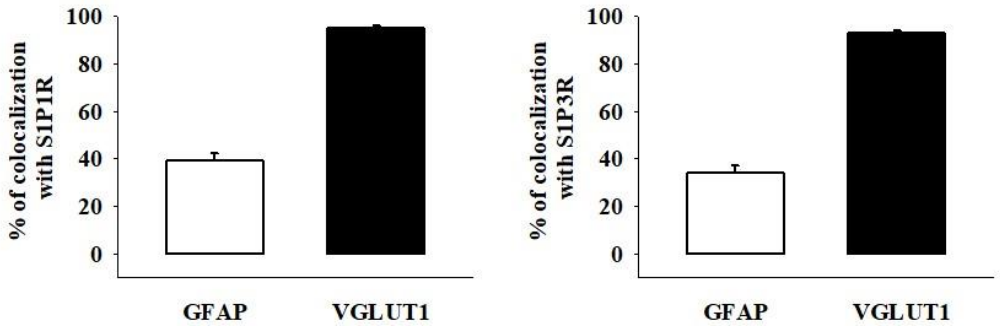

% of colocalization with S1P1R
100
80
60
40
20
0
GFAP
VGLUT1
% of colocalization with S1P3R
100
80
60
40
20
0
GFAP
VGLUT1

Figure 2. Colocalization of S1P1R and S1P3R with VGLUT1, STX1A and GFAP in mouse cortical synaptosomes and gliosomes. Confocal analysis of S1P1R and S1P3R immunoreactivity (**A** and **E** respectively, green) in STX1A -positive synaptosomes (**B** and **F**, red) and VGLUT1 -positive cortical nerve terminals (**C** and **G**, blue) and their colocalization (**D** and **H**, pink). Confocal analysis of S1P1R and S1P3R immunoreactivity (**I** and **M** respectively, green) in GFAP- positive gliosomes (**J** and **N**, red) and VGLUT1 -positive cortical nerve terminals (**K** and **O**, blue), and their colocalization (**L** and **P**, pink). The figure shows representative images of three independent experiments run on different days.

In synaptosomes, we identified large and comparable immunoreactivities of S1P1R protein (Fig. 2A) in both STX1A -positive nerve terminals (Fig.2b STX1A/S1P1R 76 ± 4% of co-localization) and VGLUT1-positive terminals (Fig.2C VGLUT1/S1P1R 76 ± 4% of co-localization), suggesting that a large part of cortical synaptosomes are glutamatergic and express S1P1R proteins. The same analysis was conducted for S1P3R (Fig. 2E), and the staining was detected in both STX1A immuno-positive synaptosomes (Fig. 2F, STX1A/S1P3R: 58 ± 3% of co-localization) and in VGLUT1 immuno-positive nerve terminals (Fig. 2G VGLUT1/S1P3R: 56 ± 3%).

The distribution of S1P1R and S1P3R was investigated also in cortical glial particles (Fig. 2I and M). In these particles, only a low amount of GFAP-positive gliosomes were immunopositive for S1P1R (GFAP/S1P1R: 39 ± 3%, Fig. 2L) and S1P3R (GFAP/S1P3R: 34 ± 3%, Fig. 2P) proteins. Notably, the immunopositivity for VGLUT1 largely colocalized with both the S1P1R (VGLUT1/S1P1R: 95 ± 1% of colocalization) and the S1P3R (VGLUT1/S1P3R: 93 ± 1% of colocalization) immunostainings, suggesting that gliosomes, endowed with S1P1R and S1P3R, are mostly glutamatergic.

Pharmacological characterization of sphingosine-1-phosphate receptors controlling glutamate release from mouse cortical nerve endings and astrocytic processes

Once confirmed with biochemical analyses the existence of S1PRs in glutamatergic particles, we performed studies to confirm, by a functional point of view, the role of the receptors in controlling glutamate release. To this aim, we performed a pharmacological characterization by using selective ligands for both S1P1R and S1P3R. We used S1P (a broad-spectrum S1PRs agonist), CS2100 (a selective S1P1R agonist), and CYM5541 (a selective S1P3R agonist).

Cortical synaptosomes and gliosomes isolated from the cortex of control healthy mice were labelled with [^{3}H]D-aspartate, a radioactive glutamate analogue that mimics the endogenous glutamate allowing a reliable measure of the release of this aminoacid [224–227]. Synaptosomes and gliosomes were exposed, for 90 seconds, in superfusion to a high KCl solution (NaCl substitutes for an equimolar concentration of KCl). Specifically, synaptosomes were exposed to a 12 mM-KCl solution while gliosomes to a 15 mM-KCl solution, in order to release a comparable amount of the radioactive tracer from both preparations (for experimental details concerning the choice of use different stimuli see Di Prisco et al., 2012 [222]).

The data from superfusion studies are reported in Fig. 3 and unveiled a complex scenario. Fig. 3A and B show the effect of S1P (a broad spectrum agonist of S1PRs) on the high KCl-induced [^{3}H]D-aspartate overflow in cortical synaptosomes (A) and gliosomes (B). The compound (0.01-10 nM) slightly but not significantly reduced the [^{3}H]D-aspartate release from cortical synaptosomes (Fig. 3A) when added up to 1 nM. Differently, S1P failed to affect tritium exocytosis from cortical gliosomes (Fig. 3B). Additionally, the efficiency of S1P in modifying the spontaneous release of tritium was investigated in both cortical preparations. In these experiments, S1P did not alter the neurotransmitter release from both synaptosomes and gliosomes (data not shown).

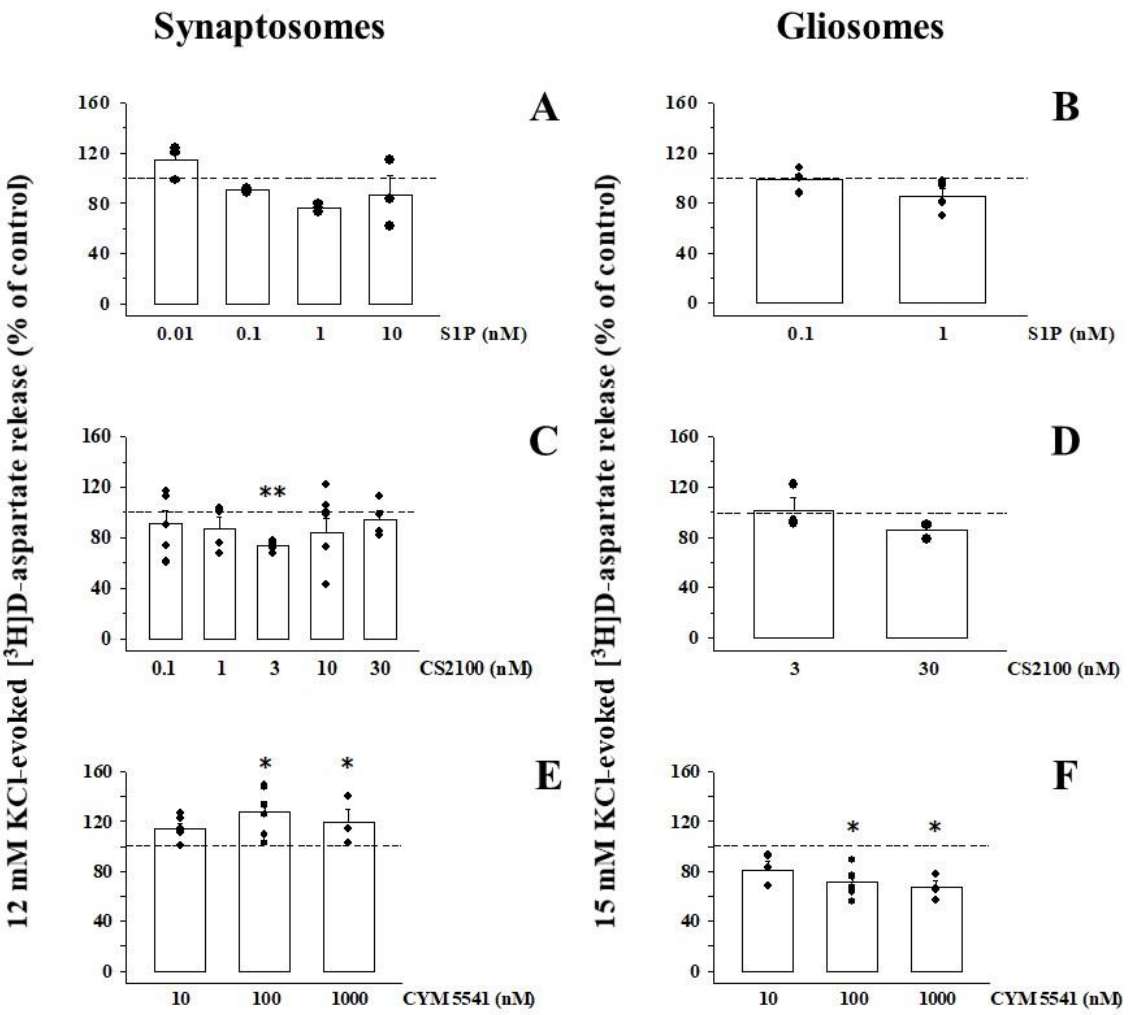


Figure 3. Effect of S1PRs ligands on KCl-induced [^{3}H]D-aspartate from mouse cortical synaptosomes and gliosomes. Cortical synaptosomes from control mice were exposed in superfusion to the 12 mM KCl-enriched medium in the absence or in the presence spingosine-1-phosphate (S1P; **A**), of the S1P1R agonist (CS2100; **C**) and of the S1P3R agonist (CYM5541; **E**) at the concentrations indicated in the X axes. Cortical gliosomes from control mice were exposed in superfusion to the 15 mM KCl-enriched medium in the absence or in the presence S1P (**B**), CS2100 (**D**) and CYM5541 (**F**), concentrations as indicated. Data are expressed as mean ± SEM of at least 4 experiments run in triplicate. (*p< 0.05 versus control; **p< 0.01 versus control).

Then experiments were carried out to verify the activity of CS2100, a selective S1P1R agonist and CYM5541, a selective agonist of S1P3R. The results unveiled that CS2100 (0.1-30 nM) significantly inhibited 12 mM KCl-evoked [^{3}H]D-aspartate overflow from cortical synaptosomes (Fig. 3C), but it failed to inhibit the tritium exocytosis from gliosomal particles (Fig. 3D). In synaptosomes, the concentration-effect relationship was a bell-shaped curve, and

the maximal activity was detected at 3 nM (Fig. 3C). Differently, CS2100 failed to alter the 15 mM KCl-evoked [^{3}H]D-aspartate overflow from gliosomal particles even when added at the maximal concentrations (3-30 nM; Fig. 3D).

A more complex scenario emerged when studying the impact of the selective S1P3R agonist (CYM5541). In cortical synaptosomes (Fig. 3E), the agonist facilitated glutamate exocytosis in a dose-dependent-manner (10-1000 nM) causing the maximal enhancement when added at 100 nM, but it significantly reduced the tritium release from cortical gliosomes (Fig. 3F), exerting the maximum inhibition at 100-1000 nM. Both the selective S1PR agonists failed to modulate the basal tritium release from both synaptosomal and gliosomal particles (data not shown).

Taken together, the results suggested the presence of release-regulating S1PRs in both cortical synaptosomes and gliosomes, which may preferentially couple different G-protein and therefore activate intracellular pathways exerting opposite effects on glutamate release.

S1P1R and S1P3R proteins are not present in cortical synaptosomal and gliosomal plasma membranes.

In general, in superfusion experiments, the finding that an agonist modifies transmitter exocytosis from superfused particles suggest the presence of a receptor at the outer side of the plasma membrane. Accordingly, the efficiency of CS2100 and CYM5541 in modifying the glutamate exocytosis would be expected to imply the presence of S1P1R and S1P3R proteins in both synaptosomal and gliosomal plasma membranes. To verify the conclusion, biotinylation studies were carried out on both preparations. In these experiments, β-tubulin, a protein having a cytosolic distribution, was used as a negative control, while the GluA2 subunit of the AMPA receptors which is highly expressed in both synaptosomes and gliosomes particles in membranes and intracellularly as well [228] was used as positive control of the biotinylation process.

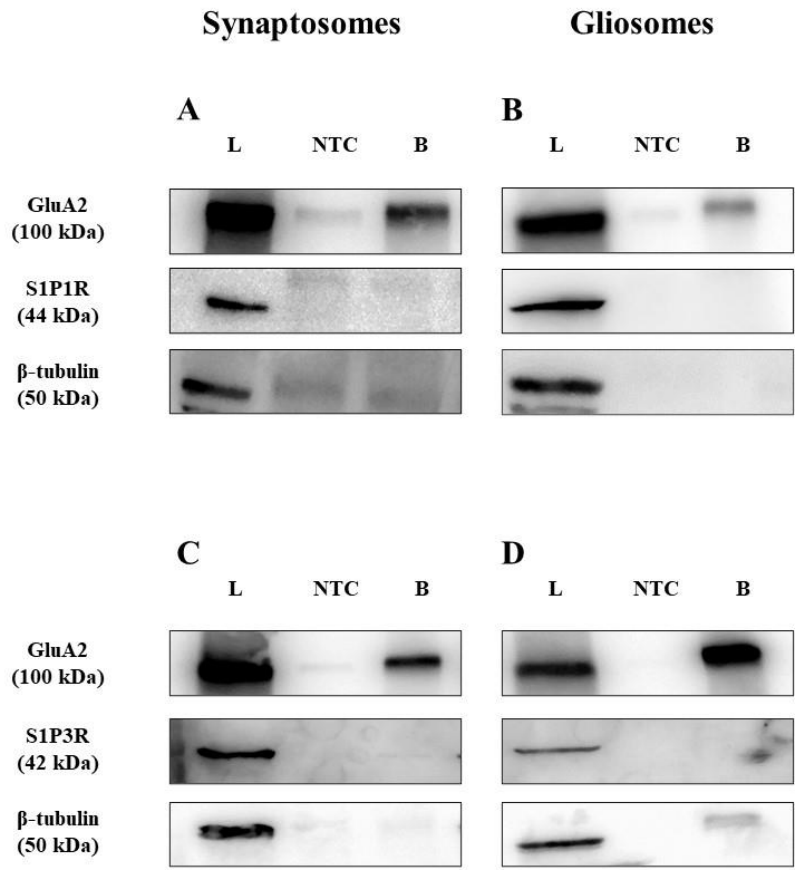


Figure 4. Western blot analysis of S1P1R and S1P3R densities in plasma membranes of cortical synaptosomes and gliosomes: biotinylation studies. The Western blot performed on cortical synaptosomes (**A** and **C**) and gliosomes (**B** and **D**) compares S1P1R and S1P3R density in total lysates (lysates, **L**), lysates untreated with biotin but subjected to streptavidin pulldown (negative control, **NTC**), and lysates incubated with biotin and subject to streptavidin pulldown (biotinylated, **B**). The blot is representative of three experiments carried out on different days.

Representative images of these experiments are reported in Fig. 4. As expected, β-tubulin immunostaining was absent in biotinylated samples but present in synaptosomal and gliosomal lysates (Fig. 4A to D). Furthermore, a clear immunopositivity for the GluA2 subunit protein was detected in the biotinylated samples as well as in the synaptosomal and gliosomal lysates (Fig. 4A to D). Interestingly, and unexpectedly, in both synaptosomes and gliosomes isolated from healthy mice, S1P1R and S1P3R immunostaining was not detected in the biotinylated particles but clearly evident in the synaptosomal and gliosomal homogenates (Fig. 4A to D).

Altogether, the data suggest that the largest part of S1PR proteins is not localized at the outer side of both synaptosomal and gliosomal plasma membranes, but rather it is not accessible to biotin consistent with conclusion that S1P1R and S1P3R are preferentially located at the inner side of the plasma membrane or even have an internal, cytosolic, distribution.

Effect of oral administration of FTY720 on the clinical score of EAE mice

Young female C57BL/6 mice (6 weeks old) were randomly assigned to the following two groups: untreated-EAE mice and FTY720-treated EAE mice. The drug was administered orally, dissolved in drinking water starting from the onset of the first clinical signs (11 ±1 d.p.i) following a therapeutic (14 days) protocol already applied in a previous work (see. Bonfiglio *et al.*, 2017 [212]).

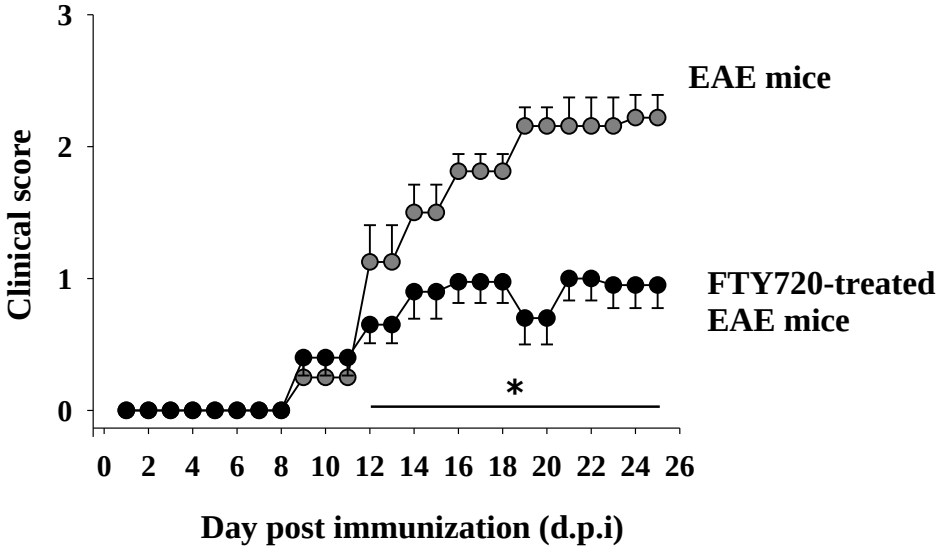


Figure 5. Impact of *in vivo* FTY720 administration on clinical score of EAE mice. Animal score was detected daily in FTY720-untreated (grey circles, n=15) and FTY720-treated EAE mice (black circles, n=12) and it was expressed as mean ± SEM. (* $p < 0.05$ versus daily clinical score in FTY720-untreated EAE mice).

EAE mice show the first clinical score at about 11 ± 1 d.p.i. and the symptoms undergo a time-dependent worsening that peaks around 25 ± 1 d.p.i. (total clinical score = 2.12 ± 0.29 at 24 d.p.i.) which correspond to the chronic phase of the disease. The EAE mice that were treated

with FTY720 show a comparable trend in the onset and the development of the clinical signs that however were characterized by a lower gravity.

Impact of therapeutic oral FTY720 on the density of S1P1R and S1P3R in cortical synaptosomes and gliosomes

It has been demonstrated that FTY720 can cross the BBB and then it is phosphorylated by the CNS-resident cells [187]. This observation suggests that, beyond the largely recognized immunomodulatory effects, the compound may directly influence the functions of central resident cells, including neurons and astrocytes. If confirmed, these events could account for part of the therapeutic activity of the drug in MS patients as well as in EAE mice. Taking in consideration the positive effect of FTY720 on glutamatergic transmission in different brain regions [115,212] it was suggested that FTY720 could target S1PRs expressed in neurons and astrocytes and recover the changes in the expression and function that these receptors might develop during the course of the disease. To verify the hypothesis, I performed Western blot analyses to investigate first the effect of the MOG-immunization on the densities of S1P1R and S1P3R in cortical synaptosomal and gliosomal particles and then to analyse whether FTY720 treatment could reverse the EAE-induced adaptation at these receptors.

The density of the S1P1R in total cortical homogenate (Fig. 6A and B), cortical synaptosomal lysate (Fig. 6C and D), and cortical gliosomal lysate (Fig. 6E and F) from control mice, FTY720-untreated-EAE mice and FTY720-treated EAE was analysed.

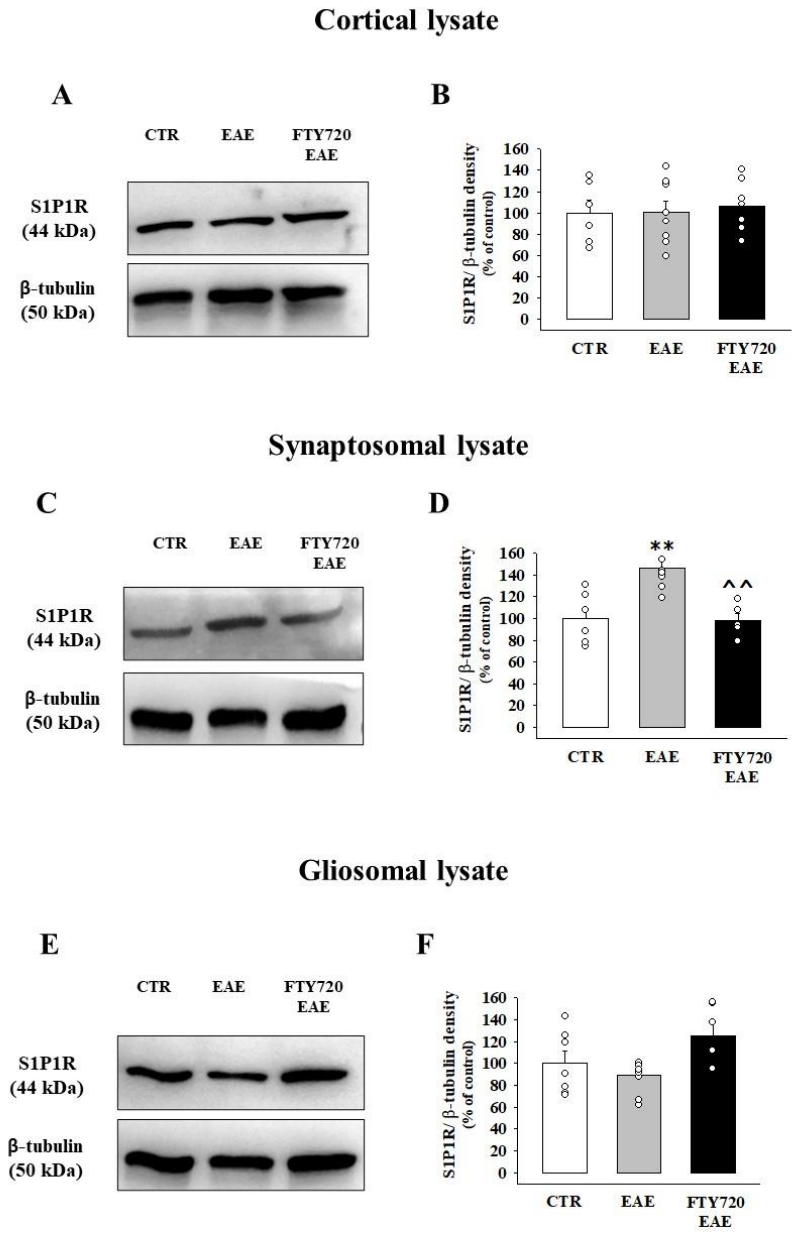


Figure 6. Western blot analysis of S1P1R density in cortical synaptosomes and gliosomes from EAE mice: impact of therapeutic administration of FTY720. The density of S1P1R proteins was quantified by Western blot analysis in the total cortical homogenate (**A**) and in cortical synaptosomal (**B**) and gliosomal (**C**) lysates. The analysis compares samples from control mice (white bar), FTY720-untreated-EAE mice (grey bar) and FTY720-treated EAE mice (black bar). The blot images are representative of at least 4 blots carried out on different days using animals from different immunizations. β-tubulin was used as internal standard and the content of S1P1Rs was expressed as S1P1/ β-tubulin ratio (**B**, **D**, **F**). Data represent the mean ± S.E.M. (** $p < 0.01$ vs control mice; ^^ $p < 0.01$ vs FTY720 untreated-EAE mice).

The immunopositivity of S1P1R was normalized on β-tubulin, here used as internal standard. The results from control, untreated-EAE, and FTY720-treated EAE mice were compared to assess the impact of MOG-immunization and of FTY720 treatment on S1P1R density (Fig. 6B, D and F). The analysis showed a significant increase of the S1P1R/ β-tubulin density in cortical synaptosomes from FTY720-untreated EAE mice compared to controls (Fig. 6C and D). Interestingly, the increase recovered in synaptosomes obtained from FTY720- treated EAE mice to a level largely comparable to that observed in control animals (Fig. 6C and D). Conversely, the results of Western blot analysis in total cortical homogenate and in cortical gliosomal lysate unveiled no significant changes in S1P1R expression in FTY720-untreated EAE mice and in EAE animals treated with FTY720 (Fig. 6A, B, E, and F).

Comparable results were obtained when analysing the S1P3R immunostaining in the cortical samples (total cortical homogenate, synaptosomal and gliosomal lysates; Fig.7 A, C and E respectively) from control, FTY720-untreated EAE and FTY720-treated EAE mice.

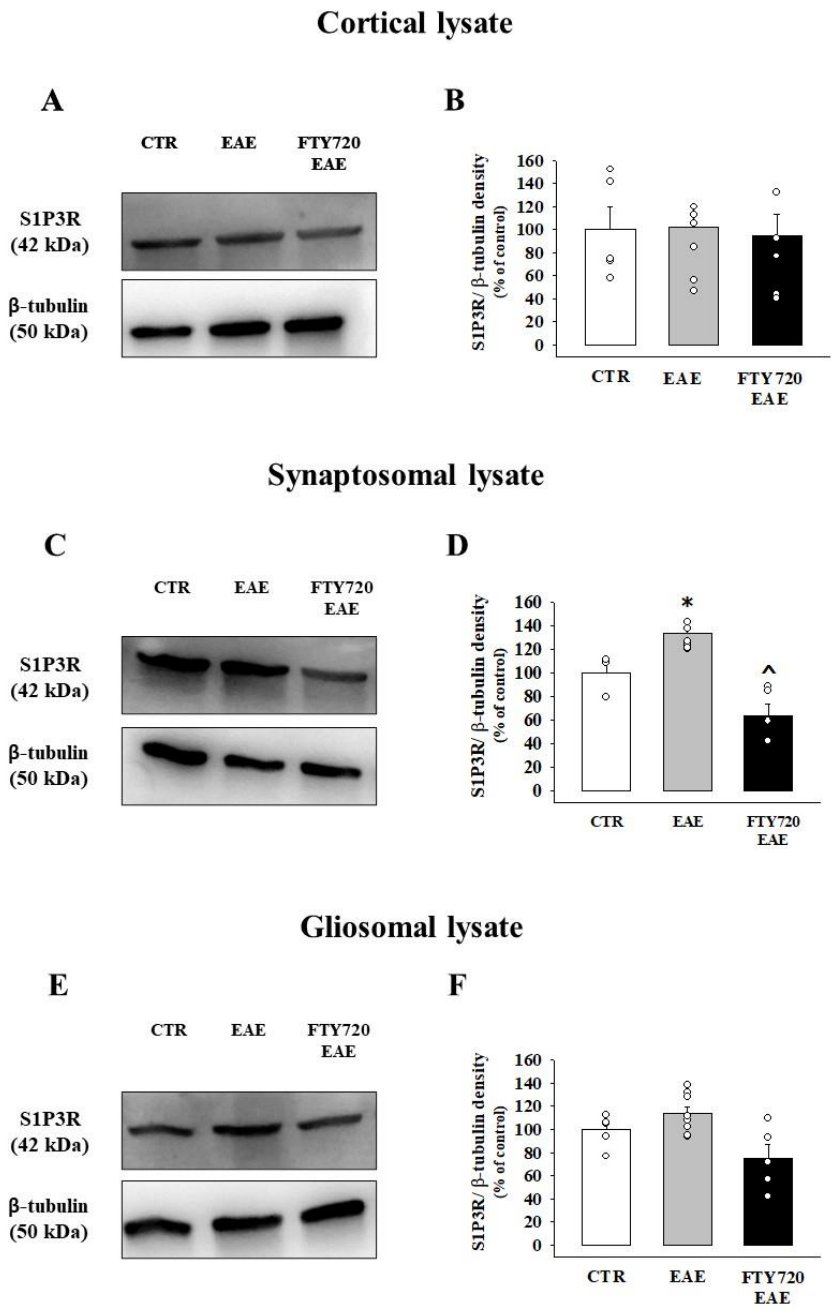


Figure 7. Western blot analysis of S1P3R density in cortical synaptosomes and gliosomes from EAE mice: impact of therapeutic administration of FTY720 The S1P3R protein density was quantified by Western blot analysis in the total cortical homogenate (**A**) and in cortical synaptosomal (**B**) and gliosomal (**C**) lysates. Samples isolated from control mice (white bar), FTY720-untreated EAE mice (grey bar) and FTY720-treated mice (black bar) were analysed and compared for S1P3R content. β-tubulin was used as internal standard. The S1P3Rs content was expressed as S1P3/ β-tubulin ratio (**B**, **D**, **F**) The blot images are representative of at least 4 blots carried out on different days using animals from different immunizations. Data represents the mean ± S.E.M. (*$p < 0.05$ vs control mice; ^ $p < 0.05$ vs FTY720 untreated-EAE mice).

The anti-S1P3R antibody recognizing the N-terminal domain of the S1P3R protein unveiled immunostainings in all the three samples with a mass consistent with the molecular weight of the protein (42 kDa). Again β-tubulin was used as internal standard and S1P3R values were normalized with respect to the housekeeping protein (S1P3R/β-tubulin ratio). The effect of EAE induction on S1P3R density are represented in Fig. 7B, D and F. In FTY720-untreated EAE mice a significant increase of the density of the S1P3R protein was detected in cortical synaptosomal lysate (Fig. 7C and D) but not in total cortical homogenate (Fig. 7A and B) and in gliosomal lysates (Fig. 7E and F). As already reported for the S1P1R protein, the administration of FTY720 reduced the immunostaining of the S1P3R protein to level comparable to that observed in control mice (Fig. 7D).

In a whole, these results unveil a significant increase of S1P1R and S1P3R immunopositivity in cortical synaptosomal particles from FTY720-untreated EAE mice but not in cortical gliosomes. Interestingly, this pathological-related adaptation recovered in EAE mice therapeutically treated with FTY720.

Starting from these results, we asked whether the increase of S1P1R and S1P3R synaptosomal expression observed in the cortical synaptosomal plasma membranes from FTY720-untreated EAE-mice may cause a re-distribution of the proteins in the plasma membranes of isolated nerve endings. To answer the question, biotinylation studies were carried out to compare the immunopositivity for S1P1R and S1P3R proteins in cortical synaptosomes from control and from FTY720-untreated EAE mice (Fig. 8). Again, a clear immunopositivity of the S1P1R was detected only in the synaptosomal lysates from both control (Fig. 9A) and FTY720-untreated EAE mice (Fig. 8B) but not in the corresponding biotinylated samples. Comparable results were obtained when analysing the immunostaining of the S1P3R protein (control vs FTY720-untreated EAE-mice. Fig. 8C and Fig. 8D).

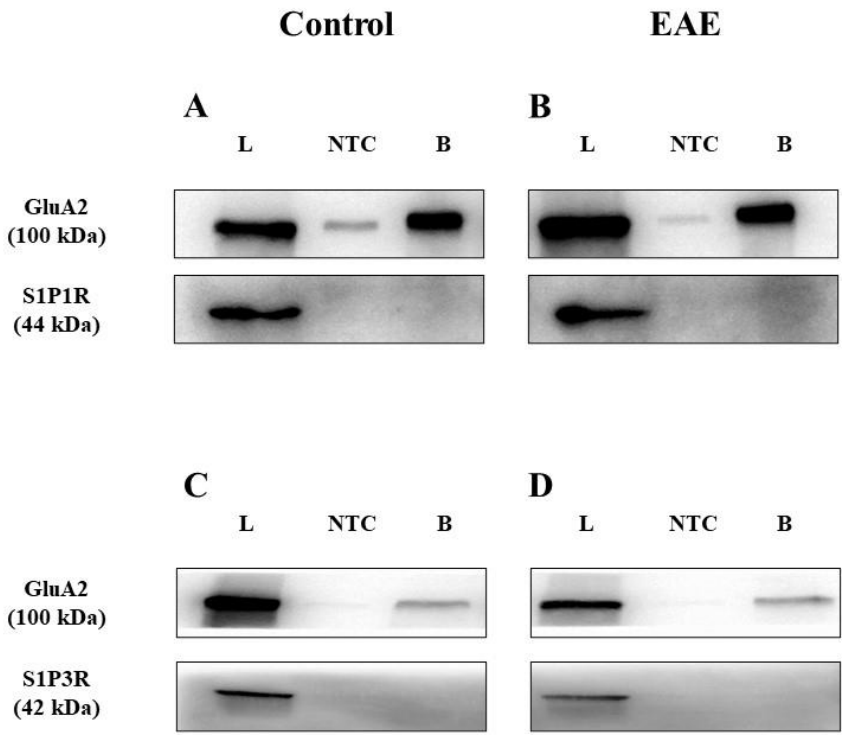


Figure 8. Western blot analysis of S1P1R and S1P3R densities in plasma membranes of cortical synaptosomes and gliosomes from control and FTY720-untreated EAE mice: biotinylation studies. Western blot analysis was carried out in cortical synaptosomal preparations from control (**A** and **C**) and FTY720-untreated EAE mice (**B** and **D**) to compare S1P1R and S1P3R density in total synaptosomal lysates (lysates, **L**), in lysates untreated with biotin but subjected to streptavidin pulldown (negative control, **NTC**), and in lysates incubated with biotin and subject to streptavidin pulldown (biotinylated, **B**). The blots in the figure are representative of three experiments carried out on different days.

Effect of MOG immunization and of FTY720 therapeutic oral administration on the presynaptic release-regulating S1P1R and S1P3R in cortical synaptosomes

Finally, we asked whether the EAE-induction and the consequent changes in S1PRs expression on cortical nerve endings may influence the efficiency of their mediated control of glutamate exocytosis and if FTY720 may recovers the pathological functional adaptations. To answer the question, release experiments were performed in cortical synaptosomes isolated from control, from FTY720-untreated EAE and FTY720-treated EAE mice, to quantify the efficiency of

CS2100 and CYM5541 in controlling the 12 mM KCl-evoked release of [^{3}H]D-aspartate. The drugs were applied at the concentration that in control cortical synaptosomes elicits the maximal functional effect (CS2100 3nM; CYM5541 100nM see Fig.3).

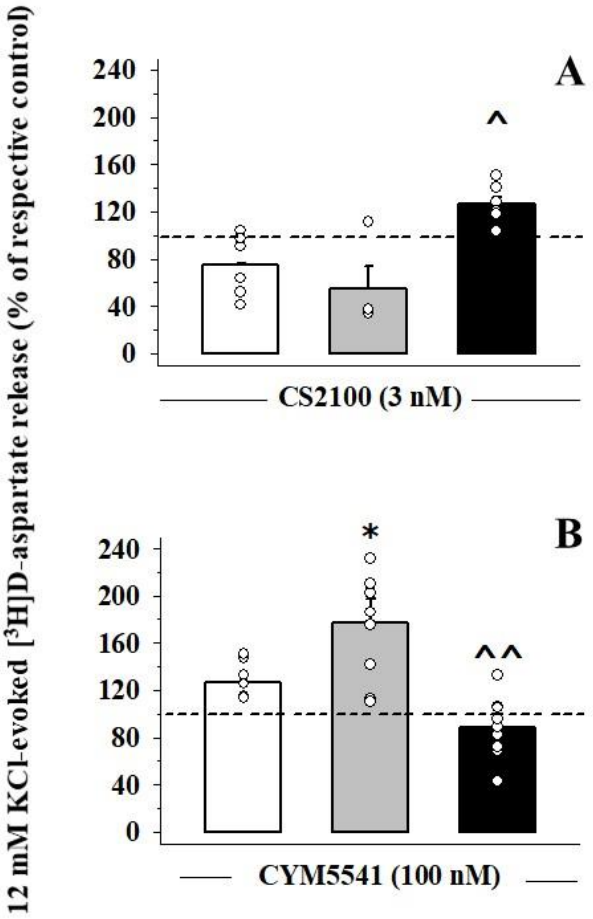


Figure 9. Effect of CS2100 and CYM5541 on the 12 mM KCl-induced [^{3}H]D-aspartate in cortical synaptosomes from control, EAE and FTY720-treated EAE mice. Isolated nerve terminals obtained from the cortex of control (white bar) , FTY720-untreated EAE (grey bar) and FTY720-treated EAE (black bar) mice were exposed in superfusion to the 12 mM KCl-enriched medium in presence of CS2100 (3 nM) (A) and CYM5541 (100nM) (B) and the ligand-induced changes of the [3H]D-aspartate exocytosis were expressed as percent of the respective control (dotted line; 12 mM KCl- induced [3H]D-aspartate release in the absence of S1PR agonist). Data are expressed as mean ± SEM of at least 4 experiments run in triplicate (* $p< 0.05$ versus control; ^ $p< 0.05$ and ^^ $p< 0.01$ versus FTY720-untreated EAE mice).

The results from the release experiments unveiled that the inhibitory activity of the S1P1R agonist (CS2100) on the [^{3}H]D-aspartate exocytosis in cortical synaptosomes from untreated

EAE mice largely increased when compared to control (Fig. 9A), well consistent with the increase of the S1P1R density observed in the Western blot analysis (Fig. 6C and D). Furthermore, the experiments unveiled that the therapeutic administration of FTY720 recovers the S1P1-mediated control of glutamate exocytosis to a physiological level, comparable to that observed in control mice (Fig. 9A). In a similar way, a significant enhancement of the efficiency of CYM5541 (100 nM) in controlling the tritium exocytosis was observed in cortical synaptosomes from FTY720-untretaed EAE mice, which again was counteracted by FTY720 administration (Fig. 9B). Also in this case, the functional results are in accordance with the changes in receptor densities that emerged in the Western blot data described in Fig. 6E and F suggesting that therapeutic administration of FTY720 can recover the EAE-induced maladaptation of both S1P1R and S1P3R.

Evaluation of sphingosine-1-phosphate receptors mRNA expression in murine primary oligodendrocyte precursor cells

As a first approach, we decided to evaluate the expression level of S1PR mRNA in both primary murine OPCs (0 days) and oligodendrocytes (OPCs cultured in differentiation medium for 12 days). The experiments were performed using the primer pairs listed in the material and method section after validation of their efficiency (data not shown). Since subtype 4 is known to be expressed mainly in the immune cells but not in the CNS [229], we decided to study the expression of four of the five S1PRs. The results (Fig. 10) show the relative mRNA expression level of S1P1,2,3 and 5 receptor mRNA by comparing OPCs (white bar) and oligodendrocytes (black bar).

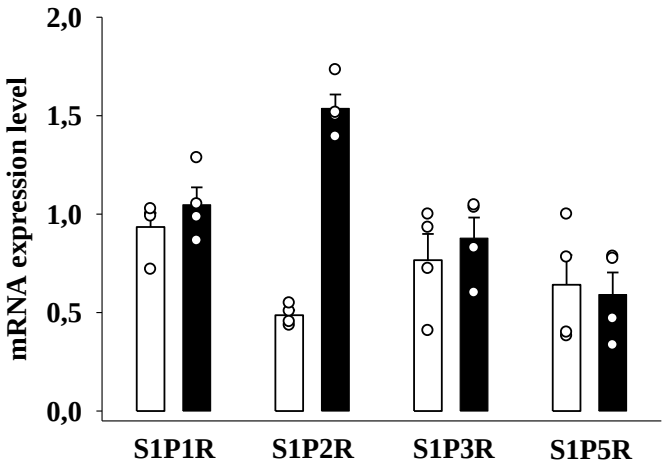


Figure 10. S1PRs mRNA is detectable in both OPCs and oligodendrocytes. Gene expression analysis in primary murine OPCs (day 0; white bar) and differentiated oligodendrocytes (day 12; black bar) of four out of five S1PRs. Data are corrected for the most stable housekeeping genes (*Pgk-1* and *Cypa*). The graph is representative of one experiment run in quadruplicate.

Although preliminary, the data unveiled that the mRNA of all the four receptor subtypes is present and detectable in both OPCs and oligodendrocytes. Surprisingly, no differences were found in the amount of S1P1,3, and 5 receptor mRNA a substantial increase in the level of S1P2R mRNA was observed in the primary oligodendroglial cells.

Effect of BAF312 on oligodendrocytes precursor cells differentiation

Once confirmed the presence of S1PRs mRNA in both cells, we decided to investigate whether the pharmacological *in vitro* treatment with BAF312 was able to induce OPC differentiation by performing an immunohistochemical analysis. Primary murine OPC were obtained following the protocol described in the "material and method section" and after a 6 days-treatment (with different concentrations of the compound) an immunostaining analysis was performed. To identify changes in differentiation stages, the OPCs were immunoassayed with myelin basic protein (MBP; red) and O_4 (green) two markers of differentiated oligodendrocytes. In particular, O_4 is considered a marker of early differentiation while MBP a marker of myelin-producing (and thus more differentiated) cells. Interestingly, although the statistical analysis could not be performed due to the lack of a sufficient amount of data, the results (represented in Fig. 11) morphologically showed a possible dose-dependent effect of BAF312 on OPC differentiation.

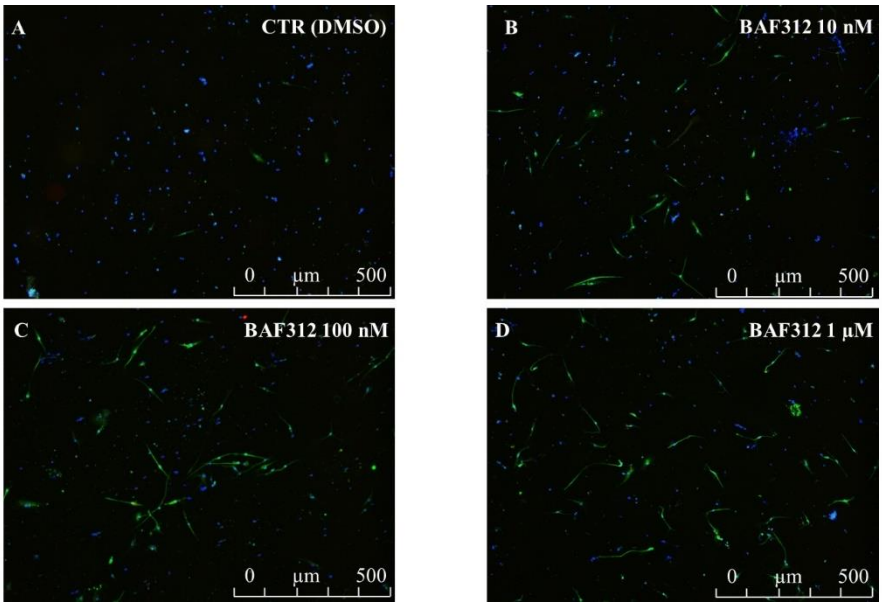


Figure 11. 6 days-treatment with BAF312 increases O_4 immunopositivity in primary murine OPCs. Murine OPCs (day 0) were treated for 6 days with three different concentrations of BAF312: 10 nM, 100 nM, and 1µM (**B**, **C** and **D** respectively) or vehicle [dimethyl sulfoxide (DMSO); **A**] At the end of the treatment, the immunostaining was carried out to investigate the immunopositivity to MBP (signal not found) and O_4 (green).

DAPI (blue) was used to stain the nuclei. The images are representative of two experiments run in triplicate and conducted on different days.

The data revealed that after 6 days neither control nor the treated cells show any immunopositivity for MBP (red signal) suggesting that probably the treatment time and the concentrations used are not sufficient to induce OPC differentiation to the myelinated stages. Otherwise, some variations were observed in the number and morphology of O_4+ cells (green). Treatment with BAF312 at all the three concentrations appears to increase the amount of O_4 immunofluorescence compared to cells treated with vehicle (DMSO; A). The effect is notable also at the lower concentration (B), but it becomes more evident when the compound is applied at the higher ones (C and D). Overall, morphological assessment of the cells showed that DMSO-treated OPCs retained undifferentiated morphology, while BAF312-treated OPCs displayed longer process extensions and more O_4 positivity correlating with a preliminary indication of a possible effect on cell differentiation.

Activation of TrkB, p-CREB and BDNF pathway in BAF312-treated Olineu cells

In literature, some studies reported the ability of S1PRs modulator to induce the release of neurotrophic factors from both neuronal and glial cells [188]. In this context, we wanted to evaluate whether, following the exposure to BAF312, OPCs are induced to increase transcription of TrkB mRNA, a BDNF receptor known to be involved in mechanisms of neuroprotection. Since the compound is highly selective for S1P1R and S1P5R we also study the mRNA expression level the two receptors.

The OPCs were treated with BAF312 for 48h with the three concentrations mentioned above. The result, shown in Fig.12, unveiled a dose-dependent increase in S1P1R after treatment while an opposite trend can be observed for the S1P5R mRNA expression level. Starting from the findings that S1P5R is associate to a more differentiated stage and S1P1R to a more immature one [102,126,230], the data suggest that, at the lowest concentration applied (10 nM; grey bar),

the compound stimulates OPC differentiation. Interestingly, the qPCR analysis performed on the same samples unveiled that at the same low concentration (10 nM) BAF312 enhances TrkB mRNA expression, consistently with the results observed on S1P5R. Like the latter receptor, the amount of TrkB mRNA also decreases in a dose-dependent manner, further supporting the hypothesis of a BDNF/TrkB-dependent neuroprotective role of BAF312 on oligodendroglial cells.

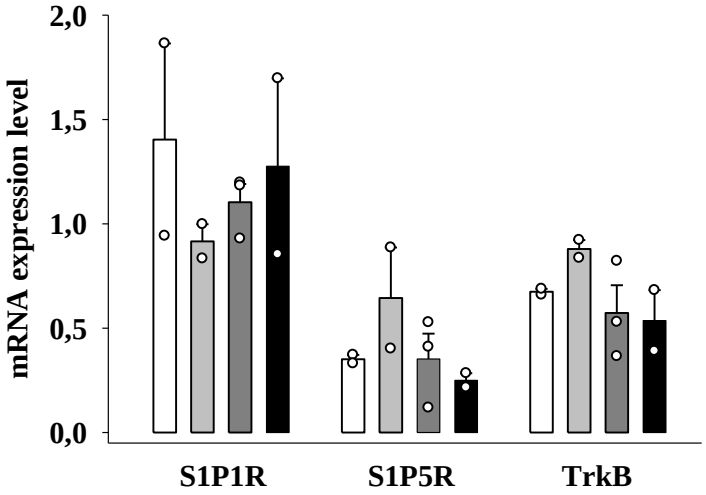


Figure 12. Low (10 nM) BAF312 concentration increases the mRNA expression level of S1P5 and TrkB receptors in primary murine OPC. The primary murine OPCs (0 day) were treated for 48h with BAF312 (10nM grey bar, 100 nM dark grey bar, and 1µM black bar) or vehicle (DMSO; white bar). Data are corrected for the most stable housekeeping genes (*Pgk-1* and *Cypa*). The graph is representative of one experiment run in duplicate/triplicate.

Based on the gene analysis, we decided to further investigate the involvement of BDNF pathway by performing a Western blot analysis. Due to the lack of time, these preliminary experiments were conducted on OPCs cell line (Olineu) rather than primary cells. Olineu were treated for 48h with BAF312 and immunopositivity for pro-BDNF, p-TrkB and p-CREB was investigated. Interestingly, the results (Fig. 13A) unveiled a higher expression of p-TrkB in samples treated with the lowest concentration of BAF312 (10nM). Since TrkB phosphorylation is positively correlated with receptor activation, the data are consistent with the resulting increased TrkB mRNA expression in qPCR analysis (Fig. 12). Conversely, both pro-BDNF and

p-CREB expression appear to be slightly reduced in a dose dependent manner (Fig. 13B and C respectively).

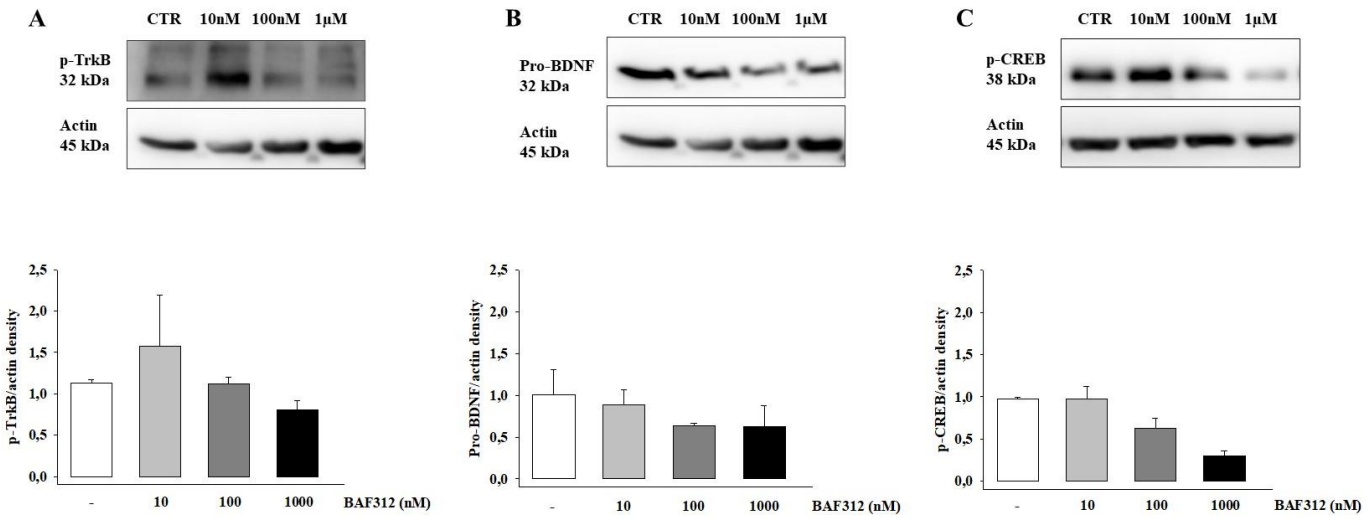


Figure 13. BAF312 (10 nM) increases the density of the p-TrkB protein in Olineu. Olineu were treated with BAF312 (10nM grey bar, 100 nM dark grey bar, and 1µM black bar) or with vehicle (DMSO; white bar). Data are the results of one experiment run in triplicate.,

DISCUSSION

Studies with synaptosomes and gliosomes

Over the past decade, several disease-modifying drugs targeting S1PRs (e.g., FTY720 and derivatives) have been proposed in the clinic for the treatment of MS. The mechanism of action of FTY720 and related drugs (i.e., BAF312) relies on their ability to inhibit T-lymphocytes egression from secondary lymph nodes [183], consequently reducing the inflammatory infiltration into the CNS, which is one of the hallmarks of the disease. The results of the research I carried out during my PhD, however, provide new insights that support the hypothesis of a direct effect of FTY720 on S1PRs in neurons in selected regions of the CNS.

Together with aspartate, glutamate is the main excitatory neurotransmitter in the CNS [231,232]. Dysregulation of the amount of glutamate available in the synaptic cleft leads to an over-activation of ionotropic receptors and consequent hyper-mobilization of intracellular Ca^{2+} ions which is deleterious to cellular functions and survival. Actually the consequent activation of Ca^{2+}-dependent intracellular pathways causes the overproduction of free radicals leading to mitochondrial damage and cell membrane disruption which culminate in neuronal death and, axonal loss [233–236]. Collectively these conditions are known as "glutamate excitotoxicity" and has been recognized as one of the hallmarks of several neurodegenerative disease including MS, Alzheimer's disease and Huntington disease [237].

In 2010, FTY720, which is a wide-spectrum agonist of S1PRs, was the first oral treatment approved for the cure of the RRMS [238] and during this time beside its immunomodulatory effect, an increasing number of studies have also been dedicated to highlight the potential role of FTY720 and derivatives (i.e., BAF312) to prevent glutamate excitotoxicity. The hypothesis was supported by preclinical findings suggesting that glutamate system could be the target of these drugs. Accordingly, the research group headed by Anna Pittaluga demonstrated that oral

administration of FTY720 recovers glutamate impairments that occur in different areas of the CNS of mice suffering from the EAE at different stages of the disease [212].

Despite the clear evidences supporting the neuroprotective role of this drug, the exact molecular mechanism involved is still a matter of debate, although the ability of the compound to cross the BBB and to reach high concentrations in the CNS [187] supports the hypothesis that FTY720 and others S1PRs modulators could interact directly with S1PRs expressed at glutamatergic synapses, particularly at the presynaptic level. The hypothesis is even more attracting considering that, beside the main role of demyelination and autoimmune-dependent inflammatory responses, MS is recognized as a synaptopathy, i.e., a pathology typed by alterations in synaptic transmission which occur even at the early stage of the disease and in different areas of the CNS, including the cortex [170,212].

To address the question and provide evidence that the restoration of glutamatergic exocytosis efficiency elicited by *in vivo* therapeutic FTY720 might also involve direct control of glutamate exocytosis, we first investigated the presence of release-regulating S1PRs in glutamatergic nerve endings and astrocytic processes in the cortex of healthy mice.

Although the spinal cord is the central region where demyelination and inflammation occur mainly during the course of EAE [239–241], we focused on the cortex as this is one of the brain regions where synaptic defects and astrocytosis preferentially develop starting from the early stage of the disease, being therefore a suitable model to study the impact of FTY720 on glutamate-mediated events that could elicit and subserve synaptopathy.

As a first approach, experiments have been carried out to investigate the expression S1PRs under physiological conditions, i.e., in nerve endings (synaptosomes) and astrocytic processes isolated from the cortex of healthy mice. In this regard, Wang and colleagues in 2021 provided evidence of the existence of S1P1Rs in cortical synaptosomes [112]. Our results from Western blot and confocal analyses in cortical synaptosomes and gliosomes confirmed their

observations, but also unveiled the presence of S1P3R in glutamatergic synaptosomes and of both S1P1R and S1P3R in cortical gliosomes, in line with previous observation showing the presence of these proteins in cortical neuron and astrocytes [242]. Based on these results, we asked whether these receptors could modulate glutamate release. To address the question, we applied an experimental approach (i.e., the up-down superfusion of a thin layer of particles) that was set up in the laboratory of the Pharmacology and Toxicology Section of DIFAR and which is widely recognized as an approach of choice to address this aspect. Very recently, FTY720 has been shown to inhibit, in concentration-dependent manner, the 4-AP-induced glutamate release from rat cortical synaptosomes [112]. The authors suggested that FTY720 acts primarily at S1P1Rs, reducing the mobilization of intraterminal Ca^{2+} ions and then the probability of glutamate exocytosis [112]. Consistent with this view, we found that S1P1R agonist (CS2100) significantly inhibited [^{3}H]D-aspartate release from mice cortical synaptosomes but had no effect on glutamate exocytosis from cortical gliosomes. In contrast, the selective S1P3R agonist CYM5541 significantly enhanced the neurotransmitter overflow in cortical synaptosomes but inhibited in a concentration-dependent manner the release of glutamate elicited by high K^+ ions from glial particles. The different impacts on glutamate exocytosis can be explained by assuming that S1P3R couples different G-proteins in nerve endings and astrocytic processes, which in turn generate opposite intracellular signaling pathways. A similar conclusion can also be proposed when considering the opposite outcomes of the ligand-mediated activation of S1P1R and S1P3R in isolated nerve endings. The activation of the former receptor efficiently inhibits glutamate exocytosis in cortical synaptosomes while that of the latter one potentiates it. Again, the two receptors could bind different GPCRs, but the opposite outcomes elicited by the two receptors in isolated nerve endings also might give the rationale for the lack of efficacy of S1P (which activates both the S1P1R and the S1P3R) in controlling glutamate exocytosis from cortical synaptosomal particles. I propose that the lack of efficacy of S1P is apparent and determined by the sum of the respective effects of the agonist at the presynaptic release-

regulating S1P1Rs and S1P3Rs, which compensate each other in controlling the transmitter outflow [216,243]. Unfortunately, the data so far available do not allow to conclude whether the two receptors co-exist on the same terminals or if they are expressed in different subpopulation of cortical synaptosomes. Nonetheless, whatever their reciprocal distribution, their concomitant activation would lead to an additive effect as observed.

The heterogeneity of the impact of S1P1R and S1PR3 ligands on glutamate overflow also accounts for the contradictory results of studies aimed at investigating the mechanisms of neuroprotection elicited by FTY720. The drug, which targets four out of the five S1PRs [244] has been reported to mediate either inhibition [115,245] or facilitation [246] of glutamate release depending on the receptor subtype involved and the targeted CNS region [108,109,247].

Another unexpected observation of the present thesis concerns the preferential cytosolic location of S1PRs in both synaptosomal and gliosomal particles. To decipher the location of the two receptors, biotinylation studies have been performed. The results from these experiments were largely unexpected and showed that S1P1R and S1P3R immunopositivity is almost undetectable in biotinylated samples from both cortical synaptosomes and gliosomes. The lack of immunostaining can be explained by assuming that the receptors are inaccessible to biotin, which indirectly implies that both proteins are poorly expressed on the outer side of the synaptosomal/gliosomal membranes, but preferentially present at the inner side of membranes or even widely present in the cytosol. These observations shed new light on the FTY720-receptor interaction in nerve endings and/or gliosomes, also considering that this aspect is matter of debate and that several scenarios have been proposed in the literature. Among these, a functional antagonism of FTY720 at S1PRs has been proposed to occur in cells, particularly the T-lymphocytes that would favour the receptors internalization and subsequent degradation [183,248]. This cascade of event is proposed to mainly account for the reduced release of the T-lymphocytes into the blood stream. A similar hypothesis involves the binding and consequent activation of the S1PRs in neurons, microglia, oligodendrocytes, and astrocytes.

Also in this case, it was proposed that the ligand-receptor interaction leads to a rapid internalization of the receptor complex, the implication of which are so far poorly investigated [249]. Last, a third scenario proposes the activation of receptor-independent mechanism(s) triggered by the interaction of the drug with cytosolic proteins to modulate intracellular pathways or affect epigenetic changes [184,250,251]. The latter case is proposed not to involve GPCR-mediated events. Interestingly, the first and the second scenarios imply a significant and rapid internalization of S1PRs while the third one implies the presence of so far unknown selective FTY720 targets in the cytosol of cells. As far as the first and the second hypothesis are concerned, the possibility should be envisaged that in synaptosomes and gliosomes the internalization of S1PRs is a rapid event that cannot be highlighted with the techniques so far applied, due to an in-out trafficking of receptor proteins that assure a preferential cytosolic distribution of the S1PR proteins and a low almost undetectable presence of the S1PRs in plasma membranes. Since the preferential cytosolic distribution of the receptor proteins has been observed in synaptosomes and gliosomes that have not been exposed to agonists, it also seems conceivable to propose that the in-out movements are constitutive and do not depend on the presence of the receptor ligands. Moreover, the partition coefficient of the S1P1R and the S1P3R ligands used in this study well supports the hypothesis that these drugs can access and bind proteins located at the inner part of the plasma membranes or with a cytosolic distribution. Further experiments will be carried out to verify the hypothesis.

Beside the considerations on the mode of interaction of S1PR ligand and S1P1/3R, it is worth reminding that evidence exist in the literature showing that in MS patients the S1PR expression is significantly altered [252,253]. In this perspective, it has been shown that *in vitro*, FTY720 is able to modulate the expression of both S1P1R and S1P3R mRNA and proteins [88,129,254]. Consistent with this observation, Western blot analyses confirmed a significant increase in the density of S1P1R and S1P3R in synaptosomes isolated from the cortex of EAE mice at the acute stage of the disease. The receptor overexpression however did not emerge in total cortical

homogenate, and it was limited to the synaptosomal particles, since it was not observed cortical gliosomes. The latter finding contrasts some data in literature which described an increased expression of S1P1Rs also in astrocytes [255–257]. In an attempt to reconcile the opposite findings, it could be supposed that the increased expression of S1PRs might occur in compartment(s) of the glial cells other than that from which gliosomal particles are thought to preferentially originate (i.e., the astrocytic branching).

The increased density of S1PRs in cortical synaptosomal lysate from EAE mice at the acute stage of disease was paralleled to changes in the efficiency of the receptors in controlling the glutamate exocytosis. In particular, the inhibitory activity of the S1P1R agonist (CS2100) on glutamate overflow was enhanced in cortical synaptosomes from EAE mice compared to that from control animals, well consistent with the higher receptor's density detected in the EAE cortical synaptosomes. Similarly, CYM5541-mediated facilitation of high KCl- evoked [^{3}H]D-aspartate was enhanced in EAE mice compared to control. Overall, these observations support the conclusion that alteration in the density of the S1P1R and S1P3R proteins detected presynaptically in isolated nerve endings from the cortex of EAE mice correlates to functional adaptation of these receptors in controlling the exocytosis of glutamate this brain area. Based on the evidence that FTY720 can modulate the mRNA expression encoding for S1PRs [88,117,129] we tested whether the chronic therapeutic administration of FTY720 (0.3 mg/kg) was able to restore the density and/or the functions of the presynaptic S1PRs in cortical synaptosomes and gliosomes. Western blot analyses of cortical synaptosomes isolated from FTY720-treated mice showed a significant reduction in immunopositivity for S1P1R and S1P3R compared to FTY720-untreated EAE mice, but comparable to that of controls. The results are consistent with the conclusion that the drug, administered therapeutically, recovers the altered expression of both receptors, positively reverberating on glutamate transmission from cortical glutamatergic nerve endings in EAE-mice.

In a whole, these results highlight the role S1PRs agonists in the tripartite synapses in the cortex of adult mice, proving their main role as modulator of the excitatory transmission. Furthermore, the study offers information on the complexity of the system and how deeply it is modified during the course of the disease. Third, new insights into the distribution of S1PRs in nerve terminals and astrocytic processes have been highlighted, raising new questions about the cellular distribution of these receptors and the site of action of FTY720.

Finally, the outcomes provide evidence that the S1PRs system is selectively targeted during the course of the EAE and that the therapeutic administration of FTY720 efficiently recovers this maladaptation, restoring the synaptic efficiency in the cortex of these mice.

Studies with oligodendroglia cells

The second part of the thesis aims to investigate the role of S1PRs modulation in inducing OPC differentiation and migration. Although the data are insufficient to draw significant conclusions and further investigations are needed, some preliminary assumptions may be proposed.

As a first approach, we performed qPCR analysis to study the mRNA expression of S1P1R, S1P2R, S1P3R, and S1P5R by comparing OPC and oligodendrocytes (OPC in differentiation medium for 12 days). In the literature the presence of S1PRs subtypes on oligodendroglia cells is not fully clarified. Interestingly, it has been observed that S1P1R is predominantly expressed in OPC, while S1P5R predominates in oligodendrocytes [102,126–132]. Indeed, initial studies aimed at investigating the distribution of S1PRs in the CNS revealed high levels of S1P5R mRNA expression in white matter tracts of the adult rat brains, suggesting the presence of this receptor in oligodendrocytes [253]. Although only low levels of S1P3R and S1P2R are known to be present in oligodendroglial cells [242], qPCR analysis reported detectable amount of mRNA of all the four receptor subtypes. Additionally, unlike expected, there were no differences reported in the amount of mRNA of S1P1R and S1P5R between OPCs and oligodendrocytes, but a substantial increase of S1P2R was detected. The results were obtained from a single experiment performed in quadruplicate and therefore further analyses are necessary to clarify the outcomes and confirm the S1PRs mRNA expression level.

Following the discovery of FTY720, an increasing number of studies were conducted to develop new S1PRs modulators. Since the main adverse effects of FTY720 have been associated with its binding to S1P3R [258], new-generation molecules aim to target with higher selectivity only S1P1R and S1P5R. Indeed, while interaction with the first receptor is associated to the anti-inflammatory properties of the ligands, S1PR5 binding, is thought to be involved in the survival of mature oligodendrocytes and the promotion of myelination processes [126,213,214]. BAF312, a highly selective S1PRs modulator for S1P1R and S1P5R, has been

shown to cross the BBB, stimulate remyelination and prevent synaptic degeneration that occur in EAE mice [204,215,259]. Therefore, we tested the effect of the compound on OPC differentiation. The immunohistochemical analysis, although preliminary, suggested that, at the times and concentrations used, the compound was unable to induce differentiation of primary OPC to the myelinated stages since no MBP immunopositivity was detected. However, the treatment increased the amount of O4 immunofluorescence compared to vehicle-treated cells.

Although no clear information has been provided about the role of BAF212 on OPC differentiation, new insights are emerging. It has been observed that prolonged daily treatment with BAF312 reduces the response to the external natural ligand by inhibiting ERK phosphorylation induced by BAF312 re-exposure, also stimulating the differentiation of foetal human OPC [260]. Further experiments are needed to improve our comprehension of these events.

Low levels of BNDF were found in the cuprizone-induce demyelination animal model [261] as well as in MS patients [262]. The lack of physiological amount of BDNF plays an important role in demyelinating lesions as it regulates the number of progenitors and their ability to differentiate into mature oligodendrocytes [261]. Interestingly, FTY720 has been observed to upregulate the expression level of BDNF mRNA and to increase the release of BDNF in an activity- and MAPK-dependent manner [188]. The BNDF-mediated neuroprotective effect of FTY720 has also been studied in an animal model of Alzheimer's disease [263]. In particular, oral administration of the compound has been shown to ameliorate the Aβ-induced memory impairments by up-regulation of BDNF production [263]. Based on this, experiments were performed to investigated whether BAF312 could induce OPC differentiation by activating the BDNF- TrkB signaling pathway. *In vitro* administration of 10 nM of BAF212 for 48 hours slightly increased the mRNA expression of S1P5R in primary OPC and at the same time appears to moderately reduce S1P1R mRNA expression. In parallel, TrkB protein density in Olineu cell line appears to be improved following stimulation with BAF212 (10 nm for 48 hours). Since

the expression S1P5R is associated with a more mature stage of differentiation and S1P1R with a more immature one, qPCR analysis suggests a possible role of the compound in inducing OPC differentiation. At the same concentration, BAF312 also enhanced the expression of the phosphorylated/active form of TrkB receptor (p-TrkB), suggesting possible involvement of BDNF pathway. Although further analyses need to be conducted to confirm these experimental data, the results suggest the hypothesis of a new possible neuroprotective effect of BAF312.

The finding that the lower concentration of BAF312 is more effective than the higher concentrations used in these experiments, is in line with other literature studies [14]. The EC50 of BAF312 towards S1P1R and S1P5R is less than nanomolar (0,39 nm and 0,38 nm respectively [46]). As a result, it is possible that higher concentration of the compound will generate promiscuous effects, possibly involving the concomitant activation of other S1PR subtypes. It may also be possible that low concentration promotes internalization of S1PR, which in turn can induce the transcription of other S1PRs, and, therefore, increase their membrane expression. This mechanism of re-sensitization could lead to prolonged responsiveness of the cells to endogenous and exogenous ligands.

Despite the preliminary nature of the outcomes, it is plausible that BAF312 can stimulate the production of BDNF, exerting a neuroprotective role towards neurons. At the same time, the results suggest that the compound could induce the transcription and the activation of TrkB, the BDNF receptor. Therefore, by enhancing the production of BNDF in the CNS, BAF312 could stimulate neuroprotection and concomitantly induce OPC differentiation.

CONCLUSIONS, SOCIAL IMPACT AND TRANSLATABILITY OF THIS STUDY

Beyond their immunomodulatory effect, S1PRs modulators are now being studied for their direct role on the CNS. However, most research is focusing on their effect on remyelination while less is known about their possible role in controlling glutamate transmission [108,109,115,212,247].

Glutamate impairment is known to occur in many neurological disorders, but effective treatments have not been found to date. In this context, the study aims to clarify the role of S1PRs in the control of this event and could open the possibility of the use of S1PRs modulators also in other neuropathological conditions. Interestingly, the results could also spark interest in studying the effect of the new and more selective S1PRs modulators in controlling neurotransmitter release. Since our findings suggest that the concomitant activation of several S1PR subtype leads to compensatory intracellular mechanisms, using more selective compounds could lead to a more specific and targeted results.

The second part of the thesis allows us to speculate on the ability of BAF123 to interact directly with the OPC and to activate the BDNF pathway. If confirmed, the findings could support the use of BAF312 and other S1PRs modulators in other demyelinating or neurological disorders where a lack of BDNF is observed.

FUTURE PERSPECTIVES

A few years ago, the research group led by Anna Pittaluga demonstrated that glutamate release from the spinal cord and cortical synaptosomes was impaired in EAE mice. Interestingly, while glutamate release from cortical synaptosomes was reduced, glutamate overflow from spinal cord synaptosomes isolated from EAE mice was significantly increased. However, oral treatment with FTY720 was able to recover the imbalance in glutamate transmission in both areas. In this context, it would be of interest to extend the study we performed in the cortex and investigate the role of S1PRs and their expression also in the spinal cord of EAE mice.

Interestingly, in recent years our research group has focused attention on the effect of ellagic acid (a natural molecule) on neuroinflammation. Our previous investigations unveiled positive outcomes following the administration of the nutraceutical compound in EAE mice (see. publication section). Since we have demonstrated the existence and the role of S1PRs in controlling glutamate transmission, it would be interesting to study the effect of the concomitant administration of ellagic acid with FTY720 (or derivatives) on EAE mice.

As for the studies conducted on OPC, the aim will be, first of all, to confirm the preliminary data obtained. If validated, it would be interesting to confirm the involvement of BNDF pathway in the BAF312-induced OPC differentiation by studying the impact of the compound on primary OPC, in presence of a TrkB antagonist. Subsequently the results could be further supported by testing the compound on OPC isolated from both TrkB and BDNF KO mice.

Interestingly, since S1P1R and S1P5R are known to regulate the migration of oligodendroglial cells, in future, study the effect of S1PR modulators on OPC migration.

ABBREVIATIONS

Dihydrosphingosine (DHS)

Sphingomyelin (SM)

Ceramide (Cer)

Sphingosine-1-phosphate (S1P)

Cer-1-phosphate (C1P)

Glycosphingolipids (GSLs)

Sphingomyelinases (SMase)

Sphingosine (Sph)

Sphingosine kinase-1/2 (SphK1/2)

Protein kinase c (PKC)

Tumor necrosis factor-α (TNF-α)

Extracellular signal-regulated kinase (ERK)

S1P lyase (SPL)

Red blood cells (RBCs)

Wild-type (WT)

ATP-binding cassette (ABC)

Spinster-like protein 2 (SPNS2)

Chinese hamster ovary (CHO)

Sphingosine-1-phosphate receptors (S1PRs)

Major facilitator superfamily transporter 2b (Mfsd2b)

G protein-coupled receptors (GPCRs)

Lysophosphatidic acid (LPA)

Central nervous system (CNS)

Vascular smooth muscle cells (VSMCs)

Phospholipase C (PLC),

Phosphoinositide 3-kinase (PI3K

Ras guanosine triphosphatase (GTPase)

Adenylyl cyclase (AC)

Mitogen-activated protein kinase (MAPK)

Protein kinase B (Akt)

Interleukin (IL)

Cyclic-AMP (cAMP)

Protein kinase A (PKA)

Pertussis toxin (PTX)

Oligodendrocyte precursor cells (OPCs)

Multiple sclerosis (MS)

Miniature excitatory postsynaptic currents (mEPSCs)

N-methyl-D-aspartate (NMDA)

Experimental autoimmune encephalomyelitis (EAE)

FTY720- phosphate (FTY720P)

4-aminopyridine (4-AP)

Lipopolysaccharide (LPS)

Nuclear factor-κB (NF-κB)

L-glutamate/L-aspartate transporter (GLAST)

Glutamate transporter-1 (GLT-1)

p38 mitogen-activated protein kinase (p38MAPK)

Metabotropic glutamate receptors (mGluRs)

Vesicular glutamate transporter (VGLUT)

Carbenoxolone (CBX),

L-glutamate (L-Glu)

Transient middle cerebral artery occlusion and reperfusion (tMCAO)

Clinically isolated syndrome (CIS)

Relapsing-remitting MS (RRMS)

Primary-progressive MS (PPMS)

Secondary progressive MS (SPMS)

Blood-brain barrier (BBB)

Cerebrospinal fluid (CSF)

Brain-derived neurotrophic factor (BDNF)

Dimethyl sulfoxide DMSO

Tropomyosin receptor kinase B (TrkB)

BIBLIOGRAPHY

1. Kleuser, B. The Enigma of Sphingolipids in Health and Disease. *Int J Mol Sci* **2018**, *19*, doi:10.3390/ijms19103126.
2. Patton, J.L.; Lester, R.L. Phosphatidylinositol Phosphate, Phosphatidylinositol Bisphosphate, and the Phosphoinositol Sphingolipids Are Found in the Plasma Membrane and Stimulate the Plasma Membrane H(+)-ATPase of Saccharomyces Cerevisiae. *Arch Biochem Biophys* **1992**, *292*, 70–76, doi:10.1016/0003-9861(92)90052-x.
3. McKibbin, J.M. Fucolipids. *J Lipid Res* **1978**, *19*, 131–147.
4. Patton, J.L.; Lester, R.L. The Phosphoinositol Sphingolipids of Saccharomyces Cerevisiae Are Highly Localized in the Plasma Membrane. *J Bacteriol* **1991**, *173*, 3101–3108, doi:10.1128/jb.173.10.3101-3108.1991.
5. Rao, R.P.; Acharya, J.K. Sphingolipids and Membrane Biology as Determined from Genetic Models. *Prostaglandins Other Lipid Mediat* **2008**, *85*, 1–16, doi:10.1016/j.prostaglandins.2007.10.002.
6. Quinville, B.M.; Deschenes, N.M.; Ryckman, A.E.; Walia, J.S. A Comprehensive Review: Sphingolipid Metabolism and Implications of Disruption in Sphingolipid Homeostasis. *International Journal of Molecular Sciences* **2021**, *22*, 5793, doi:10.3390/ijms22115793.
7. Degroote, S.; Wolthoorn, J.; van Meer, G. The Cell Biology of Glycosphingolipids. *Seminars in Cell & Developmental Biology* **2004**, *15*, 375–387, doi:10.1016/j.semcdb.2004.03.007.
8. Farfel-Becker, T.; Vitner, E.B.; Kelly, S.L.; Bame, J.R.; Duan, J.; Shinder, V.; Merrill, A.H., Jr; Dobrenis, K.; Futerman, A.H. Neuronal Accumulation of Glucosylceramide in a Mouse Model of Neuronopathic Gaucher Disease Leads to Neurodegeneration. *Human Molecular Genetics* **2014**, *23*, 843–854, doi:10.1093/hmg/ddt468.
9. Shayman, J.A. Sphingolipids. *Kidney International* **2000**, *58*, 11–26, doi:10.1046/j.1523-1755.2000.00136.x.
10. Sipione, S.; Monyror, J.; Galleguillos, D.; Steinberg, N.; Kadam, V. Gangliosides in the Brain: Physiology, Pathophysiology and Therapeutic Applications. *Front Neurosci* **2020**, *14*, 572965, doi:10.3389/fnins.2020.572965.
11. Takahashi, T.; Suzuki, T. Role of Sulfatide in Normal and Pathological Cells and Tissues. *Journal of Lipid Research* **2012**, *53*, 1437–1450, doi:10.1194/jlr.R026682.
12. Pruett, S.T.; Bushnev, A.; Hagedorn, K.; Adiga, M.; Haynes, C.A.; Sullards, M.C.; Liotta, D.C.; Merrill, A.H. Thematic Review Series: Sphingolipids. Biodiversity of Sphingoid Bases ("Sphingosines") and Related Amino Alcohols. *J Lipid Res* **2008**, *49*, 1621–1639, doi:10.1194/jlr.R800012-JLR200.
13. Marquês, J.T.; Marinho, H.S.; de Almeida, R.F.M. Sphingolipid Hydroxylation in Mammals, Yeast and Plants – An Integrated View. *Progress in Lipid Research* **2018**, *71*, 18–42, doi:10.1016/j.plipres.2018.05.001.
14. Hanada, K. Serine Palmitoyltransferase, a Key Enzyme of Sphingolipid Metabolism. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2003**, *1632*, 16–30, doi:10.1016/S1388-1981(03)00059-3.
15. Mullen, T.D.; Hannun, Y.A.; Obeid, L.M. Ceramide Synthases at the Centre of Sphingolipid Metabolism and Biology. *Biochem J* **2012**, *441*, 789–802, doi:10.1042/BJ20111626.
16. Michel, C.; van Echten-Deckert, G.; Rother, J.; Sandhoff, K.; Wang, E.; Merrill, A.H. Characterization of Ceramide Synthesis: A DIHYDROCERAMIDE DESATURASE INTRODUCES THE 4,5-TRANS-DOUBLE BOND OF SPHINGOSINE AT THE

LEVEL OF DIHYDROCERAMIDE*. *Journal of Biological Chemistry* **1997**, *272*, 22432–22437, doi:10.1074/jbc.272.36.22432.
17. Merrill, A.H. De Novo Sphingolipid Biosynthesis: A Necessary, but Dangerous, Pathway *. *Journal of Biological Chemistry* **2002**, *277*, 25843–25846, doi:10.1074/jbc.R200009200.
18. Ternes, P.; Franke, S.; Zähringer, U.; Sperling, P.; Heinz, E. Identification and Characterization of a Sphingolipid Δ4-Desaturase Family*. *Journal of Biological Chemistry* **2002**, *277*, 25512–25518, doi:10.1074/jbc.M202947200.
19. Futerman, A.H.; Riezman, H. The Ins and Outs of Sphingolipid Synthesis. *Trends in Cell Biology* **2005**, *15*, 312–318, doi:10.1016/j.tcb.2005.04.006.
20. Hanada, K.; Kumagai, K.; Yasuda, S.; Miura, Y.; Kawano, M.; Fukasawa, M.; Nishijima, M. Molecular Machinery for Non-Vesicular Trafficking of Ceramide. *Nature* **2003**, *426*, 803–809, doi:10.1038/nature02188.
21. Kraft, M.L. Sphingolipid Organization in the Plasma Membrane and the Mechanisms That Influence It. *Front Cell Dev Biol* **2017**, *4*, doi:10.3389/fcell.2016.00154.
22. Clarke, C.J.; Wu, B.X.; Hannun, Y.A. The Neutral Sphingomyelinase Family: Identifying Biochemical Connections. *Adv Enzyme Regul* **2011**, *51*, 51–58, doi:10.1016/j.advenzreg.2010.09.016.
23. Mizutani, Y.; Tamiya-Koizumi, K.; Nakamura, N.; Kobayashi, M.; Hirabayashi, Y.; Yoshida, S. Nuclear Localization of Neutral Sphingomyelinase 1: Biochemical and Immunocytochemical Analyses. *Journal of Cell Science* **2001**, *114*, 3727–3736.
24. Wu, B.X.; Rajagopalan, V.; Roddy, P.L.; Clarke, C.J.; Hannun, Y.A. Identification and Characterization of Murine Mitochondria-Associated Neutral Sphingomyelinase (MA-NSMase), the Mammalian Sphingomyelin Phosphodiesterase 5. *J Biol Chem* **2010**, *285*, 17993–18002, doi:10.1074/jbc.M110.102988.
25. Cheng, Y.; Nilsson, Å.; Tömquist, E.; Duan, R.-D. Purification, Characterization, and Expression of Rat Intestinal Alkaline Sphingomyelinase. *Journal of Lipid Research* **2002**, *43*, 316–324, doi:10.1016/S0022-2275(20)30174-7.
26. Duan, R.-D. Alkaline Sphingomyelinase: An Old Enzyme with Novel Implications. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2006**, *1761*, 281–291, doi:10.1016/j.bbalip.2006.03.007.
27. Dressler, K.A.; Kolesnick, R.N. Ceramide 1-Phosphate, a Novel Phospholipid in Human Leukemia (HL-60) Cells. Synthesis via Ceramide from Sphingomyelin. *Journal of Biological Chemistry* **1990**, *265*, 14917–14921, doi:10.1016/S0021-9258(18)77204-9.
28. Wijesinghe, D.S.; Massiello, A.; Subramanian, P.; Szulc, Z.; Bielawska, A.; Chalfant, C.E. Substrate Specificity of Human Ceramide Kinase. *Journal of Lipid Research* **2005**, *46*, 2706–2716, doi:10.1194/jlr.M500313-JLR200.
29. Brown, R.E. Sphingolipid Organization in Biomembranes: What Physical Studies of Model Membranes Reveal. *Journal of Cell Science* **1998**, *111*, 1–9.
30. Hoekstra, D.; Maier, O.; van der Wouden, J.M.; Slimane, T.A.; van IJzendoorn, S.C.D. Membrane Dynamics and Cell Polarity: The Role of Sphingolipids. *Journal of Lipid Research* **2003**, *44*, 869–877, doi:10.1194/jlr.R300003-JLR200.
31. Iessi, E.; Marconi, M.; Manganelli, V.; Sorice, M.; Malorni, W.; Garofalo, T.; Matarrese, P. Chapter Five - On the Role of Sphingolipids in Cell Survival and Death. In *International Review of Cell and Molecular Biology*; Spetz, J.K.E., Galluzzi, L., Eds.; Cell Death Regulation in Health and Disease - Part A; Academic Press, 2020; Vol. 351, pp. 149–195.
32. Zeidan, Y.H.; Hannun, Y.A. Translational Aspects of Sphingolipid Metabolism. *Trends in Molecular Medicine* **2007**, *13*, 327–336, doi:10.1016/j.molmed.2007.06.002.
33. Sentelle, R.D.; Senkal, C.E.; Jiang, W.; Ponnusamy, S.; Gencer, S.; Selvam, S.P.; Ramshesh, V.K.; Peterson, Y.K.; Lemasters, J.J.; Szulc, Z.M.; et al. Ceramide Targets

Autophagosomes to Mitochondria and Induces Lethal Mitophagy. *Nat Chem Biol* **2012**, *8*, 831–838, doi:10.1038/nchembio.1059.
34. Nojima, H.; Freeman, C.M.; Schuster, R.M.; Japtok, L.; Kleuser, B.; Edwards, M.J.; Gulbins, E.; Lentsch, A.B. Hepatocyte Exosomes Mediate Liver Repair and Regeneration via Sphingosine-1-Phosphate. *J Hepatol* **2016**, *64*, 60–68, doi:10.1016/j.jhep.2015.07.030.
35. Fukuda, Y.; Kihara, A.; Igarashi, Y. Distribution of Sphingosine Kinase Activity in Mouse Tissues: Contribution of SPHK1. *Biochemical and Biophysical Research Communications* **2003**, *309*, 155–160, doi:10.1016/S0006-291X(03)01551-1.
36. Wattenberg, B.W.; Pitson, S.M.; Raben, D.M. The Sphingosine and Diacylglycerol Kinase Superfamily of Signaling Kinases: Localization as a Key to Signaling Function. *Journal of Lipid Research* **2006**, *47*, 1128–1139, doi:10.1194/jlr.R600003-JLR200.
37. Pitson, S.M.; Moretti, P.A.B.; Zebol, J.R.; Lynn, H.E.; Xia, P.; Vadas, M.A.; Wattenberg, B.W. Activation of Sphingosine Kinase 1 by ERK1/2-Mediated Phosphorylation. *The EMBO Journal* **2003**, *22*, 5491–5500, doi:10.1093/emboj/cdg540.
38. Cannavo, A.; Liccardo, D.; Komici, K.; Corbi, G.; de Lucia, C.; Femminella, G.D.; Elia, A.; Bencivenga, L.; Ferrara, N.; Koch, W.J.; et al. Sphingosine Kinases and Sphingosine 1-Phosphate Receptors: Signaling and Actions in the Cardiovascular System. *Front. Pharmacol.* **2017**, *8*, doi:10.3389/fphar.2017.00556.
39. Ding, G.; Sonoda, H.; Yu, H.; Kajimoto, T.; Goparaju, S.K.; Jahangeer, S.; Okada, T.; Nakamura, S. Protein Kinase D-Mediated Phosphorylation and Nuclear Export of Sphingosine Kinase 2*. *Journal of Biological Chemistry* **2007**, *282*, 27493–27502, doi:10.1074/jbc.M701641200.
40. Igarashi, N.; Okada, T.; Hayashi, S.; Fujita, T.; Jahangeer, S.; Nakamura, S. Sphingosine Kinase 2 Is a Nuclear Protein and Inhibits DNA Synthesis. *J Biol Chem* **2003**, *278*, 46832–46839, doi:10.1074/jbc.M306577200.
41. Hait, N.C.; Allegood, J.; Maceyka, M.; Strub, G.M.; Harikumar, K.B.; Singh, S.K.; Luo, C.; Marmorstein, R.; Kordula, T.; Milstien, S.; et al. Regulation of Histone Acetylation in the Nucleus by Sphingosine-1-Phosphate. *Science* **2009**, *325*, 1254–1257, doi:10.1126/science.1176709.
42. Mizugishi, K.; Yamashita, T.; Olivera, A.; Miller, G.F.; Spiegel, S.; Proia, R.L. Essential Role for Sphingosine Kinases in Neural and Vascular Development. *Mol Cell Biol* **2005**, *25*, 11113–11121, doi:10.1128/MCB.25.24.11113-11121.2005.
43. Lee, S.; Kang, H.-G.; Jeong, P.-S.; Kim, M.J.; Park, S.-H.; Song, B.-S.; Sim, B.-W.; Kim, S.-U. Heat Stress Impairs Oocyte Maturation through Ceramide-Mediated Apoptosis in Pigs. *Sci Total Environ* **2021**, *755*, 144144, doi:10.1016/j.scitotenv.2020.144144.
44. Yang, Y.; Xu, G.; Xu, Y.; Cheng, X.; Xu, S.; Chen, S.; Wu, L. Ceramide Mediates Radiation-Induced Germ Cell Apoptosis via Regulating Mitochondria Function and MAPK Factors in Caenorhabditis Elegans. *Ecotoxicol Environ Saf* **2021**, *208*, 111579, doi:10.1016/j.ecoenv.2020.111579.
45. Petersen, M.C.; Shulman, G.I. Roles of Diacylglycerols and Ceramides in Hepatic Insulin Resistance. *Trends Pharmacol Sci* **2017**, *38*, 649–665, doi:10.1016/j.tips.2017.04.004.
46. Turpin-Nolan, S.M.; Hammerschmidt, P.; Chen, W.; Jais, A.; Timper, K.; Awazawa, M.; Brodesser, S.; Brüning, J.C. CerS1-Derived C18:0 Ceramide in Skeletal Muscle Promotes Obesity-Induced Insulin Resistance. *Cell Rep* **2019**, *26*, 1-10.e7, doi:10.1016/j.celrep.2018.12.031.
47. Kitatani, K.; Idkowiak-Baldys, J.; Hannun, Y.A. The Sphingolipid Salvage Pathway in Ceramide Metabolism and Signaling. *Cellular Signalling* **2008**, *20*, 1010–1018, doi:10.1016/j.cellsig.2007.12.006.

48. Mandala, S.M.; Thornton, R.; Galve-Roperh, I.; Poulton, S.; Peterson, C.; Olivera, A.; Bergstrom, J.; Kurtz, M.B.; Spiegel, S. Molecular Cloning and Characterization of a Lipid Phosphohydrolase That Degrades Sphingosine-1- Phosphate and Induces Cell Death. *PNAS* **2000**, *97*, 7859–7864, doi:10.1073/pnas.120146897.
49. Ogawa, C.; Kihara, A.; Gokoh, M.; Igarashi, Y. Identification and Characterization of a Novel Human Sphingosine-1-Phosphate Phosphohydrolase, HSPP2 *. *Journal of Biological Chemistry* **2003**, *278*, 1268–1272, doi:10.1074/jbc.M209514200.
50. Johnson, K.R.; Johnson, K.Y.; Becker, K.P.; Bielawski, J.; Mao, C.; Obeid, L.M. Role of Human Sphingosine-1-Phosphate Phosphatase 1 in the Regulation of Intra- and Extracellular Sphingosine-1-Phosphate Levels and Cell Viability*. *Journal of Biological Chemistry* **2003**, *278*, 34541–34547, doi:10.1074/jbc.M301741200.
51. Fyrst, H.; Saba, J.D. An Update on Sphingosine-1-Phosphate and Other Sphingolipid Mediators. *Nat Chem Biol* **2010**, *6*, 489–497, doi:10.1038/nchembio.392.
52. Zhang, B.; Tomura, H.; Kuwabara, A.; Kimura, T.; Miura, S.; Noda, K.; Okajima, F.; Saku, K. Correlation of High Density Lipoprotein (HDL)-Associated Sphingosine 1-Phosphate with Serum Levels of HDL-Cholesterol and Apolipoproteins. *Atherosclerosis* **2005**, *178*, 199–205, doi:10.1016/j.atherosclerosis.2004.08.024.
53. Andréani, P.; Gräler, M.H. Comparative Quantification of Sphingolipids and Analogs in Biological Samples by High-Performance Liquid Chromatography after Chloroform Extraction. *Anal Biochem* **2006**, *358*, 239–246, doi:10.1016/j.ab.2006.08.027.
54. Bode, C.; Sensken, S.-C.; Peest, U.; Beutel, G.; Thol, F.; Levkau, B.; Li, Z.; Bittman, R.; Huang, T.; Tölle, M.; et al. Erythrocytes Serve as a Reservoir for Cellular and Extracellular Sphingosine 1-Phosphate. *J Cell Biochem* **2010**, *109*, 1232–1243, doi:10.1002/jcb.22507.
55. Daum, G.; Winkler, M.; Moritz, E.; Müller, T.; Geffken, M.; von Lucadou, M.; Haddad, M.; Peine, S.; Böger, R.H.; Larena-Avellaneda, A.; et al. Determinants of Serum- and Plasma Sphingosine-1-Phosphate Concentrations in a Healthy Study Group. *TH Open* **2020**, *4*, e12–e19, doi:10.1055/s-0040-1701205.
56. Hla, T.; Venkataraman, K.; Michaud, J. The Vascular S1P Gradient—Cellular Sources and Biological Significance. *Biochim Biophys Acta* **2008**, *1781*, 477–482, doi:10.1016/j.bbalip.2008.07.003.
57. Mendoza, A.; Bréart, B.; Ramos-Perez, W.D.; Pitt, L.A.; Gobert, M.; Sunkara, M.; Lafaille, J.J.; Morris, A.J.; Schwab, S.R. The Transporter Spns2 Is Required for Secretion of Lymph but Not Plasma Sphingosine-1-Phosphate. *Cell Rep* **2012**, *2*, 1104–1110, doi:10.1016/j.celrep.2012.09.021.
58. Ito, K.; Anada, Y.; Tani, M.; Ikeda, M.; Sano, T.; Kihara, A.; Igarashi, Y. Lack of Sphingosine 1-Phosphate-Degrading Enzymes in Erythrocytes. *Biochem Biophys Res Commun* **2007**, *357*, 212–217, doi:10.1016/j.bbrc.2007.03.123.
59. Pappu, R.; Schwab, S.R.; Cornelissen, I.; Pereira, J.P.; Regard, J.B.; Xu, Y.; Camerer, E.; Zheng, Y.-W.; Huang, Y.; Cyster, J.G.; et al. Promotion of Lymphocyte Egress into Blood and Lymph by Distinct Sources of Sphingosine-1-Phosphate. *Science* **2007**, *316*, 295–298, doi:10.1126/science.1139221.
60. Venkataraman, K.; Lee, Y.-M.; Michaud, J.; Thangada, S.; Ai, Y.; Bonkovsky, H.L.; Parikh, N.S.; Habrukowich, C.; Hla, T. Vascular Endothelium as a Contributor of Plasma Sphingosine 1-Phosphate. *Circ Res* **2008**, *102*, 669–676, doi:10.1161/CIRCRESAHA.107.165845.
61. Nishi, T.; Kobayashi, N.; Hisano, Y.; Kawahara, A.; Yamaguchi, A. Molecular and Physiological Functions of Sphingosine 1-Phosphate Transporters. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2014**, *1841*, 759–765, doi:10.1016/j.bbalip.2013.07.012.

62. Tarling, E.J.; de Aguiar Vallim, T.Q.; Edwards, P.A. Role of ABC Transporters in Lipid Transport and Human Disease. *Trends Endocrinol Metab* **2013**, *24*, 342–350, doi:10.1016/j.tem.2013.01.006.
63. Kobayashi, N.; Nishi, T.; Hirata, T.; Kihara, A.; Sano, T.; Igarashi, Y.; Yamaguchi, A. Sphingosine 1-Phosphate Is Released from the Cytosol of Rat Platelets in a Carrier-Mediated Manner. *J Lipid Res* **2006**, *47*, 614–621, doi:10.1194/jlr.M500468-JLR200.
64. Kobayashi, N.; Kobayashi, N.; Yamaguchi, A.; Nishi, T. Characterization of the ATP-Dependent Sphingosine 1-Phosphate Transporter in Rat Erythrocytes *. *Journal of Biological Chemistry* **2009**, *284*, 21192–21200, doi:10.1074/jbc.M109.006163.
65. Lee, Y.-M.; Venkataraman, K.; Hwang, S.-I.; Han, D.K.; Hla, T. A Novel Method to Quantify Sphingosine 1-Phosphate by Immobilized Metal Affinity Chromatography (IMAC). *Prostaglandins & Other Lipid Mediators* **2007**, *84*, 154–162, doi:10.1016/j.prostaglandins.2007.08.001.
66. Kawahara, A.; Nishi, T.; Hisano, Y.; Fukui, H.; Yamaguchi, A.; Mochizuki, N. The Sphingolipid Transporter Spns2 Functions in Migration of Zebrafish Myocardial Precursors. *Science* **2009**, *323*, 524–527, doi:10.1126/science.1167449.
67. Nagahashi, M.; Kim, E.Y.; Yamada, A.; Ramachandran, S.; Allegood, J.C.; Hait, N.C.; Maceyka, M.; Milstien, S.; Takabe, K.; Spiegel, S. Spns2, a Transporter of Phosphorylated Sphingoid Bases, Regulates Their Blood and Lymph Levels, and the Lymphatic Network. *FASEB J* **2013**, *27*, 1001–1011, doi:10.1096/fj.12-219618.
68. Hisano, Y.; Kobayashi, N.; Yamaguchi, A.; Nishi, T. Mouse SPNS2 Functions as a Sphingosine-1-Phosphate Transporter in Vascular Endothelial Cells. *PLoS One* **2012**, *7*, e38941, doi:10.1371/journal.pone.0038941.
69. Vu, T.M.; Ishizu, A.-N.; Foo, J.C.; Toh, X.R.; Zhang, F.; Whee, D.M.; Torta, F.; Cazenave-Gassiot, A.; Matsumura, T.; Kim, S.; et al. Mfsd2b Is Essential for the Sphingosine-1-Phosphate Export in Erythrocytes and Platelets. *Nature* **2017**, *550*, 524–528, doi:10.1038/nature24053.
70. Kobayashi, N.; Kawasaki-Nishi, S.; Otsuka, M.; Hisano, Y.; Yamaguchi, A.; Nishi, T. MFSD2B Is a Sphingosine 1-Phosphate Transporter in Erythroid Cells. *Sci Rep* **2018**, *8*, 4969, doi:10.1038/s41598-018-23300-x.
71. Nguyen, T.Q.; Vu, T.M.; Tukijan, F.; Muralidharan, S.; Foo, J.C.; Li Chin, J.F.; Hasan, Z.; Torta, F.; Nguyen, L.N. Erythrocytes Efficiently Utilize Exogenous Sphingosines for S1P Synthesis and Export via Mfsd2b. *J Biol Chem* **2021**, *296*, 100201, doi:10.1074/jbc.RA120.012941.
72. Maceyka, M.; Harikumar, K.B.; Milstien, S.; Spiegel, S. Sphingosine-1-Phosphate Signaling and Its Role in Disease. *Trends Cell Biol.* **2012**, *22*, 50–60, doi:10.1016/j.tcb.2011.09.003.
73. Alvarez, S.E.; Harikumar, K.B.; Hait, N.C.; Allegood, J.; Strub, G.M.; Kim, E.Y.; Maceyka, M.; Jiang, H.; Luo, C.; Kordula, T.; et al. Sphingosine-1-Phosphate Is a Missing Cofactor for the E3 Ubiquitin Ligase TRAF2. *Nature* **2010**, *465*, 1084–1088, doi:10.1038/nature09128.
74. Xia, P.; Wang, L.; Moretti, P.A.B.; Albanese, N.; Chai, F.; Pitson, S.M.; D'Andrea, R.J.; Gamble, J.R.; Vadas, M.A. Sphingosine Kinase Interacts with TRAF2 and Dissects Tumor Necrosis Factor-α Signaling *. *Journal of Biological Chemistry* **2002**, *277*, 7996–8003, doi:10.1074/jbc.M111423200.
75. Hla, T.; Maciag, T. An Abundant Transcript Induced in Differentiating Human Endothelial Cells Encodes a Polypeptide with Structural Similarities to G-Protein-Coupled Receptors. *Journal of Biological Chemistry* **1990**, *265*, 9308–9313, doi:10.1016/S0021-9258(19)38849-0.
76. Rosen, H.; Gonzalez-Cabrera, P.J.; Sanna, M.G.; Brown, S. Sphingosine 1-Phosphate Receptor Signaling. *Annual Review of Biochemistry* **2009**, *78*, 743–768, doi:10.1146/annurev.biochem.78.072407.103733.

77. Takuwa, Y.; Takuwa, N.; Sugimoto, N. The Edg Family G Protein-Coupled Receptors for Lysophospholipids: Their Signaling Properties and Biological Activities. *J Biochem* **2002**, *131*, 767–771, doi:10.1093/oxfordjournals.jbchem.a003163.
78. Yamaguchi, F.; Tokuda, M.; Hatase, O.; Brenner, S. Molecular Cloning of the Novel Human G Protein-Coupled Receptor (GPCR) Gene Mapped on Chromosome 9. *Biochemical and Biophysical Research Communications* **1996**, *227*, 608–614, doi:10.1006/bbrc.1996.1553.
79. Gräler, M.H.; Bernhardt, G.; Lipp, M. EDG6, a Novel G-Protein-Coupled Receptor Related to Receptors for Bioactive Lysophospholipids, Is Specifically Expressed in Lymphoid Tissue. *Genomics* **1998**, *53*, 164–169, doi:10.1006/geno.1998.5491.
80. Im, D.-S.; Heise, C.E.; Ancellin, N.; O'Dowd, B.F.; Shei, G.; Heavens, R.P.; Rigby, M.R.; Hla, T.; Mandala, S.; McAllister, G.; et al. Characterization of a Novel Sphingosine 1-Phosphate Receptor, Edg-8 *. *Journal of Biological Chemistry* **2000**, *275*, 14281–14286, doi:10.1074/jbc.275.19.14281.
81. Zhou, H.; Murthy, K.S. Distinctive G Protein-Dependent Signaling in Smooth Muscle by Sphingosine 1-Phosphate Receptors S1P1 and S1P2. *Am J Physiol Cell Physiol* **2004**, *286*, C1130-1138, doi:10.1152/ajpcell.00429.2003.
82. O'Sullivan, C.; Dev, K.K. The Structure and Function of the S1P1 Receptor. *Trends in Pharmacological Sciences* **2013**, *34*, 401–412, doi:10.1016/j.tips.2013.05.002.
83. Siehler, S.; Manning, D.R. Pathways of Transduction Engaged by Sphingosine 1-Phosphate through G Protein-Coupled Receptors. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2002**, *1582*, 94–99, doi:10.1016/S1388-1981(02)00142-7.
84. Liu, Y.; Wada, R.; Yamashita, T.; Mi, Y.; Deng, C.-X.; Hobson, J.P.; Rosenfeldt, H.M.; Nava, V.E.; Chae, S.-S.; Lee, M.-J.; et al. Edg-1, the G Protein–Coupled Receptor for Sphingosine-1-Phosphate, Is Essential for Vascular Maturation. *J Clin Invest* **2000**, *106*, 951–961, doi:10.1172/JCI10905.
85. Allende, M.L.; Yamashita, T.; Proia, R.L. G-Protein-Coupled Receptor S1P1 Acts within Endothelial Cells to Regulate Vascular Maturation. *Blood* **2003**, *102*, 3665–3667, doi:10.1182/blood-2003-02-0460.
86. Matloubian, M.; Lo, C.G.; Cinamon, G.; Lesneski, M.J.; Xu, Y.; Brinkmann, V.; Allende, M.L.; Proia, R.L.; Cyster, J.G. Lymphocyte Egress from Thymus and Peripheral Lymphoid Organs Is Dependent on S1P Receptor 1. *Nature* **2004**, *427*, 355–360, doi:10.1038/nature02284.
87. Allende, M.L.; Dreier, J.L.; Mandala, S.; Proia, R.L. Expression of the Sphingosine 1-Phosphate Receptor, S1P1, on T-Cells Controls Thymic Emigration *. *Journal of Biological Chemistry* **2004**, *279*, 15396–15401, doi:10.1074/jbc.M314291200.
88. Lo, C.G.; Xu, Y.; Proia, R.L.; Cyster, J.G. Cyclical Modulation of Sphingosine-1-Phosphate Receptor 1 Surface Expression during Lymphocyte Recirculation and Relationship to Lymphoid Organ Transit. *Journal of Experimental Medicine* **2005**, *201*, 291–301, doi:10.1084/jem.20041509.
89. Okazaki, H.; Ishizaka, N.; Sakurai, T.; Kurokawa, K.; Goto, K.; Kumada, M.; Takuwa, Y. Molecular Cloning of a Novel Putative G Protein-Coupled Receptor Expressed in the Cardiovascular System. *Biochemical and Biophysical Research Communications* **1993**, *190*, 1104–1109, doi:10.1006/bbrc.1993.1163.
90. Yu, H. Sphingosine-1-Phosphate Receptor 2 Regulates Proinflammatory Cytokine Production and Osteoclastogenesis. *PLOS ONE* **2016**, *11*, e0156303, doi:10.1371/journal.pone.0156303.
91. Michaud, J.; Im, D.-S.; Hla, T. Inhibitory Role of Sphingosine 1-Phosphate Receptor 2 in Macrophage Recruitment during Inflammation. *The Journal of Immunology* **2010**, *184*, 1475–1483, doi:10.4049/jimmunol.0901586.

92. Takashima, S.; Sugimoto, N.; Takuwa, N.; Okamoto, Y.; Yoshioka, K.; Takamura, M.; Takata, S.; Kaneko, S.; Takuwa, Y. G12/13 and Gq Mediate S1P2-Induced Inhibition of Rac and Migration in Vascular Smooth Muscle in a Manner Dependent on Rho but Not Rho Kinase. *Cardiovascular Research* **2008**, *79*, 689–697, doi:10.1093/cvr/cvn118.
93. Du, W.; Takuwa, N.; Yoshioka, K.; Okamoto, Y.; Gonda, K.; Sugihara, K.; Fukamizu, A.; Asano, M.; Takuwa, Y. S1P $_2$, the G Protein–Coupled Receptor for Sphingosine-1-Phosphate, Negatively Regulates Tumor Angiogenesis and Tumor Growth *In Vivo* in Mice. *Cancer Res* **2010**, *70*, 772–781, doi:10.1158/0008-5472.CAN-09-2722.
94. Petti, L.; Rizzo, G.; Rubbino, F.; Elangovan, S.; Colombo, P.; Restelli, S.; Piontini, A.; Arena, V.; Carvello, M.; Romano, B.; et al. Unveiling Role of Sphingosine-1-Phosphate Receptor 2 as a Brake of Epithelial Stem Cell Proliferation and a Tumor Suppressor in Colorectal Cancer. *Journal of Experimental & Clinical Cancer Research* **2020**, *39*, 253, doi:10.1186/s13046-020-01740-6.
95. Li, Q.; Li, Y.; Lei, C.; Tan, Y.; Yi, G. Sphingosine-1-Phosphate Receptor 3 Signaling. *Clinica Chimica Acta* **2021**, *519*, 32–39, doi:10.1016/j.cca.2021.03.025.
96. Keul, P.; Lucke, S.; von Wnuck Lipinski, K.; Bode, C.; Gräler, M.; Heusch, G.; Levkau, B. Sphingosine-1-Phosphate Receptor 3 Promotes Recruitment of Monocyte/Macrophages in Inflammation and Atherosclerosis. *Circulation Research* **2011**, *108*, 314–323, doi:10.1161/CIRCRESAHA.110.235028.
97. Nussbaum, C.; Bannenberg, S.; Keul, P.; Gräler, M.H.; Gonçalves-de-Albuquerque, C.F.; Korhonen, H.; von Wnuck Lipinski, K.; Heusch, G.; de Castro Faria Neto, H.C.; Rohwedder, I.; et al. Sphingosine-1-Phosphate Receptor 3 Promotes Leukocyte Rolling by Mobilizing Endothelial P-Selectin. *Nat Commun* **2015**, *6*, 6416, doi:10.1038/ncomms7416.
98. Gräler, M.H.; Grosse, R.; Kusch, A.; Kremmer, E.; Gudermann, T.; Lipp, M. The Sphingosine 1-Phosphate Receptor S1P4 Regulates Cell Shape and Motility via Coupling to Gi and G12/13. *J Cell Biochem* **2003**, *89*, 507–519, doi:10.1002/jcb.10537.
99. Olesch, C.; Ringel, C.; Brüne, B.; Weigert, A. Beyond Immune Cell Migration: The Emerging Role of the Sphingosine-1-Phosphate Receptor S1PR4 as a Modulator of Innate Immune Cell Activation. *Mediators Inflamm* **2017**, *2017*, 6059203, doi:10.1155/2017/6059203.
100. Niedernberg, A.; Scherer, C.R.; Busch, A.E.; Kostenis, E. Comparative Analysis of Human and Rat S1P5 (Edg8): Differential Expression Profiles and Sensitivities to Antagonists. *Biochemical Pharmacology* **2002**, *64*, 1243–1250, doi:10.1016/S0006-2952(02)01289-3.
101. Wang, C.; Mao, J.; Redfield, S.; Mo, Y.; Lage, J.M.; Zhou, X. Systemic Distribution, Subcellular Localization and Differential Expression of Sphingosine-1-Phosphate Receptors in Benign and Malignant Human Tissues. *Experimental and Molecular Pathology* **2014**, *97*, 259–265, doi:10.1016/j.yexmp.2014.07.013.
102. Novgorodov, A.S.; El-Alwani, M.; Bielawski, J.; Obeid, L.M.; Gudz, T.I. Activation of Sphingosine-1-Phosphate Receptor S1P5 Inhibits Oligodendrocyte Progenitor Migration. *FASEB J.* **2007**, *21*, 1503–1514, doi:10.1096/fj.06-7420com.
103. Malek, R.L.; Toman, R.E.; Edsall, L.C.; Wong, S.; Chiu, J.; Letterle, C.A.; Van Brocklyn, J.R.; Milstien, S.; Spiegel, S.; Lee, N.H. Nrg-1 Belongs to the Endothelial Differentiation Gene Family of G Protein-Coupled Sphingosine-1-Phosphate Receptors. *J Biol Chem* **2001**, *276*, 5692–5699, doi:10.1074/jbc.M003964200.
104. Meng, H.; Lee, V.M. Differential Expression of Sphingosine-1-Phosphate Receptors 1-5 in the Developing Nervous System. *Developmental Dynamics* **2009**, *238*, 487–500, doi:10.1002/dvdy.21852.
105. Milstien, S.; Gude, D.; Spiegel, S. Sphingosine 1-Phosphate in Neural Signalling and Function. *Acta Paediatrica* **2007**, *96*, 40–43, doi:10.1111/j.1651-2227.2007.00206.x.

106. Kono, M.; Allende, M.L.; Proia, R.L. Sphingosine-1-Phosphate Regulation of Mammalian Development. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2008**, *1781*, 435–441, doi:10.1016/j.bbalip.2008.07.001.
107. Kajimoto, T.; Okada, T.; Yu, H.; Goparaju, S.K.; Jahangeer, S.; Nakamura, S. Involvement of Sphingosine-1-Phosphate in Glutamate Secretion in Hippocampal Neurons. *Mol Cell Biol* **2007**, *27*, 3429–3440, doi:10.1128/MCB.01465-06.
108. Kanno, T.; Nishizaki, T.; Proia, R.L.; Kajimoto, T.; Jahangeer, S.; Okada, T.; Nakamura, S. Regulation of Synaptic Strength by Sphingosine 1-Phosphate in the Hippocampus. *Neuroscience* **2010**, *171*, 973–980, doi:10.1016/j.neuroscience.2010.10.021.
109. Kanno, T.; Nishizaki, T. Endogenous Sphingosine 1-Phosphate Regulates Spontaneous Glutamate Release from Mossy Fiber Terminals via S1P3 Receptors. *Life Sciences* **2011**, *89*, 137–140, doi:10.1016/j.lfs.2011.05.021.
110. Weth-Malsch, D.; Langeslag, M.; Beroukas, D.; Zangrandi, L.; Kastenberger, I.; Quarta, S.; Malsch, P.; Kalpachidou, T.; Schwarzer, C.; Proia, R.L.; et al. Ablation of Sphingosine 1-Phosphate Receptor Subtype 3 Impairs Hippocampal Neuron Excitability In Vitro and Spatial Working Memory In Vivo. *Frontiers in Cellular Neuroscience* **2016**, *10*, 258, doi:10.3389/fncel.2016.00258.
111. Sim-Selley, L.J.; Goforth, P.B.; Mba, M.U.; Macdonald, T.L.; Lynch, K.R.; Milstien, S.; Spiegel, S.; Satin, L.S.; Welch, S.P.; Selley, D.E. Sphingosine-1-Phosphate Receptors Mediate Neuromodulatory Functions in the CNS. *Journal of Neurochemistry* **2009**, *110*, 1191–1202, doi:10.1111/j.1471-4159.2009.06202.x.
112. Wang, C.C.; Kuo, J.R.; Wang, S.J. Fingolimod Inhibits Glutamate Release through Activation of S1P1 Receptors and the G Protein Bγ Subunit-Dependent Pathway in Rat Cerebrocortical Nerve Terminals. *Neuropharmacology* **2021**, *185*, 108451, doi:10.1016/j.neuropharm.2021.108451.
113. Di Menna, L.; Molinaro, G.; Di Nuzzo, L.; Riozzi, B.; Zappulla, C.; Pozzilli, C.; Turrini, R.; Caraci, F.; Copani, A.; Battaglia, G.; et al. Fingolimod Protects Cultured Cortical Neurons against Excitotoxic Death. *Pharmacological Research* **2013**, *67*, 1–9, doi:10.1016/j.phrs.2012.10.004.
114. Attiori Essis, S.; Laurier-Laurin, M.-E.; Pépin, É.; Cyr, M.; Massicotte, G. GluN2B-Containing NMDA Receptors Are Upregulated in Plasma Membranes by the Sphingosine-1-Phosphate Analog FTY720P. *Brain Research* **2015**, *1624*, 349–358, doi:10.1016/j.brainres.2015.07.055.
115. Rossi, S.; Lo Giudice, T.; De Chiara, V.; Musella, A.; Studer, V.; Motta, C.; Bernardi, G.; Martino, G.; Furlan, R.; Martorana, A.; et al. Oral Fingolimod Rescues the Functional Deficits of Synapses in Experimental Autoimmune Encephalomyelitis. *British Journal of Pharmacology* **2012**, *165*, 861–869, doi:10.1111/j.1476-5381.2011.01579.x.
116. Welch, S.P.; Sim-Selley, L.J.; Selley, D.E. Sphingosine-1-Phosphate Receptors as Emerging Targets for Treatment of Pain. *Biochemical Pharmacology* **2012**, *84*, 1551–1562, doi:10.1016/j.bcp.2012.08.010.
117. O'Sullivan, S.A.; O'Sullivan, C.; Healy, L.M.; Dev, K.K.; Sheridan, G.K. Sphingosine 1-Phosphate Receptors Regulate TLR4-Induced CXCL5 Release from Astrocytes and Microglia. *Journal of Neurochemistry* **2018**, *144*, 736–747, doi:10.1111/jnc.14313.
118. Halassa, M.M.; Fellin, T.; Haydon, P.G. The Tripartite Synapse: Roles for Gliotransmission in Health and Disease. *Trends in Molecular Medicine* **2007**, *13*, 54–63, doi:10.1016/j.molmed.2006.12.005.
119. Rudy, C.C.; Hunsberger, H.C.; Weitzner, D.S.; Reed, M.N. The Role of the Tripartite Glutamatergic Synapse in the Pathophysiology of Alzheimer's Disease. *Aging Dis* **2015**, *6*, 131–148, doi:10.14336/AD.2014.0423.

120. Perea, G.; Navarrete, M.; Araque, A. Tripartite Synapses: Astrocytes Process and Control Synaptic Information. *Trends in Neurosciences* **2009**, *32*, 421–431, doi:10.1016/j.tins.2009.05.001.
121. Colombo, E.; Bassani, C.; De Angelis, A.; Ruffini, F.; Ottoboni, L.; Comi, G.; Martino, G.; Farina, C. Siponimod (BAF312) Activates Nrf2 While Hampering NFκB in Human Astrocytes, and Protects From Astrocyte-Induced Neurodegeneration. *Front. Immunol.* **2020**, *11*, doi:10.3389/fimmu.2020.00635.
122. zu Heringdorf, D.M.; Vincent, M.E.M.; Lipinski, M.; Danneberg, K.; Stropp, U.; Wang, D.; Tigyi, G.; Jakobs, K.H. Inhibition of Ca2+ Signalling by the Sphingosine 1-Phosphate Receptor S1P1. *Cellular Signalling* **2003**, *15*, 677–687, doi:10.1016/S0898-6568(03)00011-1.
123. Wu, C.; Leong, S.Y.; Moore, C.S.; Cui, Q.L.; Gris, P.; Bernier, L.-P.; Johnson, T.A.; Séguéla, P.; Kennedy, T.E.; Bar-Or, A.; et al. Dual Effects of Daily FTY720 on Human Astrocytes in Vitro: Relevance for Neuroinflammation. *J Neuroinflammation* **2013**, *10*, 814, doi:10.1186/1742-2094-10-41.
124. Healy, L.M.; Sheridan, G.K.; Pritchard, A.J.; Rutkowska, A.; Mullershausen, F.; Dev, K.K. Pathway Specific Modulation of S1P1 Receptor Signalling in Rat and Human Astrocytes. *British Journal of Pharmacology* **2013**, *169*, 1114–1129, doi:10.1111/bph.12207.
125. Bradl, M.; Lassmann, H. Oligodendrocytes: Biology and Pathology. *Acta Neuropathol* **2010**, *119*, 37–53, doi:10.1007/s00401-009-0601-5.
126. Jung, C.G.; Kim, H.J.; Miron, V.E.; Cook, S.; Kennedy, T.E.; Foster, C.A.; Antel, J.P.; Soliven, B. Functional Consequences of S1P Receptor Modulation in Rat Oligodendroglial Lineage Cells. *Glia* **2007**, *55*, 1656–1667, doi:10.1002/glia.20576.
127. Yu, N.; Lariosa-Willingham, K.D.; Lin, F.-F.; Webb, M.; Rao, T.S. Characterization of Lysophosphatidic Acid and Sphingosine-1-Phosphate-Mediated Signal Transduction in Rat Cortical Oligodendrocytes. *Glia* **2004**, *45*, 17–27, doi:10.1002/glia.10297.
128. Coelho, R.P.; Payne, S.G.; Bittman, R.; Spiegel, S.; Sato-Bigbee, C. The Immunomodulator FTY720 Has a Direct Cytoprotective Effect in Oligodendrocyte Progenitors. *J Pharmacol Exp Ther* **2007**, *323*, 626–635, doi:10.1124/jpet.107.123927.
129. Miron, V.E.; Hall, J.A.; Kennedy, T.E.; Soliven, B.; Antel, J.P. Cyclical and Dose-Dependent Responses of Adult Human Mature Oligodendrocytes to Fingolimod. *The American Journal of Pathology* **2008**, *173*, 1143–1152, doi:10.2353/ajpath.2008.080478.
130. Jaillard, C.; Harrison, S.; Stankoff, B.; Aigrot, M.S.; Calver, A.R.; Duddy, G.; Walsh, F.S.; Pangalos, M.N.; Arimura, N.; Kaibuchi, K.; et al. Edg8/S1P5: An Oligodendroglial Receptor with Dual Function on Process Retraction and Cell Survival. *J. Neurosci.* **2005**, *25*, 1459–1469, doi:10.1523/JNEUROSCI.4645-04.2005.
131. Miron, V.E.; Jung, C.G.; Kim, H.J.; Kennedy, T.E.; Soliven, B.; Antel, J.P. FTY720 Modulates Human Oligodendrocyte Progenitor Process Extension and Survival. *Annals of Neurology* **2008**, *63*, 61–71, doi:10.1002/ana.21227.
132. Miron, V.E.; Schubart, A.; Antel, J.P. Central Nervous System-Directed Effects of FTY720 (Fingolimod). *J. Neurol. Sci.* **2008**, *274*, 13–17, doi:10.1016/j.jns.2008.06.031.
133. Yasuda, K.; Maki, T.; Saito, S.; Yamamoto, Y.; Kinoshita, H.; Choi, Y.K.; Arumugam, T.V.; Lim, Y.-A.; Chen, C.L.H.; Wong, P.T.-H.; et al. Effect of Fingolimod on Oligodendrocyte Maturation under Prolonged Cerebral Hypoperfusion. *Brain Res.* **2019**, *1720*, 146294, doi:10.1016/j.brainres.2019.06.013.
134. Bieberich, E. There Is More to a Lipid than Just Being a Fat: Sphingolipid-Guided Differentiation of Oligodendroglial Lineage from Embryonic Stem Cells. *Neurochem. Res.* **2011**, *36*, 1601–1611, doi:10.1007/s11064-010-0338-5.

135. Cui, Q.L.; Fang, J.; Kennedy, T.E.; Almazan, G.; Antel, J.P. Role of P38MAPK in S1P Receptor-Mediated Differentiation of Human Oligodendrocyte Progenitors. *Glia* **2014**, *62*, 1361–1375, doi:10.1002/glia.22688.
136. Kim, H.J.; Miron, V.E.; Dukala, D.; Proia, R.L.; Ludwin, S.K.; Traka, M.; Antel, J.P.; Soliven, B. Neurobiological Effects of Sphingosine 1-Phosphate Receptor Modulation in the Cuprizone Model. *FASEB J.* **2011**, *25*, 1509–1518, doi:10.1096/fj.10-173203.
137. Dukala, D.E.; Soliven, B. S1P1 Deletion in Oligodendroglial Lineage Cells: Effect on Differentiation and Myelination. *Glia* **2016**, *64*, 570–582, doi:10.1002/glia.22949.
138. Ridley, A.J. Rho Family Proteins: Coordinating Cell Responses. *Trends Cell Biol.* **2001**, *11*, 471–477, doi:10.1016/s0962-8924(01)02153-5.
139. Deng, W.; Wang, H.; Rosenberg, P.A.; Volpe, J.J.; Jensen, F.E. Role of Metabotropic Glutamate Receptors in Oligodendrocyte Excitotoxicity and Oxidative Stress. *PNAS* **2004**, *101*, 7751–7756, doi:10.1073/pnas.0307850101.
140. Bagayogo, I.P.; Dreyfus, C.F. Regulated Release of BDNF by Cortical Oligodendrocytes Is Mediated Through Metabotropic Glutamate Receptors and the PLC Pathway. *ASN Neuro* **2009**, *1*, AN20090006, doi:10.1042/AN20090006.
141. Pitt, D.; Nagelmeier, I.E.; Wilson, H.C.; Raine, C.S. Glutamate Uptake by Oligodendrocytes: Implications for Excitotoxicity in Multiple Sclerosis. *Neurology* **2003**, *61*, 1113–1120, doi:10.1212/01.WNL.0000090564.88719.37.
142. Lynch, M.A. The Multifaceted Profile of Activated Microglia. *Mol Neurobiol* **2009**, *40*, 139–156, doi:10.1007/s12035-009-8077-9.
143. Lewis, S. Microglia Prune Inhibitory Synapses, Too. *Nat Rev Neurosci* **2021**, *22*, 517–517, doi:10.1038/s41583-021-00504-1.
144. Paolicelli, R.C.; Bolasco, G.; Pagani, F.; Maggi, L.; Scianni, M.; Panzanelli, P.; Giustetto, M.; Ferreira, T.A.; Guiducci, E.; Dumas, L.; et al. Synaptic Pruning by Microglia Is Necessary for Normal Brain Development. *Science* **2011**, *333*, 1456–1458, doi:10.1126/science.1202529.
145. Schafer, D.P.; Lehrman, E.K.; Stevens, B. The "Quad-Partite" Synapse: Microglia-Synapse Interactions in the Developing and Mature CNS. *Glia* **2013**, *61*, 24–36, doi:10.1002/glia.22389.
146. Takeuchi, H.; Mizuno, T.; Zhang, G.; Wang, J.; Kawanokuchi, J.; Kuno, R.; Suzumura, A. Neuritic Beading Induced by Activated Microglia Is an Early Feature of Neuronal Dysfunction Toward Neuronal Death by Inhibition of Mitochondrial Respiration and Axonal Transport *. *Journal of Biological Chemistry* **2005**, *280*, 10444–10454, doi:10.1074/jbc.M413863200.
147. Shijie, J.; Takeuchi, H.; Yawata, I.; Harada, Y.; Sonobe, Y.; Doi, Y.; Liang, J.; Hua, L.; Yasuoka, S.; Zhou, Y.; et al. Blockade of Glutamate Release from Microglia Attenuates Experimental Autoimmune Encephalomyelitis in Mice. *Tohoku J. Exp. Med.* **2009**, *217*, 87–92, doi:10.1620/tjem.217.87.
148. Takaki, J.; Fujimori, K.; Miura, M.; Suzuki, T.; Sekino, Y.; Sato, K. L-Glutamate Released from Activated Microglia Downregulates Astrocytic L-Glutamate Transporter Expression in Neuroinflammation: The 'Collusion' Hypothesis for Increased Extracellular L-Glutamate Concentration in Neuroinflammation. *J Neuroinflammation* **2012**, *9*, 275, doi:10.1186/1742-2094-9-275.
149. Tham, C.-S.; Lin, F.-F.; Rao, T.S.; Yu, N.; Webb, M. Microglial Activation State and Lysophospholipid Acid Receptor Expression. *International Journal of Developmental Neuroscience* **2003**, *21*, 431–443, doi:10.1016/j.ijdevneu.2003.09.003.
150. Nayak, D.; Huo, Y.; Kwang, W.X.T.; Pushparaj, P.N.; Kumar, S.D.; Ling, E.-A.; Dheen, S.T. Sphingosine Kinase 1 Regulates the Expression of Proinflammatory Cytokines and Nitric Oxide in Activated Microglia. *Neuroscience* **2010**, *166*, 132–144, doi:10.1016/j.neuroscience.2009.12.020.

151. Moon, E.; Han, J.E.; Jeon, S.; Ryu, J.H.; Choi, J.W.; Chun, J. Exogenous S1P Exposure Potentiates Ischemic Stroke Damage That Is Reduced Possibly by Inhibiting S1P Receptor Signaling. *Mediators of Inflammation* **2015**, *2015*, e492659, doi:10.1155/2015/492659.
152. Gaire, B.P.; Song, M.-R.; Choi, J.W. Sphingosine 1-Phosphate Receptor Subtype 3 (S1P3) Contributes to Brain Injury after Transient Focal Cerebral Ischemia via Modulating Microglial Activation and Their M1 Polarization. *Journal of Neuroinflammation* **2018**, *15*, 284, doi:10.1186/s12974-018-1323-1.
153. Gaire, B.P.; Lee, C.-H.; Sapkota, A.; Lee, S.Y.; Chun, J.; Cho, H.J.; Nam, T.; Choi, J.W. Identification of Sphingosine 1-Phosphate Receptor Subtype 1 (S1P1) as a Pathogenic Factor in Transient Focal Cerebral Ischemia. *Mol Neurobiol* **2018**, *55*, 2320–2332, doi:10.1007/s12035-017-0468-8.
154. Sapkota, A.; Gaire, B.P.; Kang, M.-G.; Choi, J.W. S1P2 Contributes to Microglial Activation and M1 Polarization Following Cerebral Ischemia through ERK1/2 and JNK. *Sci Rep* **2019**, *9*, 12106, doi:10.1038/s41598-019-48609-z.
155. Loma, I.; Heyman, R. Multiple Sclerosis: Pathogenesis and Treatment. *Curr Neuropharmacol* **2011**, *9*, 409–416, doi:10.2174/157015911796557911.
156. Lutton, J.D.; Winston, R.; Rodman, T.C. Multiple Sclerosis: Etiological Mechanisms and Future Directions. *Exp. Biol. Med. (Maywood)* **2004**, *229*, 12–20, doi:10.1177/153537020422900102.
157. Waxman, S.G. Conduction in Myelinated, Unmyelinated, and Demyelinated Fibers. *Archives of Neurology* **1977**, *34*, 585–589, doi:10.1001/archneur.1977.00500220019003.
158. Katz Sand, I. Classification, Diagnosis, and Differential Diagnosis of Multiple Sclerosis. *Current Opinion in Neurology* **2015**, *28*, 193–205, doi:10.1097/WCO.0000000000000206.
159. Miller, D.H.; Chard, D.T.; Ciccarelli, O. Clinically Isolated Syndromes. *The Lancet Neurology* **2012**, *11*, 157–169, doi:10.1016/S1474-4422(11)70274-5.
160. Tumani, H.; Lehmensiek, V.; Rau, D.; Guttmann, I.; Tauscher, G.; Mogel, H.; Palm, C.; Hirt, V.; Suessmuth, S.D.; Sapunova-Meier, I.; et al. CSF Proteome Analysis in Clinically Isolated Syndrome (CIS): Candidate Markers for Conversion to Definite Multiple Sclerosis. *Neuroscience Letters* **2009**, *452*, 214–217, doi:10.1016/j.neulet.2009.01.057.
161. Sevim, S. Relapses in Multiple Sclerosis: Definition, Pathophysiology, Features, Imitators, and Treatment. *tnd* **2016**, *22*, 99–108, doi:10.4274/tnd.75318.
162. Lublin, F.D.; Reingold, S.C.; Cohen, J.A.; Cutter, G.R.; Sørensen, P.S.; Thompson, A.J.; Wolinsky, J.S.; Balcer, L.J.; Banwell, B.; Barkhof, F.; et al. Defining the Clinical Course of Multiple Sclerosis. *Neurology* **2014**, *83*, 278–286, doi:10.1212/WNL.0000000000000560.
163. Fletcher, J.M.; Lalor, S.J.; Sweeney, C.M.; Tubridy, N.; Mills, K.H.G. T Cells in Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis. *Clinical & Experimental Immunology* **2010**, *162*, 1–11, doi:10.1111/j.1365-2249.2010.04143.x.
164. Pröbstel, A.-K.; Sanderson, N.S.R.; Derfuss, T. B Cells and Autoantibodies in Multiple Sclerosis. *International Journal of Molecular Sciences* **2015**, *16*, 16576–16592, doi:10.3390/ijms160716576.
165. Disanto, G.; Morahan, J.M.; Barnett, M.H.; Giovannoni, G.; Ramagopalan, S.V. The Evidence for a Role of B Cells in Multiple Sclerosis. *Neurology* **2012**, *78*, 823–832, doi:10.1212/WNL.0b013e318249f6f0.
166. Smolders, J.; Menheere, P.; Kessels, A.; Damoiseaux, J.; Hupperts, R. Association of Vitamin D Metabolite Levels with Relapse Rate and Disability in Multiple Sclerosis. *Mult Scler* **2008**, *14*, 1220–1224, doi:10.1177/1352458508094399.

167. Runia, T.F.; Hop, W.C.J.; de Rijke, Y.B.; Buljevac, D.; Hintzen, R.Q. Lower Serum Vitamin D Levels Are Associated with a Higher Relapse Risk in Multiple Sclerosis. *Neurology* **2012**, *79*, 261–266, doi:10.1212/WNL.0b013e31825fdec7.
168. Chang, J.-H.; Cha, H.-R.; Lee, D.-S.; Seo, K.Y.; Kweon, M.-N. 1,25-Dihydroxyvitamin D3 Inhibits the Differentiation and Migration of TH17 Cells to Protect against Experimental Autoimmune Encephalomyelitis. *PLoS ONE* **2010**, *5*, e12925, doi:10.1371/journal.pone.0012925.
169. Chiuso-Minicucci, F.; Ishikawa, L.L.W.; Mimura, L.A.N.; Fraga-Silva, T.F. de C.; França, T.G.D.; Zorzella-Pezavento, S.F.G.; Marques, C.; Ikoma, M.R.V.; Sartori, A. Treatment with Vitamin D/MOG Association Suppresses Experimental Autoimmune Encephalomyelitis. *PLOS ONE* **2015**, *10*, e0125836, doi:10.1371/journal.pone.0125836.
170. Mandolesi, G.; Gentile, A.; Musella, A.; Fresegna, D.; De Vito, F.; Bullitta, S.; Sepman, H.; Marfia, G.A.; Centonze, D. Synaptopathy Connects Inflammation and Neurodegeneration in Multiple Sclerosis. *Nat Rev Neurol* **2015**, *11*, 711–724, doi:10.1038/nrneurol.2015.222.
171. Brose, N.; O'Connor, V.; Skehel, P. Synaptopathy: Dysfunction of Synaptic Function? *Biochem Soc Trans* **2010**, *38*, 443–444, doi:10.1042/BST0380443.
172. Frigo, M.; Cogo, M.G.; Fusco, M.L.; Gardinetti, M.; Frigeni, B. Glutamate and Multiple Sclerosis. *Curr Med Chem* **2012**, *19*, 1295–1299, doi:10.2174/092986712799462559.
173. Arm, J.; Oeltzschner, G.; Al-Iedani, O.; Lea, R.; Lechner-Scott, J.; Ramadan, S. Altered in Vivo Brain GABA and Glutamate Levels Are Associated with Multiple Sclerosis Central Fatigue. *Eur J Radiol* **2021**, *137*, 109610, doi:10.1016/j.ejrad.2021.109610.
174. Rossi, S.; Studer, V.; Motta, C.; De Chiara, V.; Barbieri, F.; Bernardi, G.; Centonze, D. Inflammation Inhibits GABA Transmission in Multiple Sclerosis. *Mult Scler* **2012**, *18*, 1633–1635, doi:10.1177/1352458512440207.
175. Centonze, D.; Muzio, L.; Rossi, S.; Cavasinni, F.; De Chiara, V.; Bergami, A.; Musella, A.; D'Amelio, M.; Cavallucci, V.; Martorana, A.; et al. Inflammation Triggers Synaptic Alteration and Degeneration in Experimental Autoimmune Encephalomyelitis. *Journal of Neuroscience* **2009**, *29*, 3442–3452, doi:10.1523/JNEUROSCI.5804-08.2009.
176. Di Prisco, S.; Merega, E.; Milanese, M.; Summa, M.; Casazza, S.; Raffaghello, L.; Pistoia, V.; Uccelli, A.; Pittaluga, A. CCL5-Glutamate Interaction in Central Nervous System: Early and Acute Presynaptic Defects in EAE Mice. *Neuropharmacology* **2013**, *75*, 337–346, doi:10.1016/j.neuropharm.2013.07.037.
177. Marte, A.; Cavallero, A.; Morando, S.; Uccelli, A.; Raiteri, M.; Fedele, E. Alterations of Glutamate Release in the Spinal Cord of Mice with Experimental Autoimmune Encephalomyelitis. *Journal of Neurochemistry* **2010**, *115*, 343–352, doi:10.1111/j.1471-4159.2010.06923.x.
178. Mitosek-Szewczyk, K.; Sulkowski, G.; Stelmasiak, Z.; Struzyńska, L. Expression of Glutamate Transporters GLT-1 and GLAST in Different Regions of Rat Brain during the Course of Experimental Autoimmune Encephalomyelitis. *Neuroscience* **2008**, *155*, 45–52, doi:10.1016/j.neuroscience.2008.05.025.
179. Vallejo-Illarramendi, A.; Domercq, M.; Pérez-Cerdá, F.; Ravid, R.; Matute, C. Increased Expression and Function of Glutamate Transporters in Multiple Sclerosis. *Neurobiology of Disease* **2006**, *21*, 154–164, doi:10.1016/j.nbd.2005.06.017.
180. Werner, P.; Pitt, D.; Raine, C.S. Multiple Sclerosis: Altered Glutamate Homeostasis in Lesions Correlates with Oligodendrocyte and Axonal Damage. *Ann Neurol* **2001**, *50*, 169–180, doi:10.1002/ana.1077.
181. Lazo-Gomez, R.; Velázquez, G. de L.L.-G.; Mireles-Jacobo, D.; Sotomayor-Sobrino, M.A. Mechanisms of Neurobehavioral Abnormalities in Multiple Sclerosis:

Contributions from Neural and Immune Components. *Clinical Neurophysiology Practice* **2019**, *4*, 39–46, doi:10.1016/j.cnp.2019.01.004.
182. Martínez-Morales, J.C.; Romero-Ávila, M.T.; Reyes-Cruz, G.; García-Sáinz, J.A. S1P1 Receptor Phosphorylation, Internalization, and Interaction with Rab Proteins: Effects of Sphingosine 1-Phosphate, FTY720-P, Phorbol Esters, and Paroxetine. *Biosci. Rep.* **2018**, *38*, doi:10.1042/BSR20181612.
183. Chiba, K. FTY720, a New Class of Immunomodulator, Inhibits Lymphocyte Egress from Secondary Lymphoid Tissues and Thymus by Agonistic Activity at Sphingosine 1-Phosphate Receptors. *Pharmacol. Ther.* **2005**, *108*, 308–319, doi:10.1016/j.pharmthera.2005.05.002.
184. Mullershausen, F.; Zecri, F.; Cetin, C.; Billich, A.; Guerini, D.; Seuwen, K. Persistent Signaling Induced by FTY720-Phosphate Is Mediated by Internalized S1P1 Receptors. *Nature Chemical Biology* **2009**, *5*, 428–434, doi:10.1038/nchembio.173.
185. Verzijl, D.; Peters, S.L.M.; Alewijnse, A.E. Sphingosine-1-Phosphate Receptors: Zooming in on Ligand-Induced Intracellular Trafficking and Its Functional Implications. *Mol Cells* **2010**, *29*, 99–104, doi:10.1007/s10059-010-0041-z.
186. Mandala, S.; Hajdu, R.; Bergstrom, J.; Quackenbush, E.; Xie, J.; Milligan, J.; Thornton, R.; Shei, G.-J.; Card, D.; Keohane, C.; et al. Alteration of Lymphocyte Trafficking by Sphingosine-1-Phosphate Receptor Agonists. *Science* **2002**, *296*, 346–349, doi:10.1126/science.1070238.
187. Foster, C.A.; Howard, L.M.; Schweitzer, A.; Persohn, E.; Hiestand, P.C.; Balatoni, B.; Reuschel, R.; Beerli, C.; Schwartz, M.; Billich, A. Brain Penetration of the Oral Immunomodulatory Drug FTY720 and Its Phosphorylation in the Central Nervous System during Experimental Autoimmune Encephalomyelitis: Consequences for Mode of Action in Multiple Sclerosis. *J Pharmacol Exp Ther* **2007**, *323*, 469–475, doi:10.1124/jpet.107.127183.
188. Deogracias, R.; Yazdani, M.; Dekkers, M.P.J.; Guy, J.; Ionescu, M.C.S.; Vogt, K.E.; Barde, Y.-A. Fingolimod, a Sphingosine-1 Phosphate Receptor Modulator, Increases BDNF Levels and Improves Symptoms of a Mouse Model of Rett Syndrome. *PNAS* **2012**, *109*, 14230–14235, doi:10.1073/pnas.1206093109.
189. Hoffmann, F.S.; Hofereiter, J.; Rübsamen, H.; Melms, J.; Schwarz, S.; Faber, H.; Weber, P.; Pütz, B.; Loleit, V.; Weber, F.; et al. Fingolimod Induces Neuroprotective Factors in Human Astrocytes. *J Neuroinflammation* **2015**, *12*, 184, doi:10.1186/s12974-015-0393-6.
190. Bonfiglio, T.; Olivero, G.; Merega, E.; Di Prisco, S.; Padolecchia, C.; Grilli, M.; Milanese, M.; Di Cesare Mannelli, L.; Ghelardini, C.; Bonanno, G.; et al. Prophylactic versus Therapeutic Fingolimod: Restoration of Presynaptic Defects in Mice Suffering from Experimental Autoimmune Encephalomyelitis. *PLoS One* **2017**, *12*, e0170825, doi:10.1371/journal.pone.0170825.
191. David, O.J.; Kovarik, J.M.; Schmouder, R.L. Clinical Pharmacokinetics of Fingolimod: *Clinical Pharmacokinetics* **2012**, *51*, 15–28, doi:10.2165/11596550-000000000-00000.
192. David, O.J.; Behrje, R.; Pal, P.; Hara, H.; Lates, C.D.; Schmouder, R. Pharmacokinetic Interaction Between Fingolimod and Carbamazepine in Healthy Subjects. *Clin Pharmacol Drug Dev* **2018**, *7*, 575–586, doi:10.1002/cpdd.459.
193. Scott, F.L.; Clemons, B.; Brooks, J.; Brahmachary, E.; Powell, R.; Dedman, H.; Desale, H.G.; Timony, G.A.; Martinborough, E.; Rosen, H.; et al. Ozanimod (RPC1063) Is a Potent Sphingosine-1-Phosphate Receptor-1 (S1P1) and Receptor-5 (S1P5) Agonist with Autoimmune Disease-Modifying Activity. *British Journal of Pharmacology* **2016**, *173*, 1778–1792, doi:10.1111/bph.13476.
194. Al-Salama, Z.T. Siponimod: First Global Approval. *Drugs* **2019**, *79*, 1009–1015, doi:10.1007/s40265-019-01140-x.

195. Dumitrescu, L.; Constantinescu, C.S.; Tanasescu, R. Siponimod for the Treatment of Secondary Progressive Multiple Sclerosis. *Expert Opinion on Pharmacotherapy* **2019**, *20*, 143–150, doi:10.1080/14656566.2018.1551363.
196. Shakeri-Nejad, K.; Gardin, A.; Gray, C.; Neelakantham, S.; Dumitras, S.; Legangneux, E. Safety, Tolerability, Pharmacodynamics and Pharmacokinetics of Intravenous Siponimod: A Randomized, Open-Label Study in Healthy Subjects. *Clinical Therapeutics* **2020**, *42*, 175–195, doi:10.1016/j.clinthera.2019.11.014.
197. Glaenzel, U.; Jin, Y.; Nufer, R.; Li, W.; Schroer, K.; Adam-Stitah, S.; Marle, S.P. van; Legangneux, E.; Borell, H.; James, A.D.; et al. Metabolism and Disposition of Siponimod, a Novel Selective S1P1/S1P5 Agonist, in Healthy Volunteers and In Vitro Identification of Human Cytochrome P450 Enzymes Involved in Its Oxidative Metabolism. *Drug Metab Dispos* **2018**, *46*, 1001–1013, doi:10.1124/dmd.117.079574.
198. Tran, J.Q.; Hartung, J.P.; Peach, R.J.; Boehm, M.F.; Rosen, H.; Smith, H.; Brooks, J.L.; Timony, G.A.; Olson, A.D.; Gujrathi, S.; et al. Results From the First-in-Human Study With Ozanimod, a Novel, Selective Sphingosine-1-Phosphate Receptor Modulator. *The Journal of Clinical Pharmacology* **2017**, *57*, 988–996, doi:10.1002/jcph.887.
199. D'Ambrosio, D.; Freedman, M.S.; Prinz, J. Ponesimod, a Selective S1P1 Receptor Modulator: A Potential Treatment for Multiple Sclerosis and Other Immune-Mediated Diseases. *Therapeutic Advances in Chronic Disease* **2016**, *7*, 18–33, doi:10.1177/2040622315617354.
200. Gergely, P.; Nuesslein-Hildesheim, B.; Guerini, D.; Brinkmann, V.; Traebert, M.; Bruns, C.; Pan, S.; Gray, N.S.; Hinterding, K.; Cooke, N.G.; et al. The Selective Sphingosine 1-Phosphate Receptor Modulator BAF312 Redirects Lymphocyte Distribution and Has Species-Specific Effects on Heart Rate. *British Journal of Pharmacology* **2012**, *167*, 1035–1047, doi:10.1111/j.1476-5381.2012.02061.x.
201. Vogelgesang Antje; Domanska Grazyna; Ruhnau Johanna; Dressel Alexander; Kirsch Michael; Schulze Juliane Siponimod (BAF312) Treatment Reduces Brain Infiltration but Not Lesion Volume in Middle-Aged Mice in Experimental Stroke. *Stroke* **2019**, *50*, 1224–1231, doi:10.1161/STROKEAHA.118.023667.
202. Bigaud, M.; Rudolph, B.; Briard, E.; Beerli, C.; Schubart, A.; Gardin, A. Siponimod Penetrates, Distributes and Acts on the Central Nervous System: Translational Insights (3973). *Neurology* **2020**, *94*.
203. Hundehege, P.; Cerina, M.; Eichler, S.; Thomas, C.; Herrmann, A.M.; Göbel, K.; Müntefering, T.; Fernandez-Orth, J.; Bock, S.; Narayanan, V.; et al. The Next-Generation Sphingosine-1 Receptor Modulator BAF312 (Siponimod) Improves Cortical Network Functionality in Focal Autoimmune Encephalomyelitis. *Neural Regen Res* **2019**, *14*, 1950–1960, doi:10.4103/1673-5374.259622.
204. Gentile, A.; Musella, A.; Bullitta, S.; Fresegna, D.; De Vito, F.; Fantozzi, R.; Piras, E.; Gargano, F.; Borsellino, G.; Battistini, L.; et al. Siponimod (BAF312) Prevents Synaptic Neurodegeneration in Experimental Multiple Sclerosis. *Journal of Neuroinflammation* **2016**, *13*, 207, doi:10.1186/s12974-016-0686-4.
205. Lamb, Y.N. Ozanimod: First Approval. *Drugs* **2020**, doi:10.1007/s40265-020-01319-7.
206. Cohen, J.A.; Arnold, D.L.; Comi, G.; Bar-Or, A.; Gujrathi, S.; Hartung, J.P.; Cravets, M.; Olson, A.; Frohna, P.A.; Selmaj, K.W. Safety and Efficacy of the Selective Sphingosine 1-Phosphate Receptor Modulator Ozanimod in Relapsing Multiple Sclerosis (RADIANCE): A Randomised, Placebo-Controlled, Phase 2 Trial. *The Lancet Neurology* **2016**, *15*, 373–381, doi:10.1016/S1474-4422(16)00018-1.
207. R. Taylor Meadows, K. *Ozanimod (RPC1063) Reduces the Plasma Biomarker Neurofilament Light Chain in Preclinical Rodent Models of Multiple Sclerosis*; 2017;
208. Inc, M.G. Ozanimod (RPC1063) Is Potentially Neuroprotective through Direct... by Kristen R. Taylor Meadows Available online: https://onlinelibrary.ectrims-congress.eu/ectrims/2017/ACTRIMS-

ECTRIMS2017/200838/kristen.r.taylor.meadows.ozanimod.28rpc106329.is.potentially.neuroprotective.html (accessed on 13 May 2020).
209. Musella, A.; Gentile, A.; Guadalupi, L.; Rizzo, F.R.; De Vito, F.; Fresegna, D.; Bruno, A.; Dolcetti, E.; Vanni, V.; Vitiello, L.; et al. Central Modulation of Selective Sphingosine-1-Phosphate Receptor 1 Ameliorates Experimental Multiple Sclerosis. *Cells* **2020**, *9*, 1290, doi:10.3390/cells9051290.
210. Dash, R.P.; Rais, R.; Srinivas, N.R. Ponesimod, a Selective Sphingosine 1-Phosphate (S1P1) Receptor Modulator for Autoimmune Diseases: Review of Clinical Pharmacokinetics and Drug Disposition. *Xenobiotica* **2018**, *48*, 442–451, doi:10.1080/00498254.2017.1329568.
211. Pouzol, L.; Piali, L.; Bernard, C.C.; Martinic, M.M.; Steiner, B.; Clozel, M. Therapeutic Potential of Ponesimod Alone and in Combination with Dimethyl Fumarate in Experimental Models of Multiple Sclerosis. *Innov Clin Neurosci* **2019**, *16*, 22–30.
212. Bonfiglio, T.; Olivero, G.; Merega, E.; Di Prisco, S.; Padolecchia, C.; Grilli, M.; Milanese, M.; Di Cesare Mannelli, L.; Ghelardini, C.; Bonanno, G.; et al. Prophylactic versus Therapeutic Fingolimod: Restoration of Presynaptic Defects in Mice Suffering from Experimental Autoimmune Encephalomyelitis. *PLoS One* **2017**, *12*, doi:10.1371/journal.pone.0170825.
213. Miron, V.E.; Ludwin, S.K.; Darlington, P.J.; Jarjour, A.A.; Soliven, B.; Kennedy, T.E.; Antel, J.P. Fingolimod (FTY720) Enhances Remyelination Following Demyelination of Organotypic Cerebellar Slices. *Am. J. Pathol.* **2010**, *176*, 2682–2694, doi:10.2353/ajpath.2010.091234.
214. Jackson, S.J.; Giovannoni, G.; Baker, D. Fingolimod Modulates Microglial Activation to Augment Markers of Remyelination. *J Neuroinflammation* **2011**, *8*, 76, doi:10.1186/1742-2094-8-76.
215. Mannioui, A.; Vauzanges, Q.; Fini, J.B.; Henriet, E.; Sekizar, S.; Azoyan, L.; Thomas, J.L.; Pasquier, D.D.; Giovannangeli, C.; Demeneix, B.; et al. The Xenopus Tadpole: An in Vivo Model to Screen Drugs Favoring Remyelination. *Mult Scler* **2018**, *24*, 1421–1432, doi:10.1177/1352458517721355.
216. Roggeri, A.; Schepers, M.; Tiane, A.; Rombaut, B.; van Veggel, L.; Hellings, N.; Prickaerts, J.; Pittaluga, A.; Vanmierlo, T. Sphingosine-1-Phosphate Receptor Modulators and Oligodendroglial Cells: Beyond Immunomodulation. *Int J Mol Sci* **2020**, *21*, E7537, doi:10.3390/ijms21207537.
217. Zappia, E.; Casazza, S.; Pedemonte, E.; Benvenuto, F.; Bonanni, I.; Gerdoni, E.; Giunti, D.; Ceravolo, A.; Cazzanti, F.; Frassoni, F.; et al. Mesenchymal Stem Cells Ameliorate Experimental Autoimmune Encephalomyelitis Inducing T-Cell Anergy. *Blood* **2005**, *106*, 1755–1761, doi:10.1182/blood-2005-04-1496.
218. Cervetto, C.; Frattaroli, D.; Venturini, A.; Passalacqua, M.; Nobile, M.; Alloisio, S.; Tacchetti, C.; Maura, G.; Agnati, L.F.; Marcoli, M. Calcium-Permeable AMPA Receptors Trigger Vesicular Glutamate Release from Bergmann Gliosomes. *Neuropharmacology* **2015**, *99*, 396–407, doi:10.1016/j.neuropharm.2015.08.011.
219. Carney, K.E.; Milanese, M.; van Nierop, P.; Li, K.W.; Oliet, S.H.R.; Smit, A.B.; Bonanno, G.; Verheijen, M.H.G. Proteomic Analysis of Gliosomes from Mouse Brain: Identification and Investigation of Glial Membrane Proteins. *J Proteome Res* **2014**, *13*, 5918–5927, doi:10.1021/pr500829z.
220. Salamone, A.; Zappettini, S.; Grilli, M.; Olivero, G.; Agostinho, P.; Tomé, A.R.; Chen, J.; Pittaluga, A.; Cunha, R.A.; Marchi, M. Prolonged Nicotine Exposure Down-Regulates Presynaptic NMDA Receptors in Dopaminergic Terminals of the Rat Nucleus Accumbens. *Neuropharmacology* **2014**, *79*, 488–497, doi:10.1016/j.neuropharm.2013.12.014.

221. Raiteri, M.; Angelini, F.; Levi, G. A Simple Apparatus for Studying the Release of Neurotransmitters from Synaptosomes. *Eur J Pharmacol* **1974**, *25*, 411–414, doi:10.1016/0014-2999(74)90272-6.
222. Di Prisco, S.; Summa, M.; Chellakudam, V.; Rossi, P.I.A.; Pittaluga, A. RANTES-Mediated Control of Excitatory Amino Acid Release in Mouse Spinal Cord. *J Neurochem* **2012**, *121*, 428–437, doi:10.1111/j.1471-4159.2012.07720.x.
223. Manders, E.M.M.; Verbeek, F.J.; Aten, J.A. Measurement of Co-Localization of Objects in Dual-Colour Confocal Images. *Journal of Microscopy* **1993**, *169*, 375–382, doi:10.1111/j.1365-2818.1993.tb03313.x.
224. Grilli, M.; Raiteri, L.; Pittaluga, A. Somatostatin Inhibits Glutamate Release from Mouse Cerebrocortical Nerve Endings through Presynaptic Sst2 Receptors Linked to the Adenylyl Cyclase-Protein Kinase A Pathway. *Neuropharmacology* **2004**, *46*, 388–396, doi:10.1016/j.neuropharm.2003.09.012.
225. Luccini, E.; Musante, V.; Neri, E.; Brambilla Bas, M.; Severi, P.; Raiteri, M.; Pittaluga, A. Functional Interactions between Presynaptic NMDA Receptors and Metabotropic Glutamate Receptors Co-Expressed on Rat and Human Noradrenergic Terminals. *Br J Pharmacol* **2007**, *151*, 1087–1094, doi:10.1038/sj.bjp.0707280.
226. Olivero, G.; Grilli, M.; Vergassola, M.; Bonfiglio, T.; Padolecchia, C.; Garrone, B.; Di Giorgio, F.P.; Tongiani, S.; Usai, C.; Marchi, M.; et al. 5-HT2A-MGlu2/3 Receptor Complex in Rat Spinal Cord Glutamatergic Nerve Endings: A 5-HT2A to MGlu2/3 Signalling to Amplify Presynaptic Mechanism of Auto-Control of Glutamate Exocytosis. *Neuropharmacology* **2018**, *133*, 429–439, doi:10.1016/j.neuropharm.2018.02.030.
227. G, O.; F, C.; D, M.; D, D.P.; Mc, G.; M, P.; K, C.; A, P. The Depolarization-Evoked, Ca 2+-Dependent Release of Exosomes From Mouse Cortical Nerve Endings: New Insights Into Synaptic Transmission. *Frontiers in pharmacology* **2021**, *12*, doi:10.3389/fphar.2021.670158.
228. Cisani, F.; Olivero, G.; Usai, C.; Van Camp, G.; Maccari, S.; Morley-Fletcher, S.; Pittaluga, A.M. Antibodies Against the NH2-Terminus of the GluA Subunits Affect the AMPA-Evoked Releasing Activity: The Role of Complement. *Front Immunol* **2021**, *12*, 586521, doi:10.3389/fimmu.2021.586521.
229. Choi, J.W.; Chun, J. Lysophospholipids and Their Receptors in the Central Nervous System. *Biochim Biophys Acta* **2013**, *1831*, 20–32, doi:10.1016/j.bbalip.2012.07.015.
230. Healy, L.M.; Antel, J.P. Sphingosine-1-Phosphate Receptors in the Central Nervous and Immune Systems. *Curr Drug Targets* **2016**, *17*, 1841–1850, doi:10.2174/1389450116666151001112710.
231. Zhou, Y.; Danbolt, N.C. Glutamate as a Neurotransmitter in the Healthy Brain. *J Neural Transm (Vienna)* **2014**, *121*, 799–817, doi:10.1007/s00702-014-1180-8.
232. Martínez-Lozada, Z.; Ortega, A. Glutamatergic Transmission: A Matter of Three. *Neural Plast* **2015**, *2015*, 787396, doi:10.1155/2015/787396.
233. Zhao, H.; Steinberg, G.K.; Sapolsky, R.M. General versus Specific Actions of Mild-Moderate Hypothermia in Attenuating Cerebral Ischemic Damage. *J Cereb Blood Flow Metab* **2007**, *27*, 1879–1894, doi:10.1038/sj.jcbfm.9600540.
234. Hernández, D.E.; Salvadores, N.A.; Moya-Alvarado, G.; Catalán, R.J.; Bronfman, F.C.; Court, F.A. Axonal Degeneration Induced by Glutamate Excitotoxicity Is Mediated by Necroptosis. *Journal of Cell Science* **2018**, *131*, jcs214684, doi:10.1242/jcs.214684.
235. Chung, R.S.; McCormack, G.H.; King, A.E.; West, A.K.; Vickers, J.C. Glutamate Induces Rapid Loss of Axonal Neurofilament Proteins from Cortical Neurons in Vitro. *Exp Neurol* **2005**, *193*, 481–488, doi:10.1016/j.expneurol.2005.01.005.
236. Belov Kirdajova, D.; Kriska, J.; Tureckova, J.; Anderova, M. Ischemia-Triggered Glutamate Excitotoxicity From the Perspective of Glial Cells. *Frontiers in Cellular Neuroscience* **2020**, *14*.

237. Lewerenz, J.; Maher, P. Chronic Glutamate Toxicity in Neurodegenerative Diseases—What Is the Evidence? *Frontiers in Neuroscience* **2015**, *9*.
238. Sharma, S.; Mathur, A.G.; Pradhan, S.; Singh, D.B.; Gupta, S. Fingolimod (FTY720): First Approved Oral Therapy for Multiple Sclerosis. *J Pharmacol Pharmacother* **2011**, *2*, 49–51, doi:10.4103/0976-500X.77118.
239. Kearney, H.; Miller, D.H.; Ciccarelli, O. Spinal Cord MRI in Multiple Sclerosis—Diagnostic, Prognostic and Clinical Value. *Nat Rev Neurol* **2015**, *11*, 327–338, doi:10.1038/nrneurol.2015.80.
240. Thompson, A.J.; Baranzini, S.E.; Geurts, J.; Hemmer, B.; Ciccarelli, O. Multiple Sclerosis. *Lancet* **2018**, *391*, 1622–1636, doi:10.1016/S0140-6736(18)30481-1.
241. Ciccarelli, O.; Cohen, J.A.; Reingold, S.C.; Weinshenker, B.G.; Amato, M.P.; Banwell, B.; Barkhof, F.; Bebo, B.; Becher, B.; Bethoux, F.; et al. Spinal Cord Involvement in Multiple Sclerosis and Neuromyelitis Optica Spectrum Disorders. *The Lancet Neurology* **2019**, *18*, 185–197, doi:10.1016/S1474-4422(18)30460-5.
242. O'Sullivan, S.; Dev, K.K. Sphingosine-1-Phosphate Receptor Therapies: Advances in Clinical Trials for CNS-Related Diseases. *Neuropharmacology* **2017**, *113*, 597–607, doi:10.1016/j.neuropharm.2016.11.006.
243. Hannun, Y.A.; Obeid, L.M. Principles of Bioactive Lipid Signalling: Lessons from Sphingolipids. *Nature Reviews Molecular Cell Biology* **2008**, *9*, 139–150, doi:10.1038/nrm2329.
244. Scott, L.J. Fingolimod. *CNS Drugs* **2011**, *25*, 673–698, doi:10.2165/11207350-000000000-00000.
245. Landi, D.; Vollaro, S.; Pellegrino, G.; Mulas, D.; Ghazaryan, A.; Falato, E.; Pasqualetti, P.; Rossini, P.M.; Filippi, M.M. Oral Fingolimod Reduces Glutamate-Mediated Intracortical Excitability in Relapsing-Remitting Multiple Sclerosis. *Clin Neurophysiol* **2015**, *126*, 165–169, doi:10.1016/j.clinph.2014.05.031.
246. Darios, F.D.; Jorgacevski, J.; Flašker, A.; Zorec, R.; García-Martinez, V.; Villanueva, J.; Gutiérrez, L.M.; Leese, C.; Bal, M.; Nosyreva, E.; et al. Sphingomimetic Multiple Sclerosis Drug FTY720 Activates Vesicular Synaptobrevin and Augments Neuroendocrine Secretion. *Sci Rep* **2017**, *7*, 5958, doi:10.1038/s41598-017-05948-z.
247. Kajimoto, T.; Okada, T.; Yu, H.; Goparaju, S.K.; Jahangeer, S.; Nakamura, S. Involvement of Sphingosine-1-Phosphate in Glutamate Secretion in Hippocampal Neurons. *Molecular and Cellular Biology* **2007**, *27*, 3429–3440, doi:10.1128/MCB.01465-06.
248. Maeda, Y.; Seki, N.; Sato, N.; Sugahara, K.; Chiba, K. Sphingosine 1-Phosphate Receptor Type 1 Regulates Egress of Mature T Cells from Mouse Bone Marrow. *Int Immunol* **2010**, *22*, 515–525, doi:10.1093/intimm/dxq036.
249. Choi, J.W.; Gardell, S.E.; Herr, D.R.; Rivera, R.; Lee, C.-W.; Noguchi, K.; Teo, S.T.; Yung, Y.C.; Lu, M.; Kennedy, G.; et al. FTY720 (Fingolimod) Efficacy in an Animal Model of Multiple Sclerosis Requires Astrocyte Sphingosine 1-Phosphate Receptor 1 (S1P1) Modulation. *PNAS* **2011**, *108*, 751–756, doi:10.1073/pnas.1014154108.
250. Hait, N.C.; Avni, D.; Yamada, A.; Nagahashi, M.; Aoyagi, T.; Aoki, H.; Dumur, C.I.; Zelenko, Z.; Gallagher, E.J.; Leroith, D.; et al. The Phosphorylated Prodrug FTY720 Is a Histone Deacetylase Inhibitor That Reactivates ERα Expression and Enhances Hormonal Therapy for Breast Cancer. *Oncogenesis* **2015**, *4*, e156–e156, doi:10.1038/oncsis.2015.16.
251. Hait, N.C.; Wise, L.E.; Allegood, J.C.; O'Brien, M.; Avni, D.; Reeves, T.M.; Knapp, P.E.; Lu, J.; Luo, C.; Miles, M.F.; et al. Active, Phosphorylated Fingolimod Inhibits Histone Deacetylases and Facilitates Fear Extinction Memory. *Nat Neurosci* **2014**, *17*, 971–980, doi:10.1038/nn.3728.
252. Van Doorn, R.; Van Horssen, J.; Verzijl, D.; Witte, M.; Ronken, E.; Van Het Hof, B.; Lakeman, K.; Dijkstra, C.D.; Van Der Valk, P.; Reijerkerk, A.; et al. Sphingosine 1-

Phosphate Receptor 1 and 3 Are Upregulated in Multiple Sclerosis Lesions. *Glia* **2010**, *58*, 1465–1476, doi:10.1002/glia.21021.
253. Brana, C.; Frossard, M.J.; Pescini Gobert, R.; Martinier, N.; Boschert, U.; Seabrook, T.J. Immunohistochemical Detection of Sphingosine-1-Phosphate Receptor 1 and 5 in Human Multiple Sclerosis Lesions. *Neuropathology and Applied Neurobiology* **2014**, *40*, 564–578, doi:10.1111/nan.12048.
254. Mousseau, Y.; Mollard, S.; Richard, L.; Nizou, A.; Faucher-Durand, K.; Cook-Moreau, J.; Qiu, H.; Baaj, Y.; Funalot, B.; Fourcade, L.; et al. Fingolimod Inhibits PDGF-B-Induced Migration of Vascular Smooth Muscle Cell by down-Regulating the S1PR1/S1PR3 Pathway. *Biochimie* **2012**, *94*, 2523–2531, doi:10.1016/j.biochi.2012.07.002.
255. Dusaban, S.S.; Chun, J.; Rosen, H.; Purcell, N.H.; Brown, J.H. Sphingosine 1-Phosphate Receptor 3 and RhoA Signaling Mediate Inflammatory Gene Expression in Astrocytes. *J Neuroinflammation* **2017**, *14*, 111, doi:10.1186/s12974-017-0882-x.
256. Fischer, I.; Alliod, C.; Martinier, N.; Newcombe, J.; Brana, C.; Pouly, S. Sphingosine Kinase 1 and Sphingosine 1-Phosphate Receptor 3 Are Functionally Upregulated on Astrocytes under Pro-Inflammatory Conditions. *PLOS ONE* **2011**, *6*, e23905, doi:10.1371/journal.pone.0023905.
257. Janssen, S.; Schlegel, C.; Gudi, V.; Prajeeth, C.K.; Skripuletz, T.; Trebst, C.; Stangel, M. Effect of FTY720-Phosphate on the Expression of Inflammation-Associated Molecules in Astrocytes in Vitro. *Molecular Medicine Reports* **2015**, *12*, 6171–6177, doi:10.3892/mmr.2015.4120.
258. Subei, A.M.; Cohen, J.A. Sphingosine 1-Phosphate Receptor Modulators in Multiple Sclerosis. *CNS Drugs* **2015**, *29*, 565–575, doi:10.1007/s40263-015-0261-z.
259. O'Sullivan, C.; Schubart, A.; Mir, A.K.; Dev, K.K. The Dual S1PR1/S1PR5 Drug BAF312 (Siponimod) Attenuates Demyelination in Organotypic Slice Cultures. *J Neuroinflammation* **2016**, *13*, doi:10.1186/s12974-016-0494-x.
260. Inc, M.G. Siponimod (BAF312)-Induced Differentiation of Fetal Human... by QIAO-LING Cui Available online: https://onlinelibrary.ectrims-congress.eu/ectrims/2013/copenhagen/34501/qiao.ling.cui.siponimod.28baf31229-induced.differentiation.of.fetal.human.html (accessed on 25 February 2022).
261. VonDran, M.W.; Singh, H.; Honeywell, J.Z.; Dreyfus, C.F. Levels of BDNF Impact Oligodendrocyte Lineage Cells Following a Cuprizone Lesion. *J Neurosci* **2011**, *31*, 14182–14190, doi:10.1523/JNEUROSCI.6595-10.2011.
262. Azoulay, D.; Urshansky, N.; Karni, A. Low and Dysregulated BDNF Secretion from Immune Cells of MS Patients Is Related to Reduced Neuroprotection. *Journal of Neuroimmunology* **2008**, *195*, 186–193, doi:10.1016/j.jneuroim.2008.01.010.
263. Fukumoto, K.; Mizoguchi, H.; Takeuchi, H.; Horiuchi, H.; Kawanokuchi, J.; Jin, S.; Mizuno, T.; Suzumura, A. Fingolimod Increases Brain-Derived Neurotrophic Factor Levels and Ameliorates Amyloid β-Induced Memory Impairment. *Behavioural Brain Research* **2014**, *268*, 88–93, doi:10.1016/j.bbr.2014.03.046.

PUBLICATIONS

Review: Sphingosine-1-Phosphate Receptor Modulators and Oligodendroglial Cells: Beyond Immunomodulation

Roggeri A., *Schepers M., Tiane A., Rombaut B., van Veggel L., Hellings N., Prickaerts J., Pittaluga A., Vanmierlo T. Int J Mol Sci. 2020. doi: 10.3390/ijms21207537.*

The second part of the thesis sought to investigate the role of S1PRs and its modulators in oligodendroglial cells. Demyelination, axonal loss, and synaptic impairment are considered hallmarks of MS. In this pathological condition alterations in both OPC migration and differentiation have been recognized as key factors in the development and progression of the disease. While the early stages of the disease are mainly characterized by inflammation, at the chronic phases, failure in endogenous remyelination is associated with the impairment of OPCs to migrate and differentiate into mature myelinating oligodendrocytes. In this context, the study of novel therapeutic approaches aimed at enhancing differentiation of OPCs into myelinating oligodendrocytes has become one of the main goals. Many disease-modifying therapies targeting S1PRs have been approved or are in development for the treatment of different forms of MS. Besides their recognized immunomodulatory effects, a growing body of evidence now suggests that direct targeting S1PRs in the CNS may involve mechanisms that, beyond immunomodulation, stimulate remyelination. Staring from these observations we decided to investigate the effect of BAF312 (also known as siponimod) on OPC differentiation. The experiments were preceded by a large study of the current literature that gave rise to the review title “Sphingosine-1-Phosphate Receptor Modulators and Oligodendroglial Cells: Beyond Immunomodulation” written in collaboration with the research group of Tim Vanmierlo and published in 2020. The review summarizes the current knowledge of the effect of S1PRs modulators on oligodendroglial cells. After a brief introduction on OPC and oligodendrocytes it resumes all the information on S1PRs expression in the different stages of OPC

differentiation. Subsequently, it collects the current knowledge on the in vitro and in vivo experiments conducted to investigate the effect of S1PRs modulators specifically on oligodendroglial cells. Finally, the review provides information on the new generation of S1PRs modulators that are currently in clinical trials for the treatment of MS.

Review: Somatostatin, a Presynaptic Modulator of Glutamatergic Signal in the Central Nervous System

Pittaluga A., ***Roggeri A.,*** *Vallarino G., Olivero G. Int J Mol Sci. 2021. https://doi.org/10.3390/ijms22115864*.

The research conducted at the University of Genoa focused primarily on the effect of S1PRs on glutamate transmission in both healthy and EAE mice. However, during the PhD I took part in other projects always focused on alteration of glutamate transmission.

In this context, based on the background of the research group of Anna Pittaluga in the study of somatostatin, we decided to write a review on the role of the peptide in controlling the efficiency of synaptic transmission. Somatostatin is present in GABA- interneurons and it is known to be release in Ca2+ dependent manner following a depolarizing stimulus. Once released in the synaptic cleft somatostatin can act locally and control glutamate transmission by presynaptically modulating glutamate exocytosis. However. it has been observed that the peptide can diffuse into the extracellular space through a mechanism known as “volume diffusion”. The diffusion lead o somatostatin the possibility to interact with neurons located extra-synaptically, in a non-synaptic form of communication. Understanding the role of somatostatin in the receptor-receptor interaction or in the mechanisms of “volume diffusion” open the way to possible new therapeutic approaches for the cure of central disorders.

Paper: Acute low dose of Trazodone recovers glutamate release efficiency and mGlu2/3 autoreceptor impairments in the spinal cord of rats suffering from chronic sciatic ligation

*Cisani F., **Roggeri A.,** Olivero G., Garrone B., Tongiani S., Di Giorgio F.P., Pittaluga A. Front. Pharmacol. 2020. https://doi.org/10.3389/fphar.2020.01108.*

In the field of altered glutamate transmission, I also participate in a project aimed at investigating the effect of trazodone (TDZ; a 5-HT2A antagonist) on mGlu2/3 receptor function in rat suffering from chronic sciatic ligation (an animal model of neuropathic pain). The project was carried out in collaboration with Angelini Pharma S.p.A. and, gave rise, in 2020, to the publication of the paper entitled "Acute low dose of Trazodone recovers glutamate release efficiency and mGlu2/3 autoreceptors impairments in the spinal cord of rats suffering from chronic sciatic ligation". The experiments were conducted based on evidence in the literature demonstrating that mGlu2/3 autoreceptors are expressed at the presynaptic level in the glutamatergic nerve endings of the spinal cord where they inhibit the release of glutamate. Other studies have also shown that 5-HT2A receptors also exist at the presynaptic level and interact with mGlu2/3 receptors in an antagonistic functional crosstalk. As there are currently no agonists in clinical capable of directly modulating the function of the mGlu2/3 receptors, our hypothesis is that using a 5-HT2A receptor antagonist, TZD, could be an innovative pharmacological approach to modulate the activity of the mGlu2/3 receptor in neurological disorders characterized by impaired glutamate transmission. To test this hypothesis, we used rats with chronic nerve ligation, an animal model of neuropathic pain which is characterized by high levels of glutamate and altered expression and function of mGlu2/3 receptors. Seven days after the operation, animals were treated orally (1 hour) with TZD (0.3 mg/kg). At the end of the treatment, synaptosomes from the spinal cord of rats in the different experimental conditions (i.e, control, injured, vehicle-treated injured and TZD-treated injured rats) were isolated and

used to perform both functional and biochemical analyses. Using the superfusion technique we investigated the impact of sciatic ligation on the presynaptic mGlu2/3 and 5-HT2A receptors controlling [3H]D-aspartate exocytosis in spinal cord synaptosomes. The particles were preloaded with [3H]D-aspartate, an analogue of glutamate, and superfused to monitor tritium release. After 39 min of superfusion, synaptosomes were transiently (90s) exposed to a mild depolarizing stimulus (15 mM KCl) and glutamate exocytosis was evaluated in absence or presence of LY379268 (mGlu2/3 agonist) or (±) DOI (5-HT2A agonist). The results unveiled that, in injured rats, the function of presynaptic release-regulating mGlu2/3 and 5-HT2A receptors is affected. In particular, the mGlu2/3 receptor agonist loses the ability to inhibit glutamate release from spinal cord synaptosomes of rats with sciatic ligation while DOI, enhances its inhibitory activity becoming effective at lower concentrations. Interestingly, the treatment with TZD was able to reduce the release of glutamate from spinal cord synaptosomes and restore the functionality of the receptors. Therefore, we hypothesized that differences in glutamate overflow were attributable to alterations of the receptor's expression at the presynaptic level. Western blot analysis showed that in the sciatic nerve ligation the 5-HT2A receptor density remained unchanged while the expression of mGlu2/3 was significantly reduced. Consistently with functional studies, administration of TZD resulted in a restoration mGlu2/3 receptors density. Overall, the outcomes support the use of 5-HT2A antagonists as an alternative therapeutic approach to modulate the mGlu2/3-mediated signalling in pathological conditions associated with neuropathic pain.

Paper: Neuroinflammation in Aged Brain: Impact of the Oral Administration of Ellagic Acid

Boggia F., Turrini F., Roggeri A., Olivero G., Cisani F., Bonfiglio T., Summa M., Grilli M., Caviglioli G., Alfei S., Zunin P., Bertorelli R., Pittaluga A. Microdispersion. Int. J. Mol. Sci. 2020. doi: 10.3390/ijms21103631.

Paper: Healthy properties of a new formulation of pomegranate peel extract in mice suffering from experimental autoimmune encephalomyelitis.

Vallarino G., Salis A., Lucarini E., Turrini F., Olivero G., Roggeri A., Damonte G., Boggia R., Di Cesare Mannelli L., Ghelardini C., Pittaluga A. Molecules. 2022 27(3), 914; https://doi.org/10.3390/molecules27030914.

In the recent years, thanks to a collaboration with Prof. Boggia from the University of Genova we dedicated part of our research on nutraceuticals. In particular, we focused on the beneficial effect of ellagic acid (EA) a compound highly present in different fruits (like pomegranates) and recognized for its antioxidant and anti-inflammatory properties. A few years ago, our research group demonstrated that aging impairs the central noradrenergic transmission, affecting the release of noradrenaline (NA). Noradrenergic defects negatively reverberate on the CNS-immune system crosstalk, favoring astrocytosis and overproduction of proinflammatory cytokines that in turn worsening the deregulation of the noradrenergic innervation. In this context, we decided to investigate whether chronic oral administration of EA microdispersion (EAm; 50 mg/Kg/day for 14 days) could recover the noradrenergic defects and reduce the inflammatory markers in the cortex of old (20 months) and young (3 months) mice. Interestingly, chronic administration of EAm did not change the high KCl-induced release of NA from cortical nerve endings of young mice but significantly enhance the amine overflow from cortical synaptosomes of old animals. In parallel to the enhanced release of noradrenaline in EAm-treated old mice, a significant reduction of the TNF-α and IL-1β mRNA contents have

been detected. Furthermore, CD45 immunopositivity (used as marker of lymphocytes infiltration in CNS) was significantly reduced in the cortex of both young and old mice, in accordance with the conclusion that EAm could interfere with the crosstalk between the immune and the central nervous system. Overall, the results suggest that EAm is beneficial and represents a potential nutraceutical ingredient for elders.

Additionally, it has recently been demonstrated that the nutraceutical properties of pomegranate fruit are not restricted to its edible portion but shared by different parts of the fruit. However, the efficacy of pomegranate extract is questioned because of its solubility. The problem was recently bypassed with a new formulation proposed by the research group of Professor Boggia from the University of Genoa. The formulation consists of a solid micro-dispersion in low methoxylated pectins formulated by spray drying technology of a pomegranate peel extract (Pomegranate Extract microdispersion, PEm) which hugely increased the oral bioavailability of EA. In this context, we investigate the effect of both EAm and PEm in EAE mice.

The oral administration of PEm displayed a significant amelioration of *in vivo* clinical symptoms in EAE mice. Although the treatment had no effect in restoring central demyelination or reducing inflammation in the spinal cord of EAE mice, it significantly affected some of the pathological hallmarks pivotal to the progression of these signs. In particular, spinal cord microgliosis and astrocytosis, were significantly reduced in PEm-treated EAE mice, particularly in the grey matter. Similarly, a reduction in the density of the CD45 protein (used as a marker of lymphocytes infiltration) was detected following the treatment. The results obtained are of considerable interest, since lymphocytes recruited from the periphery act with resident glial cells and astrocytes to dictate the progression of EAE Interestingly, EAm and PEm displayed comparable efficiencies in controlling the spinal cord pathological hallmarks in EAE mice, and this would support their used as dietary supplementation in MS patients.

The positive results obtained in both aged and EAE mice lead to the classification of pomegranate extract and its active compound EA as possible dietary supplements for the treatment of MS. Since S1PRs are involved in inflammatory processes and we confirm their alteration in the CNS of EAE mice, we would like to study the effect of concomitant administration of EA and S1PRs modulators on EAE progression in mice. In addition, the cortex, we will also extend the study to the spinal cord where the inflammatory infiltration is most remarked.

Other projects

Beyond the work conducted on the project discussed in the current thesis, during these three years as a PhD student I took part to others research projects of Prof. Anna Pittaluga's team made in collaboration with different Italian research groups.

- Thanks to a collaboration with the research group of Prof. Costa from the University "Magna Græcia" (Catanzaro, IT) we also investigated the potential CNS receptors of Ellagic Acid individuating the presynaptic Alpha2 auto-receptors as potential molecular target of the natural drug.

  Romeo I., Vallarino G., Turrini F., Roggeri A., Olivero G., Boggia R., Alcaro S., Costa G., Pittaluga A. Presynaptic Release-Regulating Alpha2 Autoreceptors: Potential Molecular Target for Ellagic Acid Nutraceutical Properties. Antioxidants (Basel). 2021. https://doi.org/10.3390/antiox10111759.

- I also took part to the project "PRIN 2017 -01779W93T_0042" which has the aim to investigate the molecular mechanism accounts for the etiology of the Major depressive disorder (MDD). In this context, our group focused on the hippocampus, aiming at studying the age and sex-related changes in GABA released from isolated nerve terminals. The first results of the project were presented to the 19th Congress of Italian Society of Neuroscience (virtual congress, 9-11 September 2021)

Participation in Congress

During these three years, the results presented in the thesis have been presented in several congress listed below:

- Roggeri A., Vergassola M., Cisani F., Olivero G., Usai C., Pittaluga A. "Pharmacological characterization of release-regulating sphingosine-1-phosphate receptors in isolated nerve endings and glial particles". "The international retreat of PhD students in immunology".Camogli, 05-06 December 2019. **(oral presentation**)

- Roggeri A., Vallarino G., Olivero G., Usai C., Pittaluga A. "Sphingosine-1-phosphate receptors in isolated nerve endings and glial particles of experimental autoimmune encephalomyelitis mice". 19th National Congress of the Italian Society for Neuroscience.Virtual congress 9-11th September 2021. (**poster**)

- Roggeri A., Vallarino G., Benatti C., Blom J.M.C., Olivero G., Tascedda F., Brunello N., Pittaluga A. "Age and gender-dependent changes in the efficiency of GABA exocytosis from isolated nerve terminals of mouse hippocampus". 19th National Congress of the Italian Society for Neuroscience.Virtual congress 9-11th September 2021. (**poster)**

- Roggeri A., Vergassola M., Cisani F., Olivero G., Minetti F., Pittaluga A. "Release-regulating sphingosine-1-phosphate receptors in nerve endings and glial particles of mice central nervous system". 39th National Congress of the Italian Society of Pharmacology. Firenze,19-23th November 2019. (**poster**)

- Roggeri A., Vergassola M., Cisani F., Olivero G., Pittaluga A. "Sphingosine-1-phosphate receptors control glutamate release in mice cortical nerve endings and glial particles".18th National Congress of the Italian Society for Neuroscience. Perugia, 26-29th September 2019. (**poster**)

Awards

2021: Award from the Italian Society of Pharmacology for the best oral poster presentation: "Trazodone recovers the impaired glutamate transmission of spinal cord nerve endings of rats suffering from chronic sciatic ligation". (40th National Congress of the Italian Society of Pharmacology. Virtual congress)