SPHINGOSINE 1-PHOSPHATE ENHANCES EXCITABILITY OF SENSORY NEURONS THROUGH SPHINGOSINE 1-PHOSPHATE RECEPTORS 1 AND/OR 3

Chao Li

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Michael R. Vasko, Ph.D., Chair

Theodore R. Cummins, Ph.D.

Doctoral Committee

Andy Hudmon, Ph.D.

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Grant D. Nicol, Ph.D.

Lawrence Quilliam, Ph.D.

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Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid that has proven to be an important signaling molecule both as an extracellular primary messenger and as an intracellular second messenger. Extracellular S1P acts through a family of five S1P receptors, S1PR1-5, all of which are G protein-coupled receptors associated with different G proteins. Previous work from our laboratory shows that externally applied S1P increases the excitability of small-diameter sensory neurons by enhancing the action potential firing. The increased neuronal excitability is mediated primarily, but not exclusively, through S1PR1. This raises the question as to which other S1PRs mediate the enhanced excitability in sensory neurons.

To address this question, the expression of different S1PR subtypes in small-diameter sensory neurons was examined by single-cell quantitative PCR. The results show that sensory neurons express the mRNAs for all five S1PRs, with S1PR1 mRNA level significantly greater than the other subtypes. To investigate the functional contribution of other S1PRs in augmenting excitability, sensory neurons were treated with a pool of three individual siRNAs targeted to S1PR1,

R2 and R3. This treatment prevented S1P from augmenting excitability, indicating that S1PR1, R2 and/or R3 are essential in mediating S1P-induced sensitization.

To study the role of S1PR2 in S1P-induced sensitization, JTE-013, a selective antagonist at S1PR2, was used. Surprisingly, JTE-013 by itself enhanced neuronal excitability. Alternatively, sensory neurons were pretreated with FTY720, which is an agonist at S1PR1/R3/R4/R5 and presumably downregulates these receptors. FTY720 pretreatment prevented S1P from increasing neuronal excitability, suggesting that S1PR2 does not mediate the S1P-induced sensitization.

To test the hypothesis that S1PR1 and R3 mediate S1P-induced sensitization, sensory neurons were pretreated with specific antagonists for S1PR1 and R3, or with siRNAs targeted to S1PR1 and R3. Both treatments blocked the capacity of S1P to enhance neuronal excitability. Therefore my results demonstrate that the enhanced excitability produced by S1P is mediated by S1PR1 and/or S1PR3.

Additionally, my results indicate that S1P/S1PR1 elevates neuronal excitability through the activation of mitogen-activated protein kinase kinase. The data from antagonism at S1PR1 to regulate neuronal excitability provides insight into the importance of S1P/S1PR1 axis in modulating pain signal transduction.

Michael R. Vasko, Ph.D., Chair

TABLE OF CONTENTS

INTRODUCTION

1. Overview

A number of inflammatory mediators secreted after inflammation can augment the excitability of nociceptive sensory neurons. Studies have demonstrated that sphingosine 1-phosphate (S1P) is released by immune cells, such as mast cells and platelets, during the inflammatory response. However, the role of S1P in regulating nociception is yet to be discovered.

S1P is a bioactive sphingolipid ubiquitously found in various cell types. It is an important signaling molecule both as an extracellular primary messenger and as an intracellular second messenger. Extracellular S1P activates a family of S1P receptors (S1PRs) in an autocrine or paracrine fashion. There are five S1PR subtypes, S1PR1-5, all of which are G protein-coupled receptors (GPCRs) in association with different G proteins and corresponding signaling cascades.

Previous work from our laboratory shows that S1P increases the excitability of nociceptive sensory neurons by enhancing action potential (AP) firing (Zhang et al., 2006a; Zhang et al., 2006b). The increased number of APs due to the external application of S1P is mediated primarily, but not exclusively, through S1PR1 (Chi and Nicol, 2010). The understanding of the role of the other S1PRs in regulating S1P-induced enhancement of neuronal excitability is very limited.

The overall goal of my research is to examine the S1PR subtypes in sensory neurons and their contributions to the enhanced neuronal excitability. My work will provide a basic understanding of which S1PRs in sensory neurons regulate the heightened neuronal excitability induced by S1P. The increased excitability serves as a way to augment nociception. Therefore, my work will also have implications in the role of S1P and S1PRs in the modulation of pain signal transduction.

2. S1P biogenesis

Sphingosine 1-phosphate (S1P) belongs to a complex family of sphingolipids. Sphingolipids are a class of lipids containing a long-chain backbone of sphingoid bases. They are found essentially in all eukaryotic cells. They exist mostly in cell membranes, but are also major constituents of lipoproteins (Kolter and Sandhoff, 2006). Sphingolipids contribute to the formation of lipid rafts and play critical roles in mediating cell signaling and thus influencing cellular functions and activities. Sphingolipids encompass a wide and complex family of lipids that can be roughly divided into two groups: simple sphingolipids which include sphingoid bases (2-amino-1,3-dihydroxy-alkanes) and ceramides; and complex sphingolipids which include phosphosphingolipids and glycosphingolipids. There are more than 300 different types of complex sphingolipids reported.

The de novo biosynthesis of sphingolipids initiates in the endoplasmic reticulum (ER) from the condensation of palmitate and serine, and the product of this process in the ER is ceramide (Hannun and Obeid, 2008; Merrill, 2002). Ceramide is composed of a sphinganine and a fatty acid. The fatty acid is typically 16 to 26 carbons in length. Newly synthesized ceramide is then transported to the Golgi apparatus where it is converted to sphingomyelin, which is the major phosphosphingolipid in mammalian tissues (Hirschberg et al., 1993). Sphingomyelin on the cell membrane can be broken down into ceramide by sphingomyelinase, and ceramide can be further degraded by ceramidase to

produce sphingosine. Sphingosine can be phosphorylated by sphingosine kinase 1 and 2 (SphK1 and SphK2) to generate S1P. Eventually, S1P will be broken down into hexadecenal and phosphoethanolamine (Fyrst and Saba, 2010). This ceramide-to-S1P-biosynthesis pathway can also be reversed under the catalysis of a different set of enzymes (Figure 1), and ceramide is at the center hub of the sphingolipids metabolism.

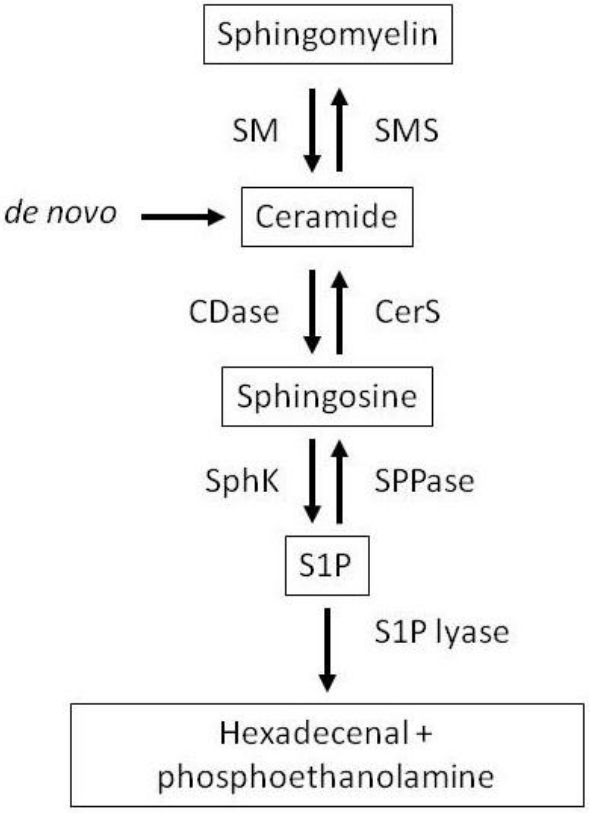

Figure 1. The metabolism pathways of S1P. SM, sphingomyelinase; SMS, sphingomyelin synthase; CDase, ceramidase; CerS, ceramide synthase; SphK, sphingosine kinases; SPPase, S1P phosphatases;

3. Sphingosine kinases

There are two isoforms of sphingosine kinase, SphK1 and SphK2. SphK1 was first cloned and purified from the rat kidney as a 49-kDa polypeptide (Olivera et al., 1998). Subsequently, mouse and human SphK2 were cloned. SphK2 contains five conserved domains found in all SphKs, but it is larger in size (~65 kDa) and diverges in its amino terminus and central region compared to SphK1 (Liu et al., 2000a; Maceyka et al., 2005). The two isoforms have distinct kinetic properties and also differ in tissue distribution and developmental expression, suggesting they have distinct physiological functions.

SphK1 and SphK2 have similar endogenous substrate specificities, and these enzymes phosphorylate sphingosine and dihydrosphingosine (Kohama et al., 1998). SphK1 promotes cell growth; for example, forced expression of SphK1 increased intracellular S1P levels and blocked breast adenocarcinoma MCF-7 cell death induced by anti-cancer drugs, conferring a growth advantage (Nava et al., 2002). On the contrary, SphK2 can inhibit cell growth and enhance apoptosis in diverse cell types through its putative BH3 domain, a peptide segment that allows it to interact with pro-survival Bcl-2 family members to trigger apoptosis. This also suggests SphK2 can exhibit its unique function independently of S1P production and S1P receptor activation (Liu et al., 2003).

Despite these differences, SphK1 and SphK2 have some similar functions. SphK1$^{-/-}$ mice were viable and fertile and lacked any severe phenotypes, although total SphK activity was substantially, but not completely, reduced (Allende et al., 2004). In contrast, the S1P level in serum from SphK2$^{-/-}$ mice was not significantly reduced (Mizugishi et al., 2005). However, SphK1/SphK2 double-knockout mice are embryonically lethal; all embryos exhibited cranial hemorrhage and none survived beyond E13.5. These results indicate that expression of either SphK1 or SphK2 is essential for development and survival.

4. Location of S1P in cells

The phosphorylation of sphingosine to form S1P requires the enzymatic activity of SphKs, SphK1 or SphK2. These two distinct SphK isoforms have different intracellular locations, and therefore, S1P generated intracellularly by these two enzymes are further translocated to different cellular compartments and trigger various downstream signaling pathways.

Several previous studies have shown that the endogenous SphK1 is mainly cytosolic (Hait et al., 2002; Olivera et al., 1999). An additional study shows that phosphorylation of human SphK1 at Ser225 is necessary for activation of the enzyme, and leads to the increase in enzyme activity and translocation of the enzyme from the cytosol to the plasma membrane. Furthermore, both intracellular and exported extracellular S1P levels were elevated by agonist-stimulated increase in enzyme activity or overexpression of wild-type hSphK1 (Pitson et al., 2003). These results provide a picture as to how S1P is generated by SphK1 and where S1P translocates after its level increases (Figure 2). SphK1 catalyzes S1P formation at the plasma membrane. Some S1P generated through SphK1 stays in the cytosol and functions as a second messenger to induce a series of downstream signaling, resulting in cell growth and inhibition of apoptosis (Kolesnick and Fuks, 2003); meanwhile, some other S1P is exported out of the cell by specific transporters, and binds to a family of G protein- coupled receptors (GPCRs), S1P receptor 1-5 (S1PR1-5), to initiate downstream

signaling. This pathway is called “inside-out” signaling (Takabe et al., 2008), which is crucial for many cellular functions, such as the migratory response and chemotaxis (Hobson et al., 2001; Jolly et al., 2004).

SphK2 is generally localized to the ER, mitochondria and nucleus (Kunkel et al., 2013). In human embryonic kidney (HEK) 293 cells, ectopically expressed SphK2 is also present in all membrane fractions and in the cytosol (Maceyka et al., 2005). In the nucleus, S1P produced by SphK2 specifically binds to the histone deacetylases HDAC1 and HDAC2 and inhibits their enzymatic activity to epigenetically regulate gene expression (Hait et al., 2009). At the ER, S1P is degraded by S1P lyase or dephosphorylated by S1P phosphatases to sphingosine, which is reused for the synthesis of ceramide (Maceyka et al., 2005). Also, one study has shown that ceramide transferred to mitochondria is converted to S1P by SphK2, and S1P is broken down to produce hexadecenal. Hexadecenal binds to and directly promotes BAX activation and oligomerization to promote apoptosis (Chipuk et al., 2012).

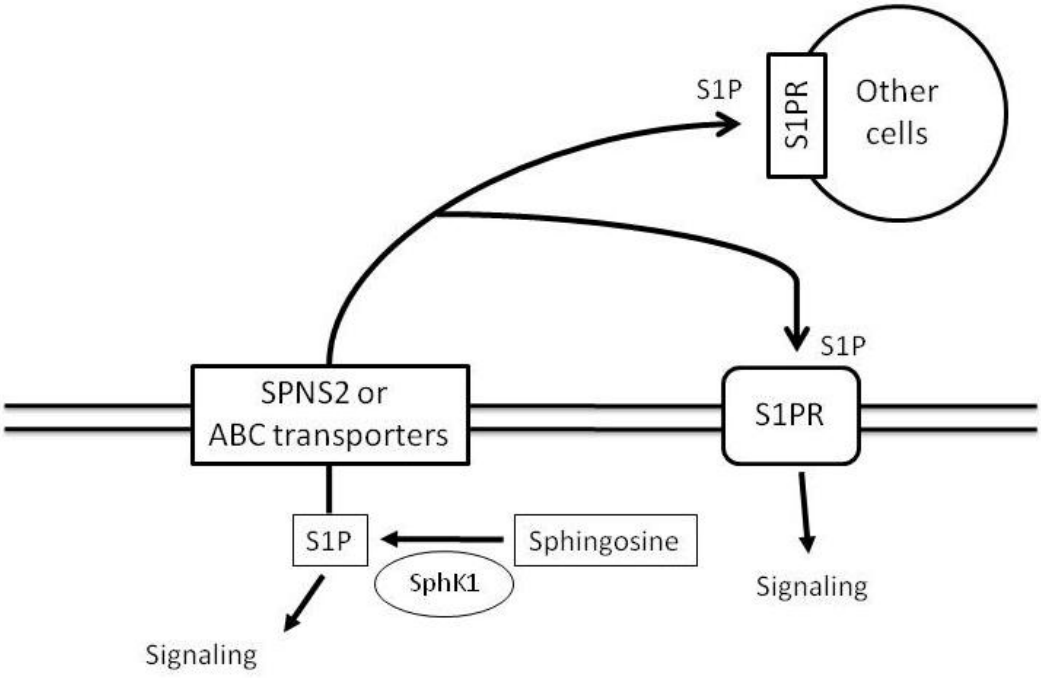

Figure 2. S1P functions both as an extracellular first messenger and an intracellular second messenger.

SphK1 catalyzes S1P formation at the plasma membrane. Some S1P stays in the cytosol and functions as a second messenger to initiate downstream signaling. Some other S1P is exported out of the cell by specific transporters, functions as a first messenger by binding to S1PRs to activate signaling cascade. This pathway is called “inside-out” signaling. If extracellular S1P activates S1PRs located on the same cell generated S1P, this is called autocrine signaling. If extracellular S1P activates S1PRs located on other cells, this is called paracrine signaling.

5. S1P as an intracellular messenger

Much evidence has shown the intracellular role for S1P as a second messenger, including that it counteracts apoptotic effects mediated by its precursor ceramide, and mobilizes Ca^{2+} from internal stores via an inositol trisphosphate-indepedent pathway (Maceyka et al., 2012). Recently, new evidence is emerging, pointing to novel intracellular targets of S1P.

Traditionally, S1P is thought to be anti-apoptotic, distinct from its precursor ceramide and sphingosine, which are thought be to pro-apoptotic. Since sphingosine and S1P, as well as some other sphingolipids metabolites, are interconvertible, it has been proposed that it is their relative levels, rather than their absolute amounts, that determine cell fate. This balance between S1P and sphingosine/ceramide is called the “sphingolipid rheostat,” which serves as a conserved stress regulation mechanism (Spiegel and Milstien, 2003).

However, another recent study shows that intracellular S1P and its metabolite hexadecenal can activate BAK/BAX to promote apoptosis (Chipuk et al., 2012). The study demonstrated that, in vitro, S1P and hexadecenal interacted specifically with BAK and BAX, respectively, to influence mitochondrial outer membrane permeabilization (MOMP), initiate BAK/BAX-dependent cytochrome c release and eventually induce apoptosis.

Besides its function in regulating cell growth and death, work from our laboratory has demonstrated that S1P functions as a second messenger downstream of the nerve growth factor (NGF) signaling pathway to increase neuronal excitability (Zhang et al., 2006b). Although the targets of intracellular S1P in NGF-induced sensitization have yet to be identified, some studies listed below have shown that S1P can alter the functions of several disparate intracellular proteins.

Histone deacetylase (HDAC)

In many cells, SphK2 is mainly localized in the nucleus, and upon phosphorylation by protein kinase D, it can be exported out of the nucleus and initiate subsequent signaling in the cytosol (Ding et al., 2007). In the nucleus, SphK2 is physically associated with histone H3 and the formation of S1P by SphK2 enhances histone acetylation at specific lysine residues and regulates gene transcription (Hait et al., 2009). Also, S1P binds to and inhibits histone deacetylases HDAC1 and HDAC2, indicating that S1P produced in the nucleus by SphK2 influences the dynamic balance of histone acetylation and thus epigenetically regulates specific gene transcription.

TNF receptor-associated factor 2 (TRAF2)

Nuclear factor kappa B (NF-κB) is a protein complex that functions as a transcriptional factor to regulate gene expression. TRAF2 belongs to a family of seven TRAF proteins that are key intermediates in nearly all NF-κB signaling pathways. TRAF2 has an N-terminal RING finger domain that functions as an E3

ubiquitin ligase to catalyze the transfer of ubiquitin to target proteins. The process of adding ubiquitin to a protein is called ubiquitination, which affects proteins in many ways such as signaling for protein degradation via the proteasome, altering protein cellular location and activity, and affecting protein-protein interactions (Hayden and Ghosh, 2008). TRAF2 binds to SphK1 (Xia et al., 2002), which generates S1P inside cells. It has been shown that the production of S1P by SphK1 is necessary for Lys 63-linked polyubiquitination of receptor interacting protein 1 (RIP1), phosphorylation of IκB kinase (IKK) and IκBα, and IκBα degradation, all leading to NF-κB activation. These responses are mediated by intracellular S1P independently of its cell surface receptors (Alvarez et al., 2010). These results highlight the role of SphK1 and its intracellular product S1P in TRAF2 signaling and the NF-κB activation pathway in response to cellular stimuli.

BACE1

Evidence from various studies has suggested that the accumulation of amyloid-β peptide (Aβ) is linked to the pathogenesis of Alzheimer's disease (AD) (De Strooper et al., 2010). Aβ is derived from amyloid precursor protein (APP) that is sequentially cleaved by two aspartate proteases, β- and γ-secretases. The major type of β-secretase is known as β-site APP cleaving enzyme 1 (BACE1). Extracellular cleavage of APP by BACE1 and then by γ-secretase within the transmembrane domain release the intracellular domain of APP and produces Aβ.

A study from Takasugi et al. discovered that a SphK inhibitor, as well as RNAi against SphK1 or 2 significantly decreased the Aβ secretion in mouse neuroblastoma N2a cells. Next, they overexpressed S1P phosphatase and S1P lyase to reduce cellular S1P level. The overexpression caused a strong reduction in the level of secreted Aβ. Further results indicate that intracellular S1P directly modulates the proteolytic activity of membrane-bound form of BACE1 (Takasugi et al., 2011).

6. S1P transporters

In order to act on S1P receptors to initiate downstream signaling pathways, S1P generated in the cytosol by SphK1 must be transported outside the cell. Studies have shown that there are two types of transporters involved in this process: ATP-binding cassette (ABC) transporters and spinster homolog 2 (SPNS2) protein (Kawahara et al., 2009; Mitra et al., 2006; Nagahashi et al., 2013; Sato et al., 2007).

ABC transporters are a family of proteins that utilize the energy from ATP hydrolysis to transport various substrates across the cellular membrane. The substrates include ions, amino acids, peptides, sugars, lipids and drugs (Holohan et al., 2013). Several studies have shown that ABC transporters export S1P out of the cell. Mitra et al (2006) found that in mast cells, constitutive and antigen-stimulated S1P release was inhibited by MK571, an inhibitor of ABC transporter C1 (ABCC1). Moreover, down-regulation of ABCC1 expression with siRNA markedly reduced S1P efflux from mast cells, demonstrating that this ABC transporter mediates S1P exportation from mast cells. In the central nervous system (CNS), Sato et al. discovered that S1P release from rat astrocytes was inhibited by siRNAs specific to ABC transporter A1 (ABCA1), and that astrocytes from Abca1$^{-/-}$ mice showed impairment of S1P release, indicating S1P release from astrocytes is through an ABC transporter (Sato et al., 2007).

Recent studies have shown that another type of protein, SPNS2, which belongs to the major facilitator superfamily of transporters, regulates S1P release from endothelial and lymphendothelial cells and controls S1P levels in plasma and lymph (Hisano et al., 2012; Nagahashi et al., 2013). In plasma, S1P exists at a high concentration level (~μM). Plasma S1P turns over rapidly with a half-life of approximately 15 minutes (Venkataraman et al., 2008), suggesting that there must be an active S1P degradation pathway in plasma, and also that the plasma S1P level must be maintained by a continuous S1P supply from S1P-producing cells. Hisano et al. showed that SPNS2 functioned as an S1P transporter in vascular endothelial cells; the plasma S1P concentration in $SPNS2^{-/-}$ mice was reduced to approximately 60% of wild-type mice (Hisano et al., 2012), demonstrating that SPNS2 is the physiological S1P transporter in mammals. Additionally, a study from Nagahashi et al. shows that overexpression of SPNS2 in human microvascular endothelium cells increases export of S1P, and that S1P is active at all cell surface S1PRs. Moreover, $SPNS2^{-/-}$ mice show defective lymphocyte trafficking and have reduced numbers of lymphocytes, suggesting SPNS2 plays a vital role in regulating S1P levels in blood, lymph node and lymph and consequently influencing lymphocyte trafficking (Nagahashi et al., 2013).

7. S1P receptors

Besides the intracellular roles of S1P as a second messenger, once secreted out of the cell, S1P can function in an autocrine and/or paracrine fashion to activate S1P receptors present on the surface of the same cell and/or on nearby cells to initiate downstream signaling cascades. This latter function of S1P as a ligand for S1P receptors suggests S1P is also a primary messenger regulating cellular functions.

S1P signals through a family of five highly specific GPCRs. These five subtypes are named S1P receptors 1-5 (S1PR1-5). All S1PRs specifically bind both S1P, dihydro-S1P (sphinganine 1-phosphate), and phytoS1P (4-hydroxysphinganine 1-phosphate) with low nanomolar affinities. Other sphingolipids may activate these receptors at very high, nonphysiological concentrations, and there is no evidence yet for additional endogenous ligands (Chun et al., 2010). Expression patterns of five S1PR subtypes vary across different tissues and cell types, as well as at various developmental and aging stages. S1PR1, R2 and R3 are ubiquitously expressed, whereas expression of S1PR4 and R5 are restricted to distinct cell types.

S1PR1, also known as endothelial differentiation gene 1 (EDG-1), was the first S1P receptor to be functionally identified (Lee et al., 1998). The S1PR1 transcript was cloned as an immediate-early gene during the induced differentiation of

human endothelial cells into capillary-like, tubular structures in 1990 (Hla and Maciag, 1990). Since then, five S1PRs, S1PR1-5, have been identified, and they are formerly known as EDG-1, EDG-5, EDG-3, EDG-6 and EDG-8, respectively (An et al., 1997; Im et al., 2000; Okamoto et al., 1999; Yamazaki et al., 2000). The EDG family of receptors also includes three receptors from lysophosphatidic acid (LPA) receptor family, LPA1 (EDG-2), LPA2 (EDG-4) and LPA3 (EDG-7) (Chun et al., 2010). In addition, the orphan receptors OGR1, GPR3, GPR4, GPR6, GPR12 and G2A are known to share some homology with S1PRs (Meyer zu Heringdorf and Jakobs, 2007). GPR3, GPR6, GPR12 and GPR63 have been characterized as GPCRs that interact with S1P (Ignatov et al., 2003; Niedernberg et al., 2003; Uhlenbrock et al., 2002).

8. Physiological functions of S1P

The first evidence to demonstrate that S1P was a bioactive lipid was from Spiegel's group in 1991 (Zhang et al., 1991). They discovered that sphingosine, a metabolite of membrane sphingolipids, could be further metabolized to S1P. S1P stimulated DNA synthesis in quiescent Swiss 3T3 cells and induced pronounced morphological alterations. Also, S1P caused mobilization of Ca^{2+} from internal stores, suggesting that S1P may be a component of intracellular second messenger.

Since then, diverse biological functions of S1P have been identified, including cell proliferation, survival, development, as well as the control of immune cell trafficking, tumor angiogenesis and vascular permeability (Abuhusain et al., 2013; Allende et al., 2003; Kunkel et al., 2013; Mandala et al., 2002; Singleton et al., 2005). Also, the link between S1P and various diseases has been identified. S1P affects the immune system, the nervous system and the cardiovascular system, and S1P is implicated in pathophysiological conditions that affect almost every organ of the human body. The study from Kim et al. found that S1P enhances the invasive/migratory phenotypes of MCF10A human breast epithelial cells, providing a molecular basis for the role of S1P in promoting breast cancer cell invasion (Kim et al., 2011). Keul et al. demonstrate that S1P has chemotactic effects on macrophages in vitro and in vivo, promotes inflammatory monocyte/macrophage recruitment, and alters smooth muscle cell behavior (Keul

et al., 2011). These results suggest that S1P plays a causal role in the pathogenesis of atherosclerosis. Moreover, another study shows that when added to the bone marrow-derived macrophage (BMM)/osteoblast cocultures, S1P greatly increased osteoclastogenesis and stimulated osteoblast migration and survival. Further evidence indicates that S1P attracts and acts on osteoblasts and T cells to augment osteoclastogenesis (Ryu et al., 2006).

S1P is also a key regulator in various cellular events in the nervous system. One study demonstrates that S1P induces proliferation and morphological changes in neural progenitor cells prepared from embryonic rat hippocampus (Harada et al., 2004). Another study shows that nerve growth factor (NGF) modulates neurite extension in a SphK1-dependent manner and through the differential transactivation of S1PRs in rat DRG neurons (Toman et al., 2004). Studies from our laboratory demonstrate that S1P functions both as a primary messenger to act on S1PRs and as a second messenger downstream of NGF signaling pathway to increase neuronal excitability (Zhang et al., 2006a; Zhang et al., 2006b). Cerebellar granule cells and astrocytes can synthesize S1P intracellularly and release S1P extracellularly, supporting the role of S1P as an autocrine/paracrine physiological messenger in the cerebellum (Anelli et al., 2005). Two studies indicate that S1P produces thermal antinociception in the CNS (Coste et al., 2008b; Sim-Selley et al., 2009); however, several other studies indicate that S1P is pronociceptive in the PNS (Camprubi-Robles et al., 2013; Doyle et al., 2011a; Doyle et al., 2011b; Xie et al., 2012).

9. S1PR1 signaling and functions

Initially after its discovery, S1PR1 was considered a prototypical member of an orphan receptor subfamily, because its ligands, signaling pathways and function had not been discovered. It was not until 1998 that S1P was confirmed as the ligand for EDG-1. Lee et al. showed that S1P bound to EDG-1 with a high affinity, with a dissociation constant of 8.1 nM. Treatment of cells with 100 nM S1P caused translocation of EDG-1 into intracellular vesicles, a phenomenon observed in GPCRs being exposed to their ligands for a prolonged period of time. Moreover, overexpression of EDG-1 induced exaggerated cell-cell aggregation and formation of adherens junctions in a manner that was dependent on S1P (Lee et al., 1998).

To date, the S1PR1 subtype is reported to exclusively couple to the Gi/o-types of heterotrimeric G proteins. The Gi α subunit inhibits adenylyl cyclase (AC), leading to a reduction of the cellular cyclic AMP (cAMP) production. There are two isoforms of Goα, Goα1 and Goα2. Goα1 has no inhibitory effect on AC activity, while Goα2 can inhibit AC similar to the Gi α subunit (Katada et al., 1986; Kobayashi et al., 1990; Wong et al., 1992). Interestingly, studies have demonstrated that Goα directly interacts with protein kinase A (PKA) to inhibit its kinase activity and interfere with PKA nuclear translocation (Ghil et al., 2006; Jiang and Bajpayee, 2009). The βγ subunits from both Gi and Go can stimulate the rat sarcoma (Ras) family of small GTPases to enhance proliferation. βγ

subunits also can activate the phosphatidylinositide 3-kinase (PI3K)/Akt (protein kinase B) pathways to inhibit apoptosis and to promote cellular migration (O'Sullivan and Dev, 2013). Moreover, agonists of Gi/o-coupled receptors have been shown to activate the phosphoinositide phospholipase C (PLC) signaling pathway to increase intracellular Ca^{2+} concentration (Murthy et al., 2004). Interestingly, one study shows that S1P can promote lymphangiogenesis through the S1PR1/Gi/PLC signaling pathway to increase intracellular Ca^{2+} mobilization in human lymphatic endothelial cells (Yoon et al., 2008). A simplified S1PR1 intracellular signaling network is summarized in Figure 3.

Recently, the structure of S1PR1 was determined (Hanson et al., 2012). This provides a detailed view of the molecular recognition that activates S1PR1 and results in the modulation of various cellular responses. The S1PR1 has an N-terminal fold that occludes access to the binding pocket from the extracellular environment as well as orthosteric and bitopic ligands with various physicochemical properties (Rosen et al., 2013).

Functionally, S1PR1 plays a major role in the development, angiogenesis, and trafficking of lymphocytes. Constitutive loss of S1PR1 leads to embryonic lethality, and $S1PR1^{-/-}$ mice exhibit embryonic hemorrhage leading to death between E12.5 and E14.5. Vascular maturation is incomplete due to a deficiency of vascular smooth muscle cells. Also, embryos demonstrate a defect in blood vessel maturation and fibroblasts do not display a significant migratory response

to S1P due to the disruption of Rac activation (Liu et al., 2000b). This is the first example of a lysophospholipid receptor that was revealed to be essential for blood vessel formation and mammalian development.

S1PR1 is also required for neural development. S1PR1$^{-/-}$ mouse embryos revealed massive cell loss in the forebrain at E12.5, and cell proliferation was also affected. A significant increase in cell death was observed in earlier E11.5 S1PR1$^{-/-}$ embryos; mitotic cell numbers were significantly decreased in the telencephalon regions. These results indicate that the effect of S1P on neural development is likely mediated by signaling from S1PR1 receptor (Mizugishi et al., 2005).

A breakthrough in the field of S1PRs happened when the role of S1PR1 in normal immune cell trafficking was established. Mice whose haematopoietic cells lack S1PR1 have an almost complete absence of peripheral T cells as well as reduced numbers of B cells, because mature T and B cells are accumulated in secondary lymphoid organs and fail to exit. Further cell transfer experiments established an intrinsic requirement for S1PR1 in the egress of T and B cells from lymphoid organs. These findings suggest that lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1PR1 (Matloubian et al., 2004). Importantly, the transmembrane C-type lectin CD69, a type of membrane glycoprotein, interacts with S1PR1. CD69 acts downstream of interferon α/β (IFN-α/β) signaling to selectively downregulate S1PR1 surface

expression and to functionally inhibit S1P to promote lymphocyte retention in lymphoid organs (Shiow et al., 2006).

S1PR1 also plays a role in decreasing vascular leakage. During inflammation, tumor angiogenesis and atherosclerosis, increased vascular permeability and organ dysfunction are observed due to the endothelial cell barrier disruption (Spiegel and Kolesnick, 2002). S1P derived from platelets exhibits multiple effects on endothelial cells, including promoting barrier integrity, and this is mediated by the activation of the small GTPase, Rac1 (Garcia et al., 2001). A study shows that, in human pulmonary artery endothelial cells, S1P recruits S1PR1 to caveolin-enriched microdomains and promotes PI3K-mediated Rac1 activation, and this signaling causes actin rearrangement and endothelial cell barrier enhancement (Singleton et al., 2005). Therefore, S1PR1 regulates S1P-induced endothelial cytoskeletal rearrangement and barrier enhancement.

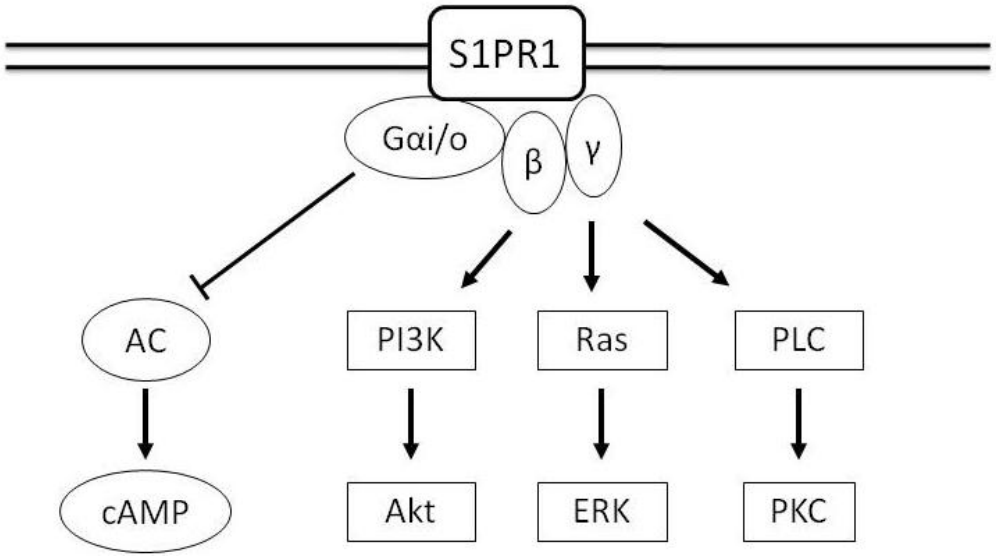

Figure 3. S1PR1 downstream signal transduction pathways.

The S1PR1 subtype exclusively couples to the Gi/o-type heterotrimeric G proteins. The Gi α subunit inhibits AC, leading to a reduction of the cAMP production. The βγ subunits can stimulate Ras family of small GTPases and extracellular signal-regulated kinase (ERK) to enhance proliferation, can activate the PI3K/Akt pathway to inhibit apoptosis and to promote cellular migration, and can activate the PLC signaling pathway to increase intracellular Ca^{2+} concentration to eventually activate PKC.

10. S1PR2 signaling and functions

S1PR2 was previously known as EDG-5 or AGR16. Human S1PR2 contains 353 amino acids and the sequence is highly conserved across species. Similar to S1PR1, S1PR2 is also coupled with Gi/o-type G proteins. Additionally, S1PR2 couples with G12/13 to enhance Rho activation and with Gq to activate PLC (Gonda et al., 1999; Okamoto et al., 2000; Windh et al., 1999). S1PR2 mediates diverse physiological effects on most types of cells upon agonist binding, including cell proliferation, migration, angiogenesis, and apoptosis.

Although coupled to Gαi/o like S1PR1, S1PR2 generally has opposite functions to S1PR1. It could be due to the fact the S1PR2 signaling cascades are preferentially activated. For example, S1PR1 promotes directed cell migration and trafficking, but S1PR2 inhibits the migration of various cell types (Green et al., 2011; Okamoto et al., 2000; Windh et al., 1999). In Chinese hamster ovary (CHO) cells expressing S1PR1, S1P induced chemotaxis and membrane ruffling in PI3K- and Rac-dependent manners. However, in S1PR2-expressing CHO cells, S1P did not promote migration and inhibited the basal Rac activity (Okamoto et al., 1999).

In another study, S1PR2 inhibited germinal center (GC) B cell responses to follicular chemoattractants and helped confine cells to the GC. S1PR2 overexpression promotes the centering of activated B cells in the follicle. This

growth control in chronically proliferating GCs is regulated by antagonism of the Akt kinase mediated by S1PR2 and its downstream effectors Gα12/13 and p115RhoGEF (Green et al., 2011).

The molecular mechanism of inhibiting migration through S1PR2 is illustrated in Figure 4. S1PR2 is coupled to G12/13-type G proteins. Upon ligand binding, the α subunit of G12/13 dissociates from the inactive protein complex and its intrinsic GTPase activities is stimulated by p115RhoGEF. Activated Gα13 binds tightly to RhoGEF and stimulates the capacity of RhoGEF to facilitate dissociation of GDP from Rho. However, Gα12 blocks stimulation of RhoGEF activity by Gα13, suggesting it could regulate the signal transduction, or it activates Rho through another RhoGEF (Hart et al., 1998). Activated Rho by RhoGEF can mediate cell body contraction and rear end retraction in migrating cells (Raftopoulou and Hall, 2004). Meanwhile, it has been shown that Rho can antagonize Rac activity through its effector ROCK (Rho-associated protein kinase) and activation of RacGAPs, such as FilGAP (Ohta et al., 2006). RacGAPs activate the GTP hydrolyzing activity of Rac, accelerating the transition of active Rac-GTP state to its inactive Rac-GDP state. Rho-mediated antagonism of Rac is important for the inhibitory role of S1PR2 in cell migration. The integration of positive and negative signals on the small GTPases activation downstream of S1PR2/G12/13 is an essential determinant for the regulation of cell migration. G12/13 is the preferential and most prominent G protein that S1PR2 couples to (Skoura and Hla, 2009).

In addition to Gi/o and G12/13, S1PR2 is also coupled to Gq-type G protein. In Sf9 cells expressing S1PR2 and heterotrimeric G protein subunits, Gαq is activated in an S1P-dependent manner (Windh et al., 1999). In CHO cells transfected with S1PR2 expression vector, the addition of S1P caused an increase in the intracellular Ca^{2+} concentration by mobilization of Ca^{2+} from both intra- and extra-cellular pools, and S1P-stimulated production of inositol phosphates and Ca^{2+} mobilization was only inhibited by 30% by pertussis toxin (PTX), a drug disrupting Gi/o-mediated signaling pathway by catalyzing the ADP-ribosylation of the αi/o subunit (Gonda et al., 1999). These results indicate besides Gi/o, S1PR2 also couples to Gq-type G protein.

Unlike S1PR1 null mice that are embryonically lethal, S1PR2 null mice do not exhibit significant embryonic lethality (Kono et al., 2004). Nevertheless, S1PR2 has been demonstrated to be essential for proper functioning of the auditory and vestibular systems. S1PR2-null mice are deaf by one month of age and they exhibit multiple inner ear pathologies (Kono et al., 2007).

S1PR2 also plays a major role in the wound healing response to acute liver injury by a mechanism involving enhanced proliferation of hepatic myofibroblasts (hMF). hMFs are a population of cells that expresses S1PR2 and triggers matrix remodeling during liver injury. $S1PR2^{-/-}$ mice, compared to wild-type (WT) mice, display reduced accumulation of hMF and lower induction of smooth muscle α-

actin mRNA, reflecting reduced activation of regeneration after liver injury. In vitro, S1P upregulates S1PR2 mRNA and enhances proliferation of cultured WT hMF, suggesting S1PR2 triggers hepatic would healing (Serriere-Lanneau et al., 2007).

The endothelium is the interface between blood and all tissues and plays a critical role in inflammation. S1PR2 appears to play a key role in the regulation of permeability and inflammatory responses of the vascular endothelium during endotoxemia (Zhang et al., 2013). Further investigation established the detailed mechanisms on the downstream signaling of S1PR2. The Rho-NF-κB pathway is required for S1PR2-mediated endothelial inflammatory responses.

S1PR2 is widely studied in cancer research because of its inhibitory effect on cell migration. One study has shown that S1P inhibits B16 melanoma cell migration and invasion, and this effect is completely abolished by the application of the S1PR2 antagonist JTE-013. S1P also induces inhibition and activation of Rac and RhoA, respectively, and this effect is also abrogated by JTE-013. Overexpression of S1PR2 in B16 cells sensitizes S1P inhibition of Rac and cell migration (Arikawa et al., 2003). These results provide compelling evidence that endogenously expressed S1PR2 negatively regulates cell motility and invasion through the regulation of Rac and RhoA activities. Another study confirms the inhibitory effect of S1PR2 on tumor angiogenesis and tumor growth in vivo in mice (Du et al., 2010).

A novel role of S1PR2 in the CNS has been recently discovered. Nogo-A is a membrane protein restricting structural and synaptic plasticity in mature neuronal networks of the CNS. Nogo-A has two different extracellular domains: Nogo-66 and Nogo-A-Δ20. S1PR2 was identified as a high-affinity receptor for Nogo-A-Δ20, and ligand binding leads to the initiation of downstream signaling transduction through G13/RhoA. Deleting or blocking S1PR2 prevents Nogo-A-Δ20-mediated inhibitory effects and enhances long-term potentiation (LTP) in the hippocampus (Kempf et al., 2014). This study proposes a novel signaling model in which S1PR2 functions as a receptor both for a membrane protein and a sphingolipid, and also providing insights into the role of S1PR2 in regulating synaptic plasticity.

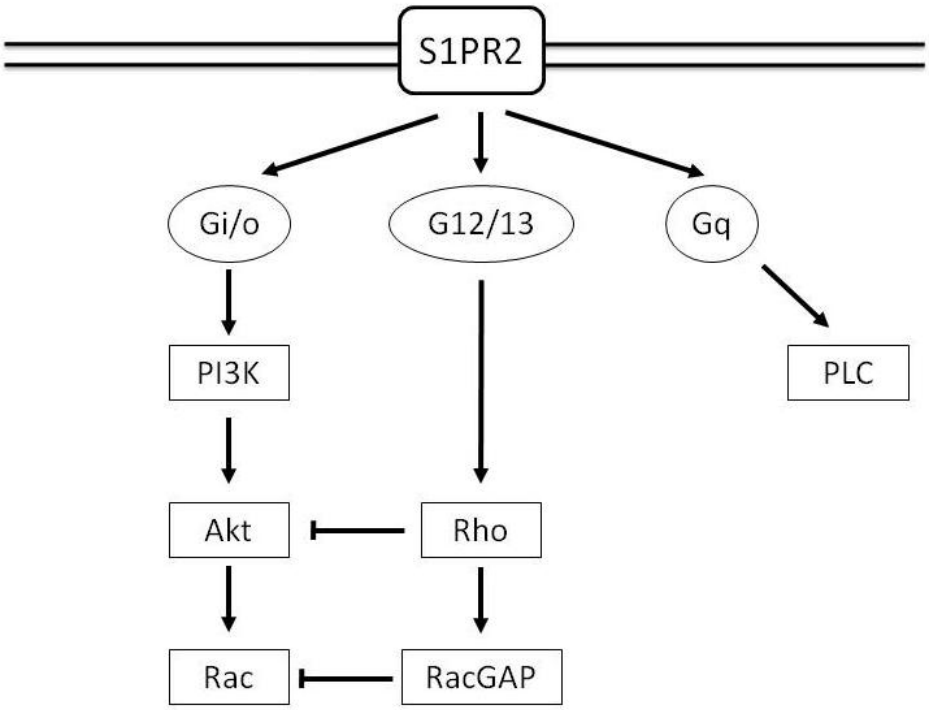

Figure 4. Molecular model for S1PR2 signaling leading to migration inhibition. S1PR2 couples with the Gi/o-, G12/13- and Gq-type G proteins. The βγ subunits from the activated Gi/o activate the PI3K/Akt/Rac pathway to promote cellular migration. The activated Gα13 binds tightly to RhoGEF and stimulates the Rho activation. Activated Rho antagonizes Rac activity through RacGAPs. Rho-mediated antagonism of Rac is important for the inhibitory role of S1PR2 in cell migration.

11. S1PR3 signaling and functions

S1PR3 was previously known as EDG-3. The human S1PR3 gene was first cloned in 1996 and was found to share a high homology with human S1PR1 (51.9% overall and 69.2% in the seven transmembrane regions) and with rat S1PR2 (47.9% overall) (Yamaguchi et al., 1996). Like S1PR2, S1PR3 couples with Gi/o-, G12/13- and Gq-type G proteins (Ancellin and Hla, 1999).

In membrane fractions prepared from Sf9 cells expressing S1PR3 and various subunits of heterotrimeric G proteins, S1P activated Gi, Gq and G13 but not Gs (Windh et al., 1999). In CHO cells overexpressing S1PR3, S1P induced inositol 1,4,5-trisphosphate (IP_3) production and an increase in intracellular Ca^{2+}. PTX partially blocked this response, which was quite different from the case of S1PR1, where S1P-induced response in the increase in intracellular Ca^{2+} was totally abolished by PTX, suggesting S1PR3 couples to other G proteins besides Gi/o. Additionally, S1PR3 mediated the activation of MAPK in a Ras-dependent manner and induced a decrease in the cellular cAMP content (Okamoto et al., 1999). Another study shows that in human TAg-Jurkat T cells transfected with S1PR3, S1P induced mobilization of intracellular Ca^{2+}, coincident with the formation of IP_3. This response was completely abolished by the PLC inhibitor U73122, and partially inhibited by PTX (An et al., 1999).

Unlike S1PR2, which activates Rho to inhibit Rac through Gα12/13, S1PR3 has been shown to mediate stimulation of Rac. In CHO cells expressing S1PR3, S1P induced chemotaxis and membrane ruffling in a PI3K and Rac-dependent manner (Okamoto et al., 2000). Interestingly, in human umbilical vein endothelial cells (HUVECs), S1P induces cell migration, and the migration is mediated by activated Rho and Rac via S1PR3 (Paik et al., 2001). It is possible that, downstream of S1PR3, both the activation of Rho via G12/13 and the activation of Rac via Gi form a dynamic regulatory network to mediate cell motility.

These results demonstrate that S1PR3 couples with Gi/o, Gq and G12/13; however, it preferentially couples to the Gq protein and stimulates the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) by PLC to form IP_3 and diacyl glycerol (DAG), thereby leading to intracellular Ca^{2+} increase and activation of protein kinase C (PKC) (Windh et al., 1999).

The initial study about S1PR3 function was done in zebrafish. A single point mutant of Mil gene, an S1PR3 ortholog, affected the migration of the heart precursors to the midline during organogenesis (Kupperman et al., 2000). The study of S1PR3 in vivo functions in mammals generated unexpected results. S1PR3-null mice are viable and fertile and develop normally without obvious phenotypic abnormalities. Mouse embryonic fibroblast (MEF) cells devoid of S1PR3 show a significant decrease in PLC activation, a slight decrease in AC inhibition, but no change in Rho activation in response to S1P (Ishii et al., 2001).

These results indicate a nonessential role for S1PR3 in mouse development, but a necessary role in S1P-induced cellular signaling mediated by S1PR3.

In HTC4 hepatoma cells transfected with human S1PR3, S1P treatment significantly increased cell proliferation and survival. This event results from S1PR3 transduction of S1P-evoked signaling to activate ERK, as well as the immediate-early induction of c-Jun and c-Fos (An et al., 2000). Conversely, in HEK 293 cells transfected with S1PR3, S1P induced rounded cell morphology, which was correlated with apoptotic cell death due to loss of attachment. Also, S1P treatment rapidly enhanced neurite retraction in PC12 cells overexpressing S1PR3 (Van Brocklyn et al., 1999).

S1PR3 has also been shown to directly regulate heart rate in mice. The sustained bradycardia induced by S1PR1 non-selective immunosuppressants in WT mice is abolished in S1PR3-null mice (Sanna et al., 2004). S1PR3 can also induce pulmonary edema by reorganizing epithelial tight junctions, compromising lung barrier integrity and respiratory function (Gon et al., 2005). Protease-activated receptor 1 (PAR1) signaling mediates a lethal inflammatory response. The S1P/S1PR3 axis is a downstream component of PAR1 signaling. In dendritic cells, PAR1-S1PR3 crosstalk regulates amplification of inflammation in sepsis syndrome. Loss of this signaling sequesters dendritic cells into lymph nodes and attenuates inflammatory responses (Niessen et al., 2008).

A recent study shows that S1PR3 suppresses cell cycle progression to regulate cell activation and function in muscle satellite cells. S1PR3 levels are high in quiescent myogenic cells, while falling during the entry into cell cycle. However, S1PR3-null mice exhibit enhanced satellite cell proliferation and muscle regeneration. Genetic deletion of S1PR3 in muscular dystrophy mouse model produces a less severe muscle dystrophic phenotype (Fortier et al., 2013). These results together indicate a critical role of S1PR3 in maintaining and regulating various physiological functions.

12. S1PR4 and R5 signaling and functions

Compared to other three S1PR subtypes, S1PR4 and R5 are less well studied. Human S1PR4 was previously known as EDG-6 and contains 384 amino acids. The expression of S1PR4 possesses a limited pattern. S1PR4 mRNA has been detected primarily in immune cells and tissues, including the thymus, spleen, bone marrow, appendix, peripheral leukocytes and lung (Graler et al., 1998).

Currently, it is believed that S1PR4 couples to the heterotrimeric G protein, Gi/o. The function and characteristics of S1PR4 were initially examined in HEK 293 cells transfected with S1PR4. The receptor demonstrates a high affinity for S1P with Kd = 63 nM, and S1P activates ERK through S1PR4 in a PTX-sensitive manner (Van Brocklyn et al., 2000). Also in K562 cells expressing S1PR4, S1P causes the elevation of Ca^{2+} concentration with a significant accumulation of IP_3, indicating the activation of PLC. This activity is markedly suppressed by PTX treatment, suggesting the Gi/o coupling of S1PR4 (Yamazaki et al., 2000).

Functionally, S1P evoked chemotaxis of mouse CD4 and CD8 T cells through S1PR4 and S1PR1 (Graeler and Goetzl, 2002). Moreover, S1P significantly induced CD4 T cell migration via S1PR4 and S1PR1 through the activation of Rho family small GTPases, Cdc42 and Rac, and this response was completed inhibited by PTX, suggesting S1PR4, as well as S1PR1, couples exclusively with Gi/o-type G protein (Matsuyuki et al., 2006). S1PR4 is specifically upregulated

during the development of human megakaryocytes (large bone marrow cells) from progenitor cells, and megakaryocytes from S1PR4-null mice show atypical and reduced formation of proplatelets (a portion of a megakaryocyte that is broken off to form a platelet) in vitro, indicating S1PR4 plays an important role in shaping the terminal differentiation of megakaryocytes (Golfier et al., 2010).

S1PR5 is the most recent member of the S1PR family to be cloned and characterized. It is also known as EDG-8, and like other members of the family, it has a high affinity for S1P with a Kd of 2 nM. In situ hybridization reveals that S1PR5 mRNA is expressed in the spleen and throughout the adult rat brain (Im et al., 2000). Further immunohistochemical experiments show that S1PR5 mRNA is detected in a subset of glial cells throughout the rat brain and spinal cord, preferentially in the oligodendrocyte lineage cells of the CNS, but not in the dorsal root ganglion (DRG) (Terai et al., 2003). However, a recent study utilizing single-cell quantitative PCR demonstrates that the mRNAs of all S1PR subtypes are expressed in cultured rat DRG neurons (Kays et al., 2012).

In rat hepatoma Rh7777 cells, the forskolin-induced rise in cAMP is reduced by 90% when cells are transfected with S1PR5 and treated with S1P. This response is fully blocked by pretreatment of PTX, thus indicating the signaling through Gi/o proteins (Im et al., 2000). GTPγS binding assay in CHO cells expressing S1PR5 demonstrates that S1PR5 activates Gi/o and G12, but not Gs or Gq/11 in response to S1P (Malek et al., 2001).

Functionally, S1PR5 is important for the homeostasis of patrolling monocytes. S1PR5-null mice lack peripheral Ly6C(-) monocytes but have these cells in the bone marrow, indicating the important role of S1PR5 in the egress of monocytes from the bone marrow, and possibly in the chemotaxis to S1P. Unexpectedly, the disruption of S1P gradients in vivo did not alter Ly6C(-) monocytes trafficking and viability, suggesting the regulation of monocytes trafficking is independent of S1P gradients (Debien et al., 2013). More work is needed to better understand the signaling cascades and functions of S1PR4 and R5. A summary of the known characteristics of S1PRs is shown in Table 1.

Receptor isoforms	Expression	G protein coupling	Signaling pathways
S1PR1	Widely distributed	Gi/o	AC inhibition; Rac activation; MAPK; PLC
S1PR2	Widely distributed	Gi/o, Gq, G12/13	Rho activation; Rac inhibition; MAPK; PLC
S1PR3	Widely distributed	Gi/o, Gq, G12/13	PLC; Rho activation; AC inhibition
S1PR4	Lymphoid tissues, lung	Gi/o	AC inhibition; MAPK; PLC
S1PR5	Nervous system	Gi, G12/13	AC inhibition

Table 1. Characteristics of S1P receptors.

AC, adenylyl cyclase; MAPK, ERK or mitogen-activated protein kinase; PLC, phospholipase C.

13. GPCR oligomerization

GPCRs play a key role in the signal transduction, not only within the organism but also between organisms. They are essential in communicating neural, endocrine and paracrine signals, as well as mediating senses, like vision, smell and taste. Specialized and fine-tuned communications require GPCRs to have an extended structural diversity. In fact, GPCRs constitute the largest and the most diverse superfamily of receptors in eukaryotic cells. GPCRs are classified into five families based on their heptahelical domain (HD) sequence: glutamate, rhodopsin, adhesion, frizzled/taste2 and secretin. They form the GRAFS classification system, and human GPCRs in the GRAFS families share a common ancestor (Fredriksson et al., 2003).

Generally speaking, specialized GPCR-mediated communication can be achieved in three ways: the specific interactions between the ligand and the receptor (ligand selectivity), the receptors (dimerization, oligomerization or transactivation), and the receptor and the intracellular components of the signaling cascade (functional selectivity). It is necessary to point out that the downstream components of GPCRs are not only limited to the heterotrimeric G proteins they couple with, but also other downstream intracellular effectors such as receptor-activity-modifying proteins (RAMPs), arrestins, rho-GTPases, scaffold proteins and so forth (Lagerstrom and Schioth, 2008). All effectors and second messengers form a complex network to diversify GPCR signaling.

Originally, all mammalian GPCRs known at the time were categorized as classes A, B or C (Kolakowski, 1994), which roughly corresponds to rhodopsin, secretin and glutamate families in GRAFS. Some GPCRs dimerize, or even oligomerize, and evidence shows that the functional role of receptor self-association is related to the modulation of ligand affinity, intrinsic efficacy and functional selectivity (Ferre et al., 2014). Currently, the ability of class A GPCRs to dimerize and the functional significance of class A GPCR dimers are still in debate, with opinions ranging from “monomeric class A GPCR as a functional unit” (Chabre and le Maire, 2005) to “class A receptor dimers and/or higher-order oligomers” (Fotiadis et al., 2006). In contrast, there is no doubt that class C GPCR oligomers exist and function as stable homodimers (Kniazeff et al., 2011), heterodimers (Doumazane et al., 2011), or heteromers (Comps-Agrar et al., 2012).

All S1PR subtypes belong to the class A, rhodopsin-type GPCR superfamily (HUGO Gene Nomenclature Committee). Several observations support the idea that class A GPCR dimers are not required for G protein activation. Studies have demonstrated that some members in the class A family, like the β_2-adrenergic (β_2AR), rhodopsin and μ-opioid receptors, function effectively as monomers (Bayburt et al., 2007; Kuszak et al., 2009; Whorton et al., 2007; Whorton et al., 2008). Also, a 1:1 stoichiometry of rhodopsin and G protein binding was observed, and one molecule of GDP was released upon 1:1 complex formation between activated rhodopsin and G protein (Bayburt et al., 2011; Ernst et al., 2007). However, these findings do not exclude that class A GPCR oligomers can

spontaneously form in living cells and be constitutively functional in transducing signaling.

One group using fluorescence resonance energy transfer (FRET) to characterize the oligomerization of purified β_2AR found that β_2AR was predominately tetrameric following reconstitution into phospholipid vesicles (Fung et al., 2009). In isolated rod outer segment membrane preparation, the rhodopsin-arrestin binding stoichiometry was increased from 1:1 to 2:1, dependent on the percentage of activated receptors (Sommer et al., 2012). Furthermore, there is some indirect biochemical data supporting heteromerization of class A GPCRs, including adenosine A_{2A} and A_1 receptor heteromers (Orru et al., 2011), and heteromultimers of homomers, as suggested for adenosine A_{2A}-dopamine D_2-cannabinoid CB_1 receptor heteromers (Navarro et al., 2010).

14. Desensitization of GPCRs

Besides the direct activation of S1PR1 signaling pathways by S1P to regulate cellular functions, the desensitization of S1PR1 upon activation also indirectly mediates certain cellular responses, especially the anti-inflammatory response. S1PR1 is a GPCR, and the activation of a GPCR by its ligand causes conformational changes in the receptor that allows it to couple to the heterotrimeric G proteins. This coupling stimulates G proteins to initiate various downstream signaling pathways through a series of effector molecules (Neer, 1995). At the same time, upon the binding of a ligand, the activation of a GPCR also initiates a process of receptor desensitization and internalization, disrupting the ongoing signaling and preventing the potentially harmful effects from persistent receptor stimulation (Kohout and Lefkowitz, 2003).

Almost every single GPCR that has been studied undergoes desensitization. The process of GPCR desensitization is defined as the physical uncoupling of the G protein from the cognate receptor, and this takes place through a universal mechanism which involves two families of proteins: the G protein-coupled receptor serine/threonine kinases (GRKs) and the arrestins (Ferguson et al., 1996; Freedman et al., 1997). GRKs phosphorylate the intracellular loops and the carboxyl terminus of activated receptors, resulting in the recruitment and the subsequent high-affinity binding of arrestins. This binding of arrestins to the activated GPCRs prevents further coupling of the receptors to G proteins. This

obstruction of coupling leads to as much as 80% reduction of receptor signaling (Lohse et al., 1992).

In humans, there are seven GRKs that are classified into three subfamilies based on the similarity in gene sequence and structure. GRK1 comprises GRK1 (rhodopsin kinase) and GRK7 (cone kinase), GRK2 comprises GRK2 and GRK3, and GRK4 comprises GRK4, GRK5 and GRK6 (Premont et al., 1999). The arrestin family includes four members; arrestin-1 and arrestin-4 are restricted to the retina, while arrestin-2 (β-arrestin-1) and arrestin-3 (β-arrestin-2) are ubiquitously expressed (Shenoy and Lefkowitz, 2003).

GRK-dependent recruitment of β-arrestins to the phosphorylated GPCRs leads to the attenuation of GPCR signal transduction. As mentioned above, this attenuation is achieved by promoting rapid receptor desensitization, the uncoupling of GPCRs from their intracellular G proteins. Furthermore, β-arrestins targeting GPCRs can interact with the components of the endocytic machinery such as clathrin, the major structural protein of coated vesicles, to initiate receptor endocytosis and the internalization process (Ferguson et al., 1996; Goodman et al., 1996). Simultaneously, β-arrestins, as scaffold proteins, interact with a continuously expanding ensemble of protein partners, like mitogen-activated protein kinase (MAPK) cascade components and non-receptor tyrosine kinases, to perform multiple functions including trafficking and signaling (Shenoy and Lefkowitz, 2011). This β-arrestin-dependent signaling has been shown to

modulate a wide range of cellular activities such as protein translation, chemotaxis and apoptosis.

Apart from being phosphorylated by GRKs, GPCRs can be phosphorylated by several other kinases to undergo desensitization as well. Theses kinases include protein kinase A and C (PKA and PKC) and s-Src (Benovic et al., 1985; Fan et al., 2001; Roth et al., 1991). This process of desensitization involves a feedback mechanism where the second messengers activated by GPCRs activate kinases to decrease receptor activity and ultimately to reduce the production of second messengers.

In addition, GPCRs are not the only targets of GRKs signaling. Evidence suggests that GRKs cause internalization of receptor tyrosine kinases (RTKs) and modulate multiple cellular responses independently of β-arrestins. In HEK 293 cells, epidermal growth factor (EGF) acts on the EGF receptor (EGFR) and activates a downstream signaling cascade that involves ERK. ERK activation translocates GRK2 from the cytoplasm to the plasma membrane and results in EGFR internalization (Gao et al., 2005).

15. The discovery and function of an S1P modulator, FTY720

The success of human organ transplantation is dependent on the use of potent immunosuppressive drugs. Cyclosporine (CsA), identified in 1972 as an antifungal peptide, is one of such drugs that have been widely used in transplant recipients to prevent organ rejection and in the treatment of autoimmune diseases (Colombo et al., 2014). In 1994, a novel compound named ISP-1 (also known as myriocin) was found to be 10- to 100-fold more potent than CsA as an immunosuppressant. ISP-1 was initially purified from the culture filtrates of the fungus *Isaria sinclairii*, and was discovered to suppress the generation of allo-reactive cytotoxic T lymphocytes in mice after intraperitoneal or oral administration (Fujita et al., 1994). However, further toxicology studies showed that ISP-1, which is a structural analog of sphingosine, produces severe digestive disorders, resulting in the death of experimental animals (Fujita et al., 1995; Osuchowski et al., 2004). To synthesize a less toxic and more specific compound, ISP-1 was chemically modified to 2 amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol hydrochloride. This newly synthesized compound was developed by Professor Tetsuro Fujita in collaboration with Yoshitomi Pharmaceuticals Industries; hence it was named FTY720.

Since its development, FTY720 has been shown to alter lymphocyte trafficking as a potent immunomodulatory agent. It produces lymphopenia in blood and thoracic duct lymph by preventing lymphocytes from entering the blood

circulation (Mandala et al., 2002). A later study found that FTY720 treatment downregulated S1PR1 in lymphocytes, causing the failure of lymphocyte egress from both thymus and peripheral lymphoid organs (Matloubian et al., 2004). The intrinsic affinity of FTY720 on S1PRs is very weak. However, FTY720 phosphate has high affinity on all S1PRs except S1PR2 (Table 2). Another study that corroborated the initial finding of the immunosuppressive function of FTY720 showed that FTY720 was phosphorylated by SphKs in the mouse blood and the phosphorylated compound was a potent agonist on S1PR1, R3, R4 and R5, and that FTY720 elicited lymphopenia due to the redistribution of lymphocytes from the circulation to the secondary lymphoid tissues (Brinkmann et al., 2002).

Further study showed that SphK2 is much more effective than SphK1 in phosphorylating FTY720 (Paugh et al., 2003), and that administration of FTY720 in SphK2-deficient mice does not induce lymphopenia (Zemann et al., 2006), suggesting SphK2 is essential in the FTY720 phosphorylation. Also, studies have demonstrated that the S1P transporter SPNS2 is capable of exporting FTY720 phosphate (Anada et al., 2007; Hisano et al., 2011).

	$S1P_1$	**$S1P_2$**	**$S1P_3$**	**$S1P_4$**	**$S1P_5$**
S1P	0.47 ± 0.34	0.31 ± 0.02	0.17 ± 0.05	95 ± 25	0.61 ± 0.39
FTY720-P	0.21 ± 0.17	>10,000	5.0 ± 2.7	5.9 ± 2.3	0.59 ± 0.27
FTY720	300 ± 51	>10,000	>10,000	>5000	2623 ± 317

Table 2. Binding affinities (nM) to S1P receptors (Mandala et al., 2002). IC_{50} measurements determined by competition of $S1^{33}P$ binding to membranes prepared from stably transfected CHO cells expressing the indicated S1P receptor. FTY720-P, FTY720 phosphate.

16. S1PR agonists and antagonists

Following the cloning of the S1PRs, the development of S1P compounds to activate or antagonize S1PRs began. These compounds, either agonists or antagonists, provide useful and effective tools to pharmacologically elucidate the functions of S1PR subtypes and the signaling mechanisms of S1PRs that mediate their functions. At the same time, the in vivo investigation of these compounds to study their efficacy and specificity in regulating a specific physiological function may provide promising results and key evidence to further develop a compound into a new therapeutic agent.

High throughput screening of commercial chemical libraries identified SEW2871 as a S1PR1-selective agonist (Jo et al., 2005). Later, using computational modeling, CYM-5442 was developed as a full S1PR1 selective agonist that caused S1PR1 internalization, phosphorylation and ubiquitination in vitro, and induced S1PR1-dependent blood lymphopenia in vivo (Gonzalez-Cabrera et al., 2008). W146 was developed as a competitive antagonist at S1PR1. S1PR1 agonism prevents capillary leakage and induces lymphopenia, both of which were reversed upon S1PR1 antagonism by W146 (Sanna et al., 2006). JTE-013, a pyrazopyridine derivative, was identified as a specific S1PR2 antagonist, and it blocked the S1P-induced inhibition of cell migration via S1PR2 (Osada et al., 2002). A prodrug approach to optimize the oral exposure of a series of S1PR1 antagonists for chronic efficacy studies led to the discovery of NIBR-15, an

S1PR1 specific antagonist shown to induce sustained peripheral blood lymphocyte reduction in rats when orally administered (Angst et al., 2012). Using a three-dimensional database search, CAY10444, was found to act as a potent S1PR3 antagonist (Koide et al., 2002), and a further study confirmed that CAY10444 blocked the promigratory effect of S1P through S1PR3 in breast cancer cells (Long et al., 2010). The modification of the FTY720 phosphate structure led to the development of VPC23019 as a selective S1PR1 and R3 antagonist, with full agonistic activity at S1PR4 and partial agonistic activity at S1PR5. The blockade of the migration of T24 cells expressing human S1PR1 obtained with the S1PR1 agonist VPC22277 was observed with 10 nM VPC23019. Cell binding assay showed that the antagonistic effect of VPC23091 at S1PR3 was about 10-fold less potent than at S1PR1 (Davis et al., 2005). VPC44116, an octyl analogue of W146 and γ-aminophosphonate analogue of VPC23019, is also a potent antagonist at S1PR1 and R3, an agonist at S1PR4 and a partial agonist at S1PR5. VPC23019 and VPC44116 are nearly indistinguishable in their affinity for the S1PR1 and R3 (Ki ~30 and 300 nM, respectively) (Foss et al., 2007; Im, 2010). Table 3 summarizes the S1PR agonists and antagonists already discussed above and used in my thesis research.

Drugs	Targets (Agonist)	Targets (Antagonist)	References
SEW2871	S1PR1		(Jo et al., 2005)
CYM-5442	S1PR1		(Gonzalez-Cabrera et al., 2008)
FTY720	S1PR1, R3, R4, R5		(Brinkmann et al., 2002; Mandala et al., 2002)
W146		S1PR1	(Sanna et al., 2006)
NIBR-15		S1PR1	(Angst et al., 2012)
JTE-013		S1PR2	(Osada et al., 2002)
CAY10444		S1PR3	(Koide et al., 2002)
VPC23019	S1PR4, R5 (partial)	S1PR1, R3	(Davis et al., 2005)
VPC44116	S1PR4, R5 (partial)	S1PR1, R3	(Foss et al., 2007)

Table 3. S1PR agonists and antagonists.

17. Small-diameter capsaicin-sensitive sensory neurons

In my research, I use cultured sensory neurons from the rat DRGs as the model to study S1PR function in mediating neuronal excitability. DRG neurons are pseudo-unipolar neurons that have bifurcated axon with peripheral and central branches. The axons of DRG neurons are known as afferent fibers. The peripheral branch of the axon has free nerve endings in the skin, muscles, and joints. The central branch of the axon projects to the secondary neurons in the dorsal horn of the spinal cord. The afferent fibers detect and conduct signals from chemical, physical or mechanical stimulations. Unlike the majority of neurons in the CNS, an action potential initiated in sensory neurons in the peripheray may bypass the cell body and continute to propagate along the central axon to reach the spinal cord.

Some sensory neuron axons are covered with myelin, a electrically insulating material that surrounds the axonal fibers and forms a layer, the myelin sheath. The myelin sheath is provided by the outgrowth of a type of glial cells, called Schwann cells. The production of the myelin sheath is called myelination. Myelination is essential for proper functions of the nervous system, because it prevents electrical currents from leaving the axon and enables fast electrical signal transmission.

DRG neurons are highly heterogeneous, in terms of the soma sizes, the types of afferent fibers, the axonal conduction velocities (CVs) and myelination, the cellular receptor expression profiles, the neurotransmiters and so forth. However, there is a positive correlation between the DRG neuron axonal CV, the afferent fiber type and the neuronal soma size. The fast-conducing, heavily myelinated Aα/β fibers have a CV >14 m/s with large-diameter soma (>40 μm), the lightly myelinated Aδ fibers have a CV between 2.2 and 8 m/s with medium-diameter soma (25-40 μm), and the non-myelinated C fibers have a CV <1.4 m/s with small-diameter soma (<25 μm) (Harper and Lawson, 1985a; Harper and Lawson, 1985b).

The physiological relevence of my thesis is to investigate the molecular mechanisms of increased nociception in sensory neurons, which directly associates with pain. Identifying moleular targets, either cell membrane receptors or intracellular effectors, that mediate neuronal excitability at the cellular level can provide insights into the mechanisms of nociceptive behavior at the systemic level and finally into the novel therapeautic targets with better effectiveness and less side effects to alleviate pain. Nociception is the detection and transduction of noxicious stimuli from the external enviroment to the brain. In the peripheral nervous system (PNS), a nociceptive stimulus is transduced through nociceptors (noxious stimulus detectors), which, therefore, are the primary interest of my research.

Nociceptors are DRG neurons which have the properties of longer AP duration and slower maximum rate of fiber firing, as well as greater expression of substance P and calcitonin gene-related peptide (CGRP) immunoreactivity (Lawson, 2002). These properties are most common in DRG neurons with C fibers, less common in those with Aδ and Aα/β fibers. Thus, all the sensory neurons I studied were small-diameter neurons with C fibers (soma diameter <25 μm), which are predomintly nociceptors.

Besides using the selection criterion based on their soma diameter, I also used the expression of a vanilloid receptor subtype 1, TRPV1, as a marker to identify nociceptive neurons. TRPV1 is a nonselective cation channel with high Ca^{2+} permeability and sensitivity to noxious heat. It is activated by capsaicin and other vanilloids. DRG neurons expressing TRPV1 and exposed to capsaicin exhibit inward currents (Helliwell et al., 1998). With high doses or prolonged exposure to capsaicin, neurons are functionally desensitized and exhibiting long-lasting loss of responsiveness to capsaicin (Michael and Priestley, 1999). Studies have indicated that most capsaicin-sensitive neurons are small-diameter sensory neurons. However, there are some small-diameter neurons not expressing TRPV1 and some TRPV1-expressing neurons are medium- and large-diameter neurons (Helliwell et al., 1998; Michael and Priestley, 1999). Also, not all nociceptive neurons are capsaicin-sensitive. That being said, for all my patch-clamp experiments to measure electrophysiological properties of sensory neurons, the diameter of neurons was measured before the experiment and the

neuronal response to capsaicin was measured at the end of each experiment. Only small-diameter, capsaicin-sensitive neurons were included in the analysis and interpretation of my study, and they were supposed to be almost exclusively nociceptors.

18. Specific aims

S1P is a bioactive sphingolipid that plays a key role in regulating neuronal excitability through S1PRs. S1P increases neuronal excitability primarily, but not exclusively, through S1PR1. This raises the question as to which other S1PRs mediate the enhanced excitability. The overall goal of my proposed research is to examine the S1PR subtypes in small-diameter sensory neurons and their contributions to the enhanced neuronal excitability caused by S1P. To accomplish this goal, three specific aims are proposed:

Specific Aim 1: To investigate the expression pattern of S1PR subtypes in small-diameter sensory neurons.

Specific Aim 2: To determine the contributions of other S1PRs, besides S1PR1, in enhancing excitability of small-diameter sensory neurons.

Specific Aim 3: To establish the signaling pathways of different S1PRs which contribute to the enhanced excitability of sensory neurons caused by S1P.

METHODS

1. Sensory neuronal culture

Sensory neurons were harvested from young adult rats (80-150 g) (Harlan, Indianapolis, IN). Specifically, male Sprague Dawley rats were placed and killed in a chamber filled with CO_2. Dorsal root ganglia (DRGs) were isolated and collected in a conical tube with sterilized Puck's solution. The tube was centrifuged for 1 min at 4,000 rpm and the pellet was resuspended in 1 ml Puck's solution containing 10 U of papain (Worthington, Lakewood, NJ). After 15-min incubation at 37°C, the tube was centrifuged for 1 min and the supernatant was replaced by 1 ml F-12 medium containing 1 mg collagenase IA (Sigma, St. Louis, MO) and 2.5 mg dispase II (Roche Diagnostics, Indianapolis, IN). The DRGs were resuspended and incubated at 37°C for 20 min. The suspension was centrifuged for 1 min and the supernatant was removed. The pellet was resuspended in F-12 medium supplemented with 30 ng/ml nerve growth factor (NGF) (Harlan, Indianapolis, IN) and mechanically dissociated with fire-polished glass pipette until all visible chunks disappeared. Isolated cells were placed onto plastic coverslips previously coated with 100 μg/ml poly-D-lysine (Sigma, St. Louis, MO) and 5 μg/ml laminin (Sigma, St. Louis, MO). Cells were maintained in culture at 37°C and 3% CO_2 for 18-24 hr before electrophysiological recording.

2. siRNA treatment

The gene sequences of S1PR2 and S1PR3 were obtained from NCBI with the accession numbers NM_017192 and XM_225216 respectively. siRNAs targeted to S1PR2 and S1PR3 were designed by the online tool provided by the Whitehead Institute (http://sirna.wi.mit.edu) (Yuan et al., 2004) and synthesized by Thermo Scientific. Both siRNAs were labeled with the fluorescent tag, fluorescein, with 3'-end modification. For S1PR2 siRNA, the sense strand was 5'-CCUUCUGGUGCUAAUCGCAUU-3', and the antisense strand was 3'-UUGGAAGACCACGAUUAGCGU-5'. For S1PR3 siRNA, the sense strand was 5'-CAUUCUGAUGUCCGGUAGGUU-3', and the antisense strand was 3'-UUGUAAGACUACAGGCCAUCC-5'. The S1PR1 siRNA was based on the sequence designed by Chi and Nicol (2010) and labeled with fluorescent tag DY547. A universal Silencer Negative Control #1 siRNA (Ambion) is used as the negative control.

DRG neurons were isolated and cultured in F-12 medium with 30 ng/ml NGF at 37°C for 24 hr. F-12 was replaced with Opti-MEM medium (Life Technologies) and the neurons were incubated at 37°C for about 5 hr for lipid transfection. Transfection reagent Metafectene (Biontex) and the siRNA complex (40 μM) were prepared in 2 ml Opti-MEM. For sensory neurons transfected with one individual siRNA, the final siRNA concentration was 200 nM; for those transfected with a pool of two or three different siRNAs, the final concentration for

each siRNA is 100 nM. Neurons were exposed to either siRNA(s), negative control or Metafectene alone, and maintained at 37°C for 48 hr. After that, Metafectene and siRNA were washed out with F-12, and neurons were supplied with F-12 medium with 30 ng/ml NGF. Neurons were incubated for another 48 hr before real-time quantitative PCR or patch-clamp experiments were performed.

3. Real-time quantitative PCR

Sensory neurons that underwent siRNA treatment were collected for real-time quantitative PCR (qPCR) experiments. F-12 medium was aspirated from the cell-culture dish, and neurons were washed with 2 ml PBS solution. Total RNA from neurons was extracted by using RNeasy Plus Mini Kit (Qiagen), following manufacturer's instruction. The concentration of total RNAs from different treatments was measured by NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). To eliminate genomic DNA contamination, 500 ng RNA was treated with 1 µl DNase I (Invitrogen) in a 10-µl reaction at room temperature for 15 min. The reaction was terminated by adding 1 µl 25 mM EDTA and the reaction mixture was incubated at 65°C for 10 min. To generate cDNA from RNA, RNA template was mixed with 1 µl iScript reverse transcriptase (Bio-Rad) in a 20-µl reaction. The reaction protocol is as follows: 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min.

qPCR was performed in a 10-μl reaction mix, containing 20 ng cDNA template, 500 nM forward/reverse primers and 5 μl Power SYBR Green PCR Master Mix (Applied Biosystems). Positive control template was a 15-fold dilution of a pooled rat lung cDNA. Negative control template was nuclease-free water. Reactions were run in triplicates on a 7500 Fast Real-Time PCR System (Applied Biosystems). The thermal-cycling condition is 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The quantification cycle (Cq) values of various genes of interest were obtained at the threshold where ΔRn = 0.3. The relative expression of different genes was calculated by the Pfaffl method (Pfaffl, 2001), using ribosomal protein large P0 (Arbp) as the reference gene.

4. Patch-clamp recording

Recordings were made using the whole cell patch-clamp technique. A coverslip with sensory neurons was placed in the center of a recording chamber filled with normal Ringer's solution, with the composition of (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose, with pH adjusted to 7.4 using NaOH. Recording pipettes were pulled from borosilicate glass tubing and filled with the following solution (in mM): 140 KCl, 5 $MgCl_2$, 0.25 $CaCl_2$, 0.5 EGTA, 10 HEPES, 4 ATP and 0.3 GTP, with pH adjusted to 7.2 using KOH. The free Ca^{2+} concentration in the pipette solution is about 150 nM, which mimics the resting intracellular Ca^{2+} level in small-diameter DRG neurons. Whole cell voltages were

recorded with an Axopatch 200 or Axopatch 200B amplifier (Molecular Devices). Data were acquired and analyzed with pCLAMP 10 (Molecular Devices). All drugs were applied with a VC-8 bath perfusion system. Neurons with a resting membrane potential between -45 and -65 mV were selected and ramps of depolarizing currents (1 sec duration) were injected to evoke 2-4 action potentials (APs) under control conditions. The same ramp protocol was used throughout the recoding period. At the end of each recording, the neuron was exposed to 1 μM capsaicin. Capsaicin sensitivity is an indication of nociceptive neurons (see Introduction). All the results in this paper were from capsaicin-sensitive neurons unless otherwise stated. All experiments were performed at room temperature.

5. Single cell isolation

A coverslip with cultured rat sensory neurons was placed in a recording chamber filled with normal Ringer's solution. The chamber was washed with Ringer's solution several times to eliminate debris. The diameter of each individual neuron was measured using a calibrated eye-piece micrometer. Each single neuron was collected either directly from the coverslip or after whole-cell patch-clamp recording.

To collect single neurons directly from coverslips for single-cell real-time qPCR study, recording pipettes were pulled from borosilicate glass tubing. The glass tubes were previously baked at 250 °C for 2 hr to eliminate possible RNase

contamination. The tip of the pipette (15-25 μm diameter) was dipped into diethylpyrocarbonate (DEPC)-treated water to draw up about 1 μl water at the tip. The pipette was positioned against a cell's membrane and negative pressure was applied. The cell was sucked into the pipette and then transferred into a 0.2 ml PCR tube prefilled with 5 μl RNase-free water. Final volume of cell lysate generally ranged from 5 to 8 μl. Cell lysates were immediately placed on ice and then processed to generate cDNA.

To collect single neurons after patch-clamp recording for conventional PCR study, the process was the same as mentioned above, with one exception that, after recording, the recording pipette was still attached to the neuron to keep the cell intact. Another pipette for collecting was brought close to the same neuron and then negative pressure was applied to suck the neuron into the glass pipette. Each neuron was labeled accordingly in order to match their PCR profiles with electrophysiology properties.

6. cDNA from single cells and preamplification of single cell cDNA

The total volume of each single cell lysate was estimated and RNase-free water was added into each sample up to a final volume of 8.5 μl. To eliminate genomic DNA contamination, each cell lysate was treated with 1 μl DNase I and 0.5 μl DNase 10X buffer (Invitrogen) in a 10-μl reaction at room temperature for 15 min. The reaction was terminated by adding 1 μl 25 mM EDTA and the reaction

mixture was incubated at 65°C for 10 min. To generate cDNA, each sample was mixed with 0.5 µl iScript reverse transcriptase (iScript cDNA Synthesis Kit, Bio-Rad) in a 15-µl reaction. The reaction protocol is as follows: 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min.

A pooled assay mix was prepared by adding 20X TaqMan Gene Expression Assay for each gene of interest (GOI, Table 4) to Tris-EDTA buffer (0.5X final concentration for each GOI). All Gene Expression Assays were labeled with the reporter dye FAM, except for HPRT, which was labeled with VIC. For each single cell preamplification reaction, 2 µl pooled assay mix was mixed with 7.5 µl cDNA template, plus 10 µl 2X Pre-Amp Master Mix (Applied Biosystems) and 0.5 µl nuclease-free water, to a final volume of 20 µl. The reaction condition was 95°C for 10 min followed by 14 cycles of 95°C for 15 sec and 60°C for 4 min. All the samples were stored at -20°C.

7. Single-cell quantitative PCR

Quantitative TaqMan PCR was performed in a 10-µl reaction mix, containing 2.5 µl diluted pre-amplified single cell cDNA template (1:12 dilution), 0.5 µl 20X TaqMan GOI Assay, 5 µl 2X Taqman Gene Expression Master Mix (Applied Biosystems) and 2 µl nuclease-free water. Positive control template was a 15-fold dilution of a pooled rat lung cDNA. Reactions were run in triplicate on a 7500 Fast Real-Time PCR System (Applied Biosystems). The thermal-cycling

condition is 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The Cq values of various genes of interest were obtained at the threshold where the value of normalized fluorescence emission generated by FAM or VIC (ΔRn) reached 0.3. The expression of different genes was calculated based on the number of copies of each gene where Number of Copies = (Primer Efficiency)$^{-Cq}$. The relative expression of GOI was determined by dividing the average copy number of GOI by that of reference gene hypoxanthine phosphoribosyltransferase 1 (HPRT). Efficiencies of each primer pair were determined from the slope of a seven-point standard curve (Table 4).

GOI	Applied Biosystems Catalog No.	Primer Efficiency
S1PR1	Rn02758712_s1	1.97
S1PR2	Rn02130568_s1	1.96
S1PR3	Rn02758880_s1	1.95
S1PR4	Rn01408085_s1	1.90
S1PR5	Rn00572952_s1	1.97
TRPV1	Rn00583117_m1	2.02
Arbp	Rn00821065_g1	1.95
HPRT	Rn01527840_m1	1.95

Table 4. Genes of interest and their primer efficiencies for TaqMan Gene Expression Assay.

The primer efficiency was calculated from the averaged slope of three standard curve experiments (Kays et al., 2012).

8. Single-cell conventional PCR

cDNA from single isolated sensory neurons after recording was used as the template to detect the expression of S1PR2 and the house-keeping gene HPRT. Amplification of S1PR2 (Accession No. NM_017192.1) used the forward primer (180…198 bp) 5'- TGAGAAGGTTCAGGAACAC-3' and the reverse primer (632...614 bp) 5'-AGAATCAGCGATATCAGCC-3'. The size of the amplification product was 453 bp. Amplification of HPRT (Accession No. NM_012583.2) used the forward primer (495...514 bp) 5'-GCAGACTTTGCTTTCCTTGG-3' and the reverse primer (772...753 bp) 5'-TACTGGCCACATCAACAGGA-3' with a product size of 278 bp. Amplification was performed in a 50-µl reaction containing 5 µl cDNA template, 2 µl forward/reverse primers and 43 µl Platinum PCR Supermix (Invitrogen). The PCR reactions were run on a PTC-100 programmable thermal controller (MJ Research, Watertown, MA). For S1PR2 detection, the protocol was: 47 cycles were run at 94°C for 15 s, 47°C for 40 s, and 72°C for 1 min; and for HPRT, 45 cycles were run at 94°C for 15 s, 58°C for 30 s, and 72°C for 40 s. Templates from rat DRG and lung tissues were used as positive controls, and nuclease-free water was used as negative control template to rule out possible contamination. The PCR products were verified by electrophoresis (Bio-Rad) on a 1% agarose gel with appropriate DNA ladders.

9. Wound healing assay

B16 mouse melanoma cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen) in 75-cm^2 cell culture flasks. Cells were plated into the wells of a six-well culture dish that had been coated with 10 μg/ml fibronectin. Cells were maintained at 37°C and 5% CO_2, allowing them to adhere onto the substrate. When the cells reached 70–80% confluence, the cell monolayer was scraped in a straight line with a 200-μl pipette tip to create a “wound.” To remove the debris and smooth the edge of the wound, cells were washed twice with 1 ml of phosphate-buffered saline followed by 1 ml of migration medium, which contained serum-free F-12 medium (Invitrogen) and 0.1% fatty acid-free bovine serum albumin. There were four different treatment groups: the untreated control group with migration medium alone, cells exposed to 100 nM S1P, cells exposed to 1 μM JTE-013, and cells exposed to 100 nM S1P and 1 μM JTE-013. A mark on the plate was placed near the wound site and used as a reference point. Images were acquired with a 10X objective at both 0 and 48 hr after creation of the wound.

10. Data analysis

Data are presented as the means ± standard error of the mean (SEM). Statistical differences at the mRNA expression level between the control groups and those

treated with siRNAs were determined by either a Student's t-test or an analysis of variance (ANOVA). Statistical differences between the control recordings and those obtained under various treatment conditions were determined by either an ANOVA or a repeated measures (RM) ANOVA whenever appropriate. When a significant difference was obtained with an ANOVA, post hoc analyses were performed using a Holm-Sidak all pairwise test. If the data set failed the normality test, a Kruskal-Wallis one-way ANOVA on ranks was performed, followed by a Tukey or a Dunn's all pairwise test. The results were considered statistically significant when the P value was < 0.05 (SigmaStat 3.5 Software).

11. Chemicals

Tissue culture supplies were purchased from Thermo Fisher Scientific (Waltham, MA). S1P and VPC23019 were obtained from Avanti Polar Lipids (Alabaster, Al), and S1P was dissolved according to the manufacturer's instructions. JTE-013, prostaglandin E2 (PGE2), W140, W146, FTY720, sphingosine kinase inhibitor 2 (SKI-2), SEW2871, and CAY10444 were purchased from Cayman Chemical (Ann Arbor, MI). Pertussis toxin (PTX) was purchased from EMD Chemicals (San Diego, CA). PD98059 was obtained from EMD Millipore (Billerica, MA). CYM-5442 was purchased from Tocris Bioscience (Bristol, UK). VPC44116 is a generous gift from Dr. Kevin R. Lynch, University of Virginia. NIBR-15 is a generous gift from Dr. Daniela Salvemini, Saint Louis University School of Medicine. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

If the chemical does not easily dissolve in water, it was dissolved in 1-methyl-2-pyrrolidinone (MPL) first. The MPL stock solutions were then diluted with normal Ringer's solution to yield the appropriate concentrations. The vehicle MPL was typically used at 1,000- to 5,000-fold dilutions. The vehicle MPL has no effect on AP firing (Figure 12).

RESULTS

1. S1PR1 has the highest expression among the five S1PR subtypes in small-diameter sensory neurons

Before investigating the functional contribution of each individual S1PR to the S1P-induced increase in excitability in small diameter sensory neurons, one critical issue to examine is the existence and the expression profile of the different S1PR subtypes. Previously, our laboratory has demonstrated that all four receptor subtypes are expressed in DRGs (Zhang et al., 2006a). However, whole DRG tissue contains small-, medium- and large-diameter sensory neurons, as well as glial cells, resident immune cells, and satellite cells. To characterize the S1PR expression in small-diameter sensory neurons, which matches the functional study obtained by patch-clamp, we needed a technique that allowed us to analyze gene expression at a more defined and morphology-specific cell population. Single-cell qPCR is such a technique that enabled us to isolate single neurons based on their size to investigate S1PR expression patterns in small-diameter sensory neurons.

1.1. S1PR expression profiles in small-diameter sensory neurons

A total of 18 small-diameter sensory neurons (diameter <25 μm) cultured in vitro were individually isolated and underwent the single-cell qPCR procedure to

measure the expression of various genes. The mRNA expression of five S1PR subtypes (S1PR1-5) was detected, and the relative expression level of each gene was calculated using the reference gene HPRT in each individual neuron (Figure 5). The expression profiles of S1PRs in the 18 sensory neurons turned out to be quite variable. There were 5 neurons expressing all five receptors (e.g., a-2 and c-3), neuron b-3 expressed only S1PR1 and neuron e-1 expressed only S1PR2, whereas other neurons expressed two to four receptor subtypes. Our patch-clamp data shows that S1P enhances the excitability in roughly 90% small-diameter sensory neurons. The fact that not every neuron expresses all five S1PR subtypes indicates that there might be functional redundancy among S1PRs or certain S1PR subtype(s) are not necessary in mediating the S1P-induced increase in excitability. This raises an interesting question as to which S1PR subtypes are involved in the S1P-induced excitatory effect, as we have shown that S1PR1 plays a major role. It is of great interest if we can correlate function with expression. More specifically, this will require us in the future to measure the excitability of a neuron in response to S1P and then to measure the S1PR expression within the same neuron, to have a better idea of the S1PR subtypes in S1P-sensitive sensory neurons.

Besides looking at S1PR expression, we also examined the expression of the vanilloid receptor subtype 1, TRPV1, as a phenotypic marker for nociceptive neurons (Caterina et al., 2000; Helliwell et al., 1998; Michael and Priestley, 1999). We found that TRPV1 mRNA was expressed in 8 out of a total of 18 small-

diameter sensory neurons. This finding is consistent with a previous study using in situ hybridization which showed that TRPV1 mRNA expression was variable in small-diameter sensory neurons, with a portion of small-sized DRG cells having detectable TRPV1 (Michael and Priestley, 1999). Interestingly, this finding does not agree with our electrophysiological data where we saw that about 90% small-diameter sensory neurons were sensitive to capsaicin, indicating that the majority of those neurons had functional TRPV1 receptors. This discrepancy may result from different endpoints we were examining, mRNA or functional protein, as the amount of mRNA is not necessarily equal to that of functional protein in a single cell. Also, it can be due to the fact that some small-diameter sensory neurons indeed express TRPV1 mRNA but the mRNA level is below the limit of detection by qPCR.

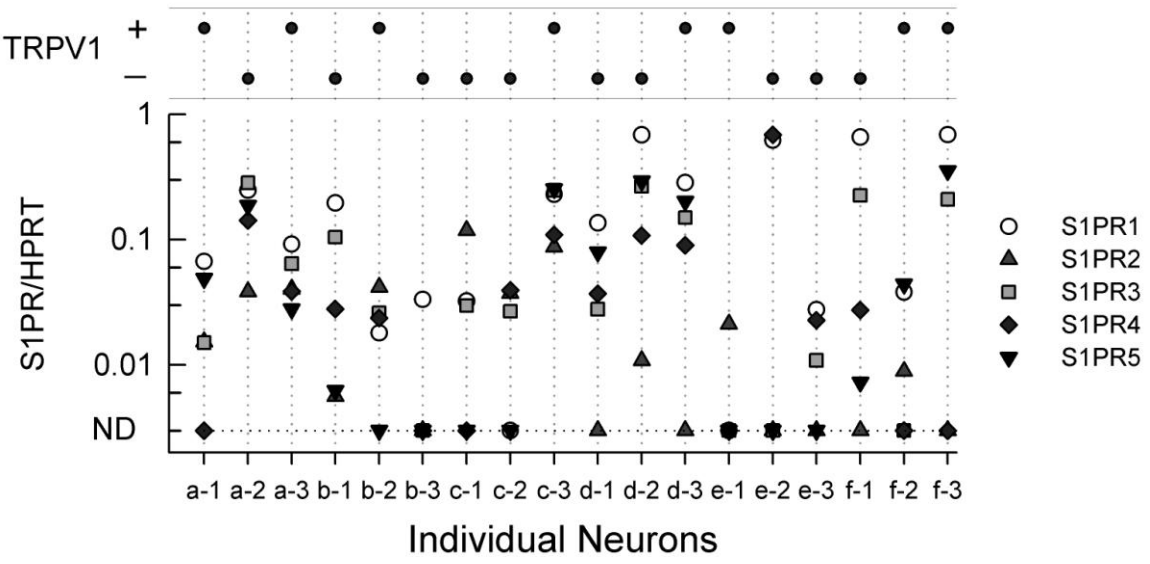

Figure 5. Expression profiles of S1PRs in 18 single isolated small-diameter sensory neurons.

Each column represents the profile of one single neuron. Upper panel indicates the expression of marker gene TRPV1, where + indicates detectable, - indicates not detectable. Lower panel shows the relative receptor expression normalized to reference gene HPRT. ND, not detected.

1.2. S1PR1 has the highest expression in small-diameter sensory neurons

Because the S1PR expression profile is variable in different individual neurons, it does not offer us much insight into the possible contributions of each S1PR subtype in the S1P-induced enhancement in excitability. To minimize the variation from cell to cell, we decided to look at the expression of different S1PRs collectively. As shown in Figure 6, the mRNA expression of each S1PR subtype across all 18 small-diameter neurons was added up and the mRNA level was calculated based on the expression of each S1PR relative to HPRT. On average, S1PR1 has the highest relative expression (0.224 ± 0.060), which is about 9 fold more than S1PR2 (0.024 ± 0.008) and 2 fold more than S1PR3, 4 and 5 (0.094 ± 0.025, 0.075 ± 0.037 and 0.083 ± 0.028 respectively). The expression of S1PR1 is significantly different from that of S1PR2, but there are no other differences between other receptor subtypes (Kruskal-Wallis one-way ANOVA on ranks followed by Tukey test, $P < 0.05$). The expression of S1PR1 is prominent among all five S1PR subtypes, and this finding is consistent with our previous finding that S1PR1 plays a predominant role in mediating S1P-induced increase in excitability. Meanwhile, this result hints that S1PR3, R4 and R5 may have a more important role in regulating excitability than S1PR2, pointing out a future direction for us to further study the functions S1PRs.

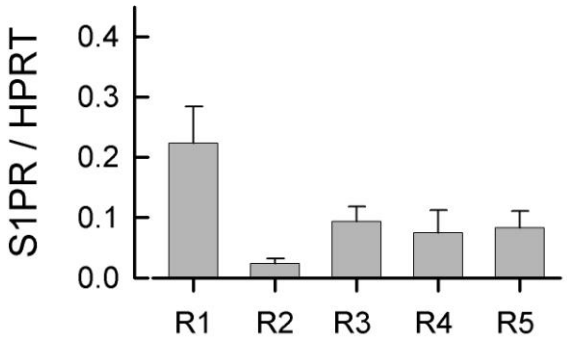

Figure 6. The S1PR expression relative to HPRT in 18 small-diameter sensory neurons.

Each individual receptor expression was normalized to reference gene HPRT. Data are presented as means ± SEM, n = 18.

1.3. S1PR1 is the most highly expressed subtype in about half of the sensory neurons

Aside from analyzing the total expression of each S1PR subtype across all 18 neurons, we analyzed the qPCR data from another perspective. Presumably, receptor expression at the mRNA level correlates with protein expression, and expressed proteins are functional in mediating the S1P-induced neuronal excitability. If one S1PR subtype has the highest mRNA expression in one single neuron, it indicates that that specific subtype has the highest level of proteins (receptor) and that receptor dominates the regulation of S1P-induce excitability enhancement.

Based on this theory, we investigated the highest expresser among five S1PR subtypes in each neuron. We found that 10 small-diameter sensory neurons had S1PR1 as the highest expresser (Figure 7), showing that S1PR1 expression dominated in about half of the neurons. This result is, again, consistent with our functional study suggesting S1PR1 is the predominant player in S1P-induced increase in excitability.

Looking further into those 10 neurons, we found that for the rest of the S1PR subtypes, S1PR3, R4 and R5 had the second highest expression, followed by R2 (Figure 7). The mRNA expression of S1PR1 and R3 is significantly different from S1PR2 (Kruskal-Wallis one-way ANOVA on ranks followed by Tukey test, $P <$

0.05). These data suggest that, functionally, S1PR3 and R5 may play an auxiliary role for S1PR1 in S1P-induced enhancement in excitability.

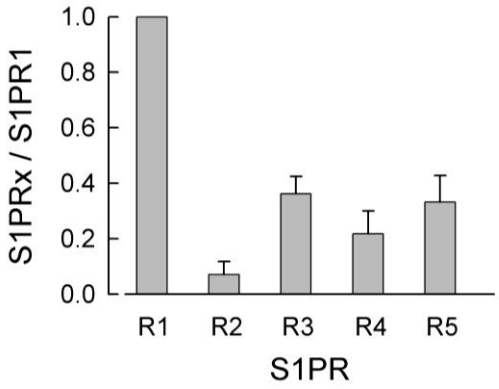

Figure 7. S1PR mRNA expression normalized to S1PR1 in 10 small-diameter sensory neurons where S1PR1 has the highest expression.

Data shows the relative expression of S1P receptor subtypes normalized to HPRT and then normalized to the highest expresser S1PR1 in 18 small-diameter sensory neurons.

1.4. Other receptor subtypes besides S1PR1 are the most highly expressed receptors in a small percentage of sensory neurons

In a total of 18 sensory neurons that underwent single cell qPCR, S1PR1 was the most highly expressed subtype in 10. In the other 8 neurons where S1PR1 was not the highest expresser, S1PR2 had the highest expression in 3 neurons, S1PR3 in 1 neuron, S1PR4 in 2 neurons and S1PR5 in 2 neurons (Figure 8). This result raises the question as to what is the role of the other subtypes (S1PR2-5), when mostly highly expressed, in regulating S1P-induced enhancement in excitability. This question cannot be easily addressed without linking the single neuron expression profile with its functional response to S1P. However, these data, together with our previous study indicating that S1PR1 is not the exclusive subtype in mediating S1P-induced increase in excitability (Chi and Nicol, 2010), emphasizes the importance of studying the functions of the other S1PRs, besides S1PR1, in terms of their contributions to the elevated neuronal excitability in response to S1P.

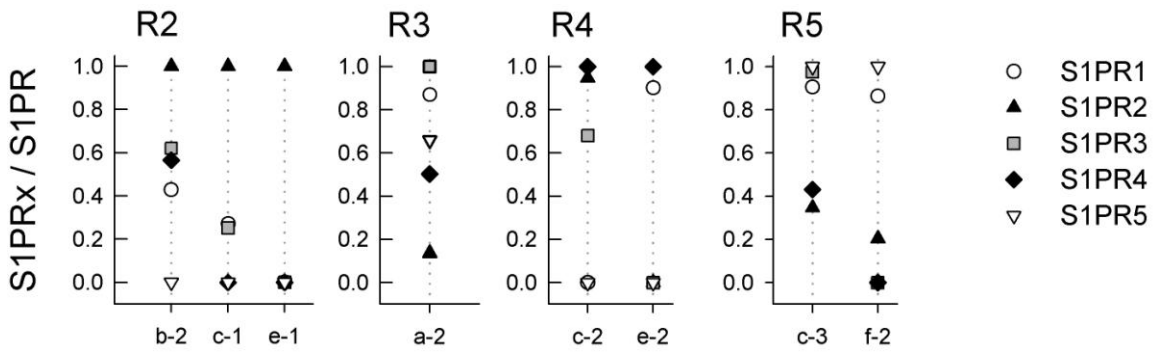

Figure 8. S1PR expression level normalized to the most highly expressed subtypes where S1PR1 was not the highest expresser.

Each column represents the relative expression of different S1PR subtypes in each single cell where S1PR was not the highest expresser. Data were normalized to HPRT first and then the highest expresser in the neuron.

2. Sensory neurons without S1PR1, R2 and R3 were insensitive to S1P

Single-cell qPCR data demonstrates that the S1PR expression profile of small-diameter sensory neurons vary from cell to cell. Therefore, at a functional level, the contribution of each S1PR subtype to the enhanced excitability may vary too. We have known S1PR1 plays a predominant but not exclusive role in S1P-induced increase in excitability. This leads me to ask the question, what functional roles do other S1PR(s) play in mediating the increased excitability in sensory neurons.

There have been several studies showing the importance of S1PR2 and R3 in the nervous system (see Introduction), while the biological functions of S1PR4 or R5 have not been well determined. Furthermore, there are selective agonists and antagonist for S1PR1, R2 and/or R3. However, the availability of pharmacological tools targeting S1PR4 and/or R5 is very limited. Therefore, I would like to first investigate the role of S1PR2 and R3 in S1P-induced enhancement of excitability.

2.1. siRNAs targeted to S1PR1, R2 and R3 significantly knock down corresponding S1PR expression

To examine the importance of S1PR2 and R3 in mediating sensory neuron excitability, I utilized siRNAs to knock down the mRNA expression of S1PR2 and

R3. In addition, I knocked down the mRNA expression of S1PR1 in order to eliminate its predominant effect. After the siRNA treatment, I measured the change in neuronal excitability in response to S1P. First of all, the effectiveness of siRNA knockdown needed to be verified. The verification can be achieved by using qPCR to measure mRNA expression, or by using Western blot to measure protein expression. Western blot experiments yielded unsatisfying results. The poor quality of the S1PR antibodies caused multiple nonspecific bands on the blot and complicated the interpretation of results. I then switched to qPCR method as an indirect measurement for the receptor knockdown.

Sensory neurons underwent a series of different treatments: a pool of three individual siRNAs targeted to S1PR1, R2 and R3 respectively, siRNA targeted to S1PR2, siRNA targeted to S1PR3, negative control siRNA, and Metafectene as a transfection reagent vehicle control. Quantitative real-time PCR demonstrated that a combination of S1PR1/R2/R3 siRNA treatment significantly reduced the mRNAs for S1PR1/R2/R3 by about 65, 75, and 75%, respectively, compared to the untreated neurons (Figure 9A, 9B and 9C), whereas the mRNA levels of S1PR4 and R5 were unaffected (Figure 9D and 9E). S1PR2 or R3 siRNA alone significantly reduced the mRNA expression for S1PR2 or R3 by about 70% (Figure 9B and 9C), but did not significantly affect the mRNA expression of the other S1PR subtypes (Figure 9). Besides, neither negative control siRNA nor vehicle control significantly reduced the mRNA level of any S1PR subtype compared to the untreated neurons (Figure 9). Therefore, siRNAs targeted to

S1PR1, R2 and R3 effectively knocked down their corresponding receptor mRNA expression, and they did not exhibit any off-target effect to reduce the mRNAs of S1PR4 and R5.

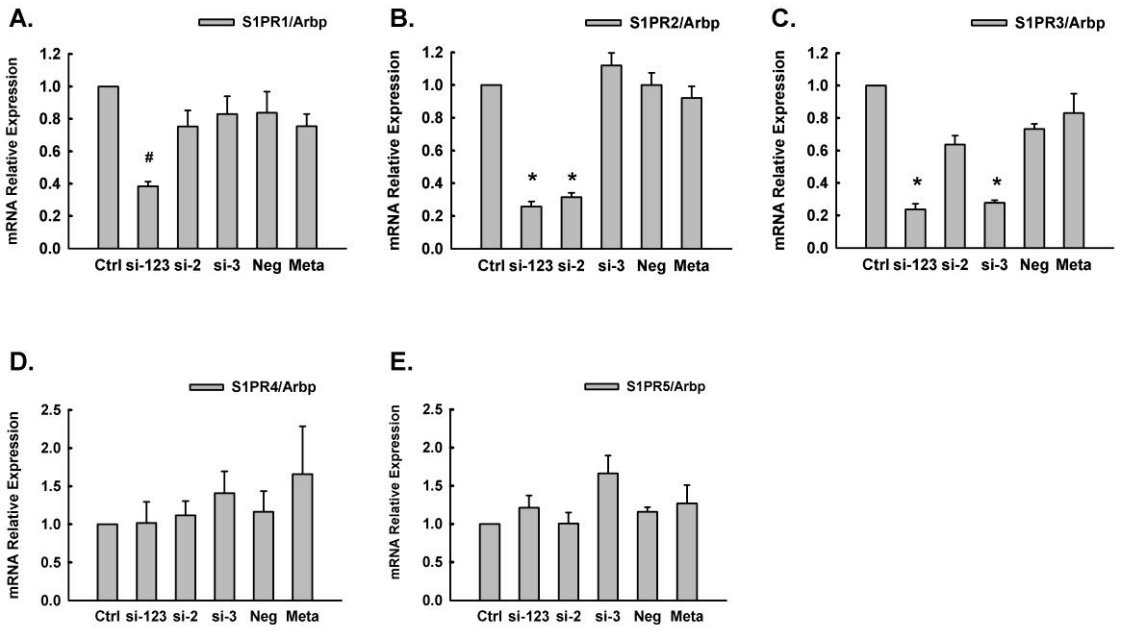

Figure 9. siRNAs targeted to S1PR1, R2 and R3 significantly knock down receptor expression.

Each individual figure represents the relative S1P receptor mRNA expression. S1PR subtype expression levels were normalized to reference gene Arbp. Then different treatment groups were normalized to the control. Data are presented as means ± SEM, n = 4. A: the combination of S1PR1/R2/R3 siRNAs significantly knocked down the expression of S1PR1 (# P =< 0.001 compared to control, t-test). B: the combination of S1PR1/R2/R3 siRNAs treatment and S1PR2 siRNA alone significantly reduced the expression of S1PR2 mRNA (* P < 0.05 compared to control, ANOVA on ranks followed by Tukey test). C: the combination of S1PR1/R2/R3 siRNAs treatment and S1PR3 siRNA alone significantly reduced the expression of S1PR3 mRNA (*P < 0.05 compared to control, ANOVA followed by Holm-Sidak method). D and E: the siRNAs targeted

to S1PR1, R2 and R3 did not significantly affect the mRNA level of either S1PR4 or R5 (ANOVA on ranks). Ctrl, control; si-123, a combination of siRNAs targeting S1PR1, R2 and R3 respectively, 100 nM each; si-2, 200 nM siRNA to S1PR2; si-3, 200 nM siRNA to S1PR3; Neg, 200 nM negative control siRNA; Meta, Metafectene alone as vehicle control.

2.2. Knocking down S1PR1/2/3 blocks the S1P-induced increase in excitability

After validating the siRNAs, sensory neurons were transfected with a pool of siRNAs targeted to S1PR1, R2 and R3 (100 nM for each siRNA). As a control, sensory neurons from the same preparation were transfected with universal negative control siRNA (300 nM). Sensory neurons in both groups underwent a 2-day siRNA treatment and an extra 2-day culture in normal medium. Following that, patch-clamp experiments were performed to determine the excitability of those sensory neurons. In the group treated with negative control siRNA, 1 μM S1P increased the number of elicited action potentials (AP) in small-diameter sensory neurons at 6 min compared to the control (0 min before S1P exposure), as shown in the representative traces (Figure 10A, top). Under control conditions before S1P treatment, the sensory neuron fired 4 APs, and 6 min after S1P exposure using the same ramp current stimulus, this neuron fired 10 APs. We define that if there is a larger than two fold increase in the number of elicited APs in sensory neurons compared to the control, these neurons are sensitized. Therefore, S1P sensitized the neuron. In contrast, in the group treated with pooled siRNAs targeted to S1PR1, R2 and R3, the number of APs generated by a sensory neuron went to 4 APs at 6 min after S1P treatment, compared to 3 APs in the control condition (Figure 10A, bottom), and the neuron was not sensitized. Averaged results from a total of 5 cells in each group demonstrate that 1 μM S1P significantly increased the number of APs in negative control

siRNA-treated neurons at 6 and 10 min compared to the control. On the other hand, 1 μM S1P failed to augment the AP firing in the pooled siRNA-treated neurons compared to the control (Figure 10B). The numbers of elicited APs before S1P exposure (the control) from both groups are not significantly different from each other (t-test).

These data demonstrate S1P is capable of increasing the excitability of small-diameter sensory neurons treated with negative control siRNA, consistent with our previous finding that the excitability of naive sensory neurons was significantly enhanced by S1P within 6 min (Zhang et al., 2006a). In contrast, a pooled siRNA treatment, which knocked down the expression of S1PR1, R2 and R3, blocked S1P from increasing the excitability in sensory neurons, suggesting S1PR1, R2 and/or R3 are essential in mediating the excitatory effect of S1P. Neurons that underwent the pooled siRNA treatment still likely had S1PR4 and/or R5 on their membrane, if they were expressed, and the fact that S1P failed to alter their excitability suggests that S1PR4 and R5 are not sufficient to regulate the excitability in response to S1P. However, it is too early to conclude that S1PR4 and R5 are not involved in the effect of S1P, because we cannot rule out the possible auxiliary or indirect role S1PR4 and R5 may play in mediating S1P-induced increase in excitability.

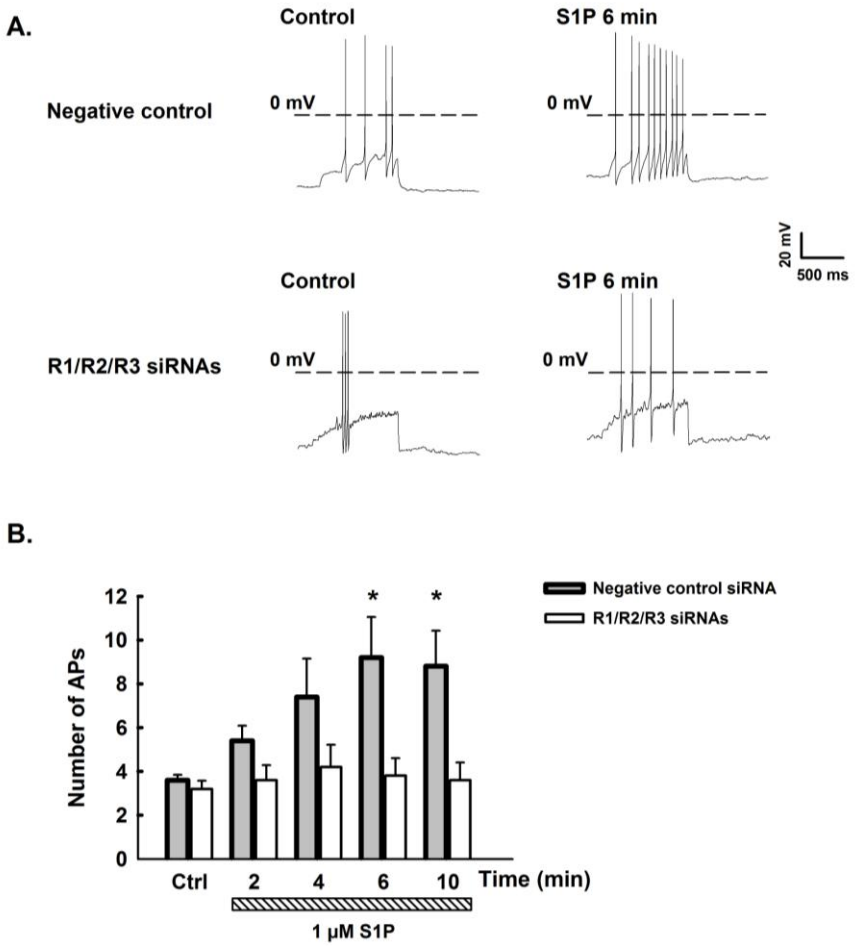

Figure 10. siRNAs targeted to S1PR1/R2/R3 blocked the S1P-induced increase in excitability in sensory neurons.

A shows representative traces from cells that underwent negative control siRNA treatment or a combination of S1PR1/R2/R3 siRNAs treatment. For negative control, the neuron generated 4 action potentials (APs) under control condition; however, 6 min after S1P exposure, the same ramp current elicited 10 APs, > 2 fold increase in the number of APs. In contrast, for R1/R2/R3 siRNAs, the neuron generated 3 APs under control condition and 4 APs 6 min after exposed to S1P. B summarizes the averaged results from 5 cells in each group. S1P significantly

increased the number of APs in negative-siRNA-treated neurons at 6 and 10 min (* $P < 0.05$ compared to control, repeated measures (RM) ANOVA followed by Holm-Sidak method), whereas the combination siRNA treatment blocked S1P from increasing the number of APs (RM ANOVA).

3. Activation of S1PR2 does not regulate S1P enhancement of excitability

My experiments show that S1PR1, R2 and/or R3 are essential in regulating S1P enhancement of excitability, while R4 and R5 are not sufficient. Therefore, my focus moved to S1PR2 and R3 as the two other S1PR subtypes, besides S1PR1, to mediate S1P-induced increase in excitability. To address this issue, I decided to study the function of S1PR2 and R3 separately.

There are few specific S1PR2 or R3 agonists or antagonists available, which creates a limitation on the pharmacological tools I am able to use for the study. There is a selective S1PR2 antagonist, JTE-013, which allows me to investigate the role of S1PR2 in S1P-induced increase of excitability.

3.1. JTE-013 increases the excitability of sensory neurons

JTE-013 is considered a selective antagonist at S1PR2. It has a high binding affinity at S1PR2 (IC_{50} ~20 nM), but not at S1PR1 or R3. Moreover, JTE-013 reversed the effect of S1P on migration inhibition through S1PR2 (Osada et al., 2002). However, there had been no reports showing JTE-013 was used as a selective S1PR2 antagonist in sensory neurons. Thus, before using JTE-013 to study S1PR2 function in sensory neurons, I decided to confirm it would not affect neuronal excitability over time.

To test this idea, small-diameter sensory neurons were treated with 100 nM JTE and their excitability was measured over a course of 15 min. To my surprise, 100 nM JTE-013 greatly augmented the number of APs that sensory neurons evoked within 10 min after treatment (Figure 11A). Further experiments showed that, in 11 out of 16 neurons, 100 nM JTE-013 produced a significant increase in the number of APs within 5 min (6.9 ± 0.6 APs) compared to the control condition (3.2 ± 0.2 APs), and the effect of enhanced excitability persisted until 10 min (7.7 ± 0.4 APs) and 15 min (8.3 ± 1.0 APs) ($P < 0.001$ compared to control, ANOVA on ranks followed by Dunn's test). However, there was a small population of neurons (5 out of 16) which were not affected by JTE-013. These neurons generated an average of 3.2 ± 0.3 APs under control conditions and after 15 min of 100 nM JTE-013 exposure, the number of APs (3.2 ± 0.6 APs) was not significantly altered (ANOVA). The finding that JTE-013 increases neuronal excitability is contradictory to the function of a supposedly specific antagonist. One possible explanation is that JTE-013 had off-target effects which altered neuronal excitability at the concentration used.

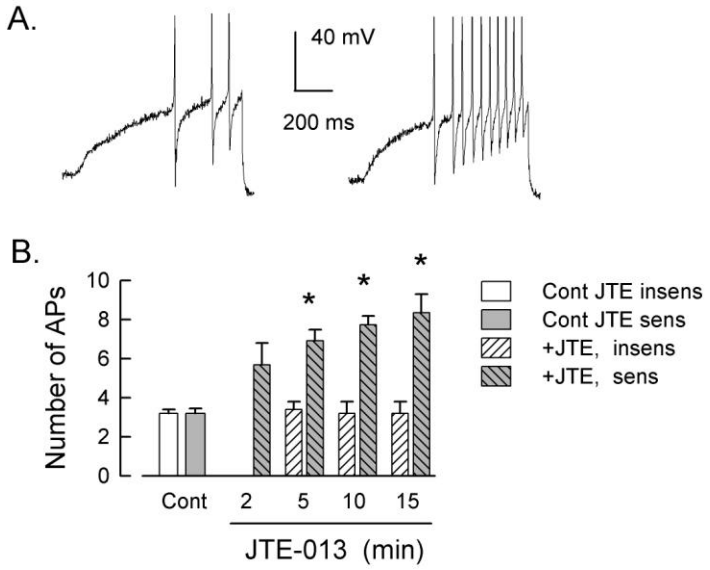

Figure 11. JTE-013 increases excitability of small diameter sensory neurons.

A shows representative traces from one neuron where under control condition, it generates 3 APs (left); after exposed to 100 nM JTE-013 for 10 min, the same neurons under the same current stimulus generated 10 APs (right). B summaries the effect of JTE-013 on sensory neurons over a 15-min recoding period. Data are presented as means ± SEM. The neurons are divided into two populations, JTE-013 sensitive and insensitive. In the sensitive group, JTE-013 greatly increased the number of APs compared to control at 5, 10 and 15 min (*$P < 0.001$ compared to control, ANOVA on ranks followed by Dunn's test). The number of neurons at each time points is: control 11, 2 min 6, 5 min 11, 10 min 11 and 15 min 9. In the insensitive group, JTE-013 did not significantly change the number of APs neurons evoked (ANOVA, n=5).

3.2. JTE-013 enhances excitability in a concentration- and time-dependent manner

To further characterize the effect of JTE-013 on the excitability of small diameter sensory neurons, various concentrations of JTE-013 were tested, and the concentration-response curve was generated indicating the relation between the JTE-013 concentration and the change of neuronal excitability over time.

Based on the JTE-013 IC_{50} value (20 nM) (Osada et al., 2002), a series of various JTE-013 concentrations was used, from 1 nM, 3 nM, 10 nM, 100 nM to 1000 nM. As a vehicle control, a 5000-fold diluted solution of 1-methyl-2-pyrrolidinone (MPL), the JTE solvent, was prepared. This MPL dilution has the highest solvent content compared to all JTE-013 solutions. Sensory neurons were then exposed to these solutions and their excitability over a course of 15 min was measure in the form of elicited APs.

The summarized results are shown in Figure 12A, demonstrating that JTE-013 augments neuronal excitability in both a time- and concentration-dependent manner. The number of APs in each treatment group was then normalized to its corresponding control value (Figure 12B). The vehicle itself did not significantly change the number of APs over time (control 3.4 ± 0.2 APs, 10 min 4.2 ± 0.6 APs, RM ANOVA). Similarly, neither 1 nor 3 nM significantly altered the number of evoked APs over a 15-min recording period (1 nM: control 4.3 ± 0.3 APs, 15 min

5.5 ± 0.9 APs, ANOVA; 3 nM: control 4.0 ± 0 APs, 15 min 5.5 ± 0.6 APs, ANOVA on ranks). In contrast, 10 nM JTE-013 significantly altered neuronal excitability in 10 out of 12 sensory neurons. The number of APs significantly increased within 5 min after JTE-013 exposure and this enhancement effect of JTE-013 lasted until 15 min (ANOVA on ranks). As for 100 nM JTE-013, the number of APs from 11 neurons (out of 16) was significantly elevated at 5, 10 and 15 min, compared to the control (Figure 12B). At the 1000 nM concentration, JTE-013 significantly increased the number of APs in 8 out of 11 sensory neurons at 5, 10 and 15 min (ANOVA on ranks).

Not only did JTE-013 enhance neuronal excitability in a timely manner, also it increased the number of APs in a concentration-dependent manner (Figure 12C). At the 10 and 15 min time points, 10 nM JTE-013 significantly enhanced AP firing compared to 1 and 3 nM ($P < 0.001$, ANOVA on ranks). There was no significant difference for the increased number of APs produced by 10, 100 or 1000 nM JTE-013 at 10 min (ANOVA), suggesting JTE-013 at these three concentrations is equally potent to alter neuronal excitability. In all, the results demonstrate that JTE-013 enhances neuronal excitability in a concentration- and time-dependent manner.

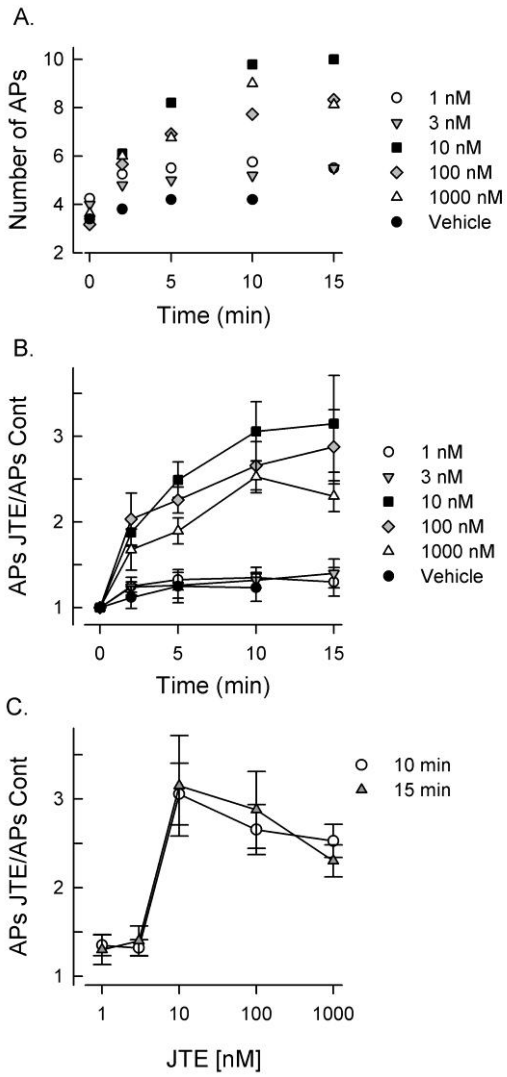

Figure 12. JTE-013 increases excitability in a concentration- and time-dependent manner.

A represents the mean value of the number of APs small diameter sensory neurons generated under control conditions, and 5, 10 and 15 min after vehicle (5000-fold dilution of MPL) or various JTE-013 concentrations treatment. The number of neurons recorded under each concentration is: vehicle 5, 1 nM 4, 3 nM 5, 10 nM 10, 100 nM 11, 1000 nM 8. B shows the data obtained from the same neurons as in A with the number of APs normalized to the control in each group, and data are represented as means ± SEM. There was no difference

either in the evoked number of APs or in the normalized number of APs for vehicle ($P = 0.20$ and $P = 0.24$ for the number of APs and the normalized number, respectively, ANOVA), for 1 nM JTE-013 ($P = 0.38$ and $P = 0.27$ for the number of APs and the normalized number, respectively, ANOVA), and for 3 nM JTE-013 ($P = 0.16$ both for the number of APs and the normalized number, ANOVA on ranks). There was a significant difference in the number of APs and normalized number of APs for 10, 100 and 1000 nM JTE-013 at 5, 10 and 15 min compared with their controls ($P < 0.001$, ANOVA on ranks followed by Dunn's test). C summarizes the increase in the normalized number of APs at 10 and 15 min as a function of different JTE-013 concentrations.

3.3. Neurons sensitized by JTE-013 are not further sensitized by S1P

The unexpected finding that JTE-013 augments neuronal excitability raises the question as to which signaling cascade mediates JTE-013-induced sensitization. Considering its binding affinity at S1PR2, it is possible that JTE-013 shares the same signaling pathways as S1P to increase neuronal excitability. If so, it is interesting to know how the effect of JTE-013 on sensory neurons affects the ability of S1P in increasing neuronal excitability. To explore this idea, in a separate experiment, a total of 9 neurons were first exposed to 100 nM JTE-013, and as shown in previous experiments, JTE-013 significantly increased the number of APs in the those neurons at 10 and 15 min (Figure 13). At this point, JTE-013 was washed away and the bath solution was replaced by 1 μM S1P. In the following 10 min after S1P exposure, the number of evoked APs was still significantly increased compared to control; nevertheless, the number of APs was not significantly different from 15 min time point ($P = 0.99$, ANOVA on ranks), indicating S1P did not further increase excitability of sensory neurons.

This result shows that S1P cannot further enhance the excitability of sensory neurons already sensitized by JTE-013, suggesting there may be an overlap in JTE-013 and S1P signaling pathways. However, we do not know yet if the effect of JTE-013 is readily reversible, so it is difficult to know whether JTE-013 masked any potential effect of S1P. It is quite possible that JTE-013 has produced an effect that maximized the number of APs sensory neurons could generate, and

therefore following exposure of S1P to the same neurons did not further alter neuronal excitability.

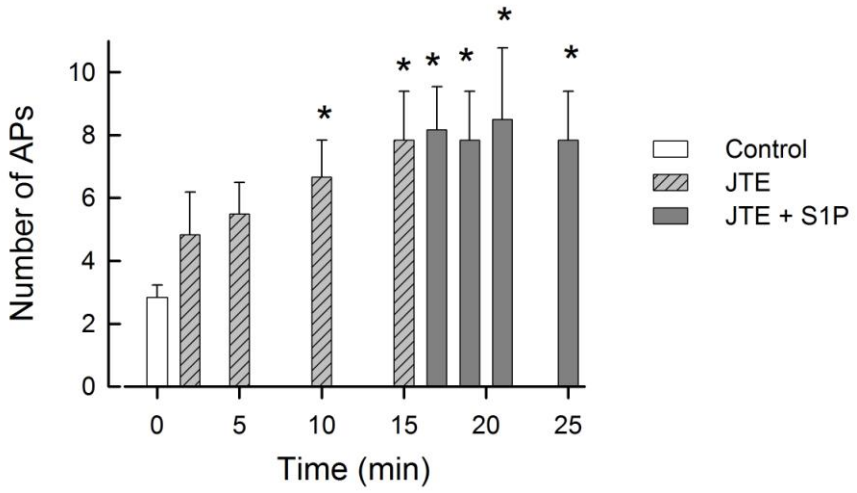

Figure 13. S1P does not cause a further increase in AP firing after JTE-013 treatment in sensory neurons.

The data represent the averaged number of APs (means ± SEM) from sensory neurons over a course of 25 min recording period. The neurons were exposed to 100 nM JTE-013 during the first 15 min. At 15 min, JTE-013 was replaced by 1 μM S1P and recording were obtained for the next 10 min. (* $P < 0.001$ compared to control, RM ANOVA, n = 7)

3.4. Guanosine 5'-O-(2-thiodiphosphate) prevents JTE-013 from altering excitability

As a selective antagonist on S1PR2, a GPCR, JTE-013 exhibits an unexpected agonistic effect. This led me to speculate the possibility that JTE-013 might act on another unidentified GPCR in sensory neurons to increase excitability. To explore this idea, I utilized guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S) to disrupt G protein signaling and then tested the agonistic effect of JTE-013. GDP-β-S is resistant to hydrolysis and phosphorylation and thus can act as a competitive inhibitor of GTP. G proteins in the GDP-β-S-binding form are not active and cannot activate downstream effectors to initiate singling cascade (Eckstein et al., 1979). Therefore, intracellularly applied GDP-β-S blocks G protein signaling pathways.

As a positive control to confirm the blocking effect of GDP-β-S, 3 mM GDP-β-S was added to the recording pipette solution and internally perfused into the sensory neurons. Sensory neurons with GDP-β-S were exposed to 1 μM prostaglandin E2 (PGE2). PGE2 augments neuronal excitability through the Gαs-cAMP-PKA signaling pathway (England et al., 1996). As shown in Figure 14A, 1 μM PGE2 significantly increased the number of APs at 2, 5 and 10 min after sensory neurons were exposed to PGE2. In contrast, neurons perfused with 3 mM GDP-β-S did not exhibit a significant increase in the number of APs even in the presence of PGE2 (Figure 14A, RM ANOVA). This result shows that GDP-β-

S blocks the Gαs-mediated PGE2 signaling cascade, indicating it can be used as an effective tool to block GPCR signaling in sensory neurons.

Next, I asked if internally perfused GDP-β-S will change neuronal excitability. So I internally applied 3 mM GDP-β-S into sensory neurons and observed the number of evoked APs for 10 min. I found GDP-β-S by itself did not significantly alter the excitability of sensory neurons (Figure 14B). Following that, 100 nM JTE-013 was externally applied to the sensory neurons in the presence of intracellular GDP-β-S. JTE-013 failed to elevate the number of APs within the next 10 min (Figure 14B, ANOVA). This finding shows that blocking the G protein signaling cascade prevents JTE-013 from increasing neuronal excitability, and suggests JTE-013 enhances neuronal excitability through a G protein-dependent mechanism.

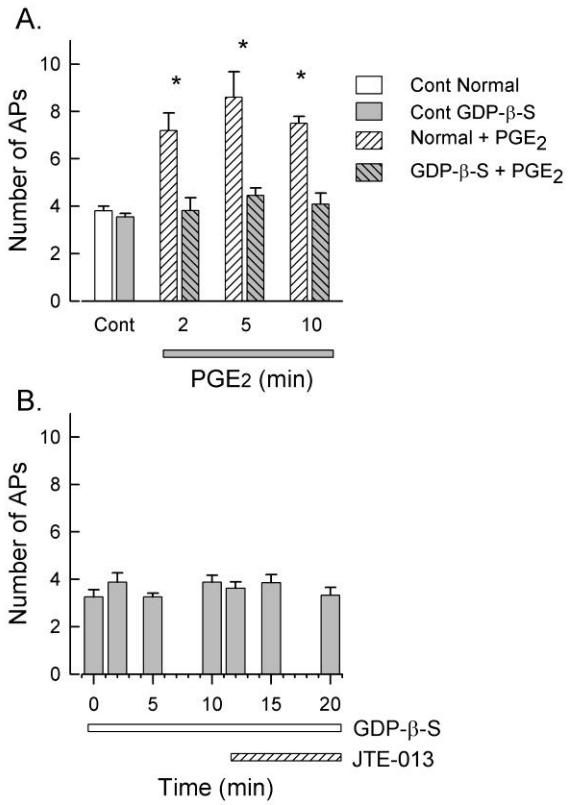

Figure 14. Internally perfused guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S) blocks the effect of JTE-013.

A represents the effect of 1 μM prostaglandin E2 (PGE2) in naive sensory neurons (normal) and in neurons internally perfused with GDP-β-S. In normal cells, 1 μM PGE2 significantly increased the number of APs at 2, 5 and 10 min (* $P < 0.001$ compared to control, RM ANOVA followed by Holm-Sidak test, $n = 5$). In contrast, internal perfusion with 3 mM with GDP-β-S prevented PGE2 from augmenting excitability (RM ANOVA, $n = 11$). B shows the number of APs evoked by neurons internally perfused with 3 mM with GDP-β-S during a 20-min recording period. The first 10 min indicated GDP-β-S itself did not cause any significant change on control AP firing. At 10-min time point, 100 nM JTE-013 was added to the bath; however, GDP-β-S blocked the effect of JTE-013 in

increasing excitability during the next 10 min (ANOVA). The numbers of neurons at each time points were: control through 12 min 8 neurons, 15 min 7 and 20 min 6. Data are presented as means ± SEM.

3.5. Pertussis toxin and a selective S1PR1 antagonist W146 block the JTE-013-induced increase in excitability

The previous result demonstrates the JTE-013-induced enhancement in neuronal excitability is G protein-dependent. This indicates it is likely that JTE-013 acts on a certain GPCR to enhance neuronal excitability. GPCRs couple to a great variety of G proteins, including Gi/o, Gq, Gs and G12/13. To test the idea that JTE-013 augments excitability through Gi/Go type of G proteins, I decided to first block the Gi/Go signaling in sensory neurons and then measure the effect of JTE-013 on those neurons. Pertussis toxin (PTX) is a drug that catalyzes the transfer of ADP ribose from NAD to the α subunit of the heterotrimeric Gi/o protein. The ADP-ribosylation of Gαi/o prevents heterotrimeric Gi/o proteins from interacting with GPCRs on the cell membrane, and thus disrupting the activation of Gαi/o and further intracellular signaling (Bokoch et al., 1983).

Sensory neurons were treated with 200 ng/ml PTX for 24 hr, and after that, the response of neurons to 10 nM JTE-013 was tested. As shown in Figure 15A, after PTX pretreatment, 10 nM JTE-013 was unable to increase the number of APs in sensory neurons. In the parallel group where neurons from the same harvest were not treated with PTX, 10 nM JTE-013 significantly elevated the number of APs in those sensory neurons (Figure 15A). The enhancement in excitability by JTE-013 in the untreated group took place at 2 min and was maintained until 10 min (ANOVA followed by Holm-Sidak test). This result

suggests JTE-013 enhances neuronal excitability through Gi/Go signaling pathway.

Following exposure to 10 nM JTE-013 for 10 min, sensory neurons from both PTX-treated and untreated groups were exposed to 1 μM S1P. Interestingly, PTX treatment also blocked S1P from increasing excitability in 6 out of 8 neurons (Figure 15A), indicating S1P activation of Gi/Go type G protein may play a critical role in S1P-induced increase in excitability. However, one caveat is that the time for S1P exposure is not long enough for us to see the effect of S1P, as indicated by previous experiments that S1P significantly increases neuronal excitability starting at 6 min (Zhang et al., 2006a; Zhang et al., 2006b). Also, for the other 2 neurons, their AP firing went from 5 and 6 APs to 12 and 11 APs, respectively, after 2 min exposure to S1P. This could be due to the fact that S1P increases neuronal excitability through other non-Gi/Go-coupled S1PR subtypes. In the untreated group, 1 μM S1P did not further increase the excitability of sensory neurons already sensitized by 10 nM JTE-013 (Figure 15A), consistent with the finding in Figure 13 where 100 nM JTE-013 was used, suggesting 10 and 100 nM JTE-013 are equally effective in altering neuronal excitability.

S1PR1-R5 share 33-51% amino acid identity (Parrill et al., 2004). Considering the sequence similarity across S1PR subtypes, it is possible that JTE-013, as an antagonist on S1PR2, could also bind to and have an agonistic effect on other S1PR subtypes. In addition, a study by Windh et al. (Windh et al., 1999) indicates

that S1PR1 is coupled to the Gi family of G protein, and our result from Figure 15A suggests the JTE-013 increase in excitability is Gi/Go dependent. This evidence suggests JTE-013 possibly enhances excitability through S1PR1.

To test this idea, neurons were pretreated with 1 μM W146, a selective S1PR1 antagonist (Sanna et al., 2006), for 30 min and then exposed to 10 nM JTE-013. As a control, neurons isolated and cultured under the same condition were pretreated with 1 μM W140, an inactive analog of W146 (Sanna et al., 2006). Interestingly, W146 pretreatment blocked the effect of JTE-013 from increasing excitability in sensory neurons, and the number of APs did not significantly change at various time points within a 15-min recording period (Figure 15B, ANOVA). However, W140 did not affect the ability of JTE-013 to increase excitability. The number of evoked APs significantly increased 2 min after JTE-013 exposure and was maintained until 15 min (Figure 15B, ANOVA followed by Holm-Sidak test).

Taken together, these results suggest that JTE-013 may act as an agonist on an unidentified GPCR that is coupled with a Gi/o-type G protein. The finding that a selective S1PR1 antagonist W146 prevents JTE-013 from increasing neuronal excitability indicates JTE-013 exerts its agonistic function perhaps through the activation of S1PR1. However, considering the poor understanding of JTE-013 metabolism, additional studies are required to fully establish the targets of JTE-013.

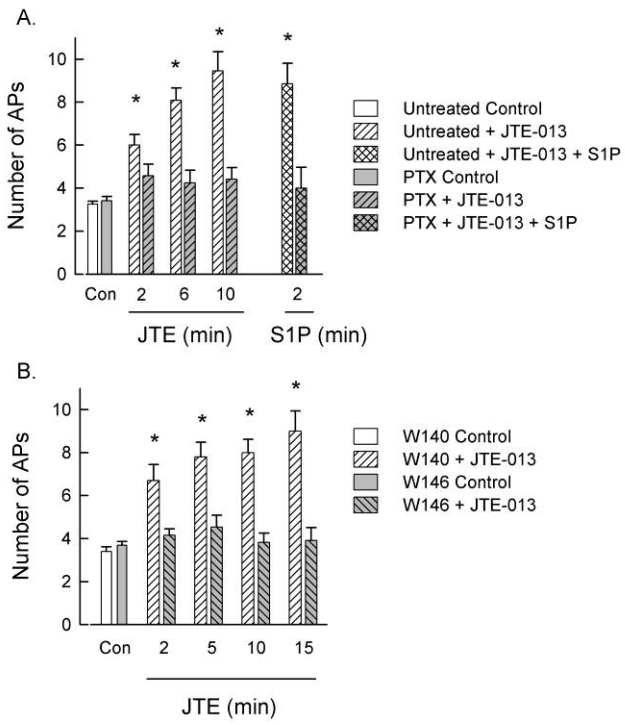

Figure 15. Pertussis toxin (PTX) and S1PR1 antagonist W146 block JTE-013 increase in excitability.

A shows two groups of neurons. One group was treated with 200 ng/ml PTX for 24 hr. The other group consisted of two subgroups: one subgroup was obtained from neurons isolated at the same time under the same condition as the PTX group, except that they were untreated; another subgroup was from the concentration-response experiments for 10 nM JTE-013. These 2 subgroups were not significantly different from each other and thus combined (ANOVA). The numbers of neurons in the untreated group were: control 12, JTE-013 12, 12 and 11 at 2, 6 and 10 min, respectively, and S1P 7. The numbers of neurons in the PTX-treated group were: control 12, JTE-013 12 and S1P 6. In the untreated group, the number of APs was significantly increase at 2, 6 and 10 min after JTE-013 exposure, as well as 2 min after 1 μM S1P exposure (* $P < 0.001$, ANOVA

followed by Holm-Sidak test). To compare between groups, an ANOVA followed by Holm-Sidak was used. There was a significant difference between the untreated neurons at 6 and 10 min compared to all time points in untreated neurons. Untreated neurons at 2 min were significantly different from PTX-treated control, but not treated neurons at other time points ($P < 0.001$). The controls in two groups were not different. B shows the results from 1 μM selective S1PR1 antagonist W146 and its inactive analog W140. 1 μM W140 significantly increased AP firing at 2, 5, 10 and 15 min time points compared to control (* $P < 0.001$, ANOVA followed by Holm-Sidak test). The numbers at each time points in W140 group were: control 10, 2 min 10, 5 min 10, 10 min 7 and 15 min 5. In the W146 pretreatment group, JTE-013 had no effect on the number of evoked APs (ANOVA). The numbers at each time points in W146 group were: control 13, 2 min 13, 5 min 13, 10 min 12, and 15 min 12. A between group comparison shows that there was no difference between controls of W140 and W146 groups (ANOVA), but in the W140 group, JTE-013 significantly increased the number of evoked APs at 5, 10 and 15 min compared with all time points from W146 treatment ($P < 0.001$, ANOVA followed by Holm-Sidak test).

3.6. JTE-013 increases neuronal excitability independently of S1PR2

JTE-013 exhibits the surprising effect of increasing neuronal excitability, and the findings with GDP-β-S and PTX suggest that JTE-013 may act as an agonist on GPCRs. One possibility is that, instead of blocking S1PR2, JTE-013 actually activates S1PR2 to increase neuronal excitability in sensory neurons. To address whether the JTE-013-induced increase in neuronal excitability is mediated through S1PR2, two neurons (C1 and C2) in which 100 nM JTE-013 significantly increased excitability were collected (Figure 16A). To determine the expression of S1PR2 mRNA, the collected contents from these two neurons underwent RT-PCR, and the presence of S1PR2 and housekeeping gene HPRT is shown in Figure 16B. Unexpectedly, these two neurons did not have detectable levels of mRNA for S1PR2, while they both expressed HPRT. DRG and liver tissues from rats were used as positive controls and they both showed expression of S1PR2 and HPRT. In the negative control (blank), nuclease-free water was used as the PCR template, and it exhibited no sign of contamination during the process of the experiment. These results show that sensory neurons not expressing S1PR2 can still be sensitized by JTE-013, suggesting that JTE-013 can augment the excitability of sensory neurons independently of S1PR2.

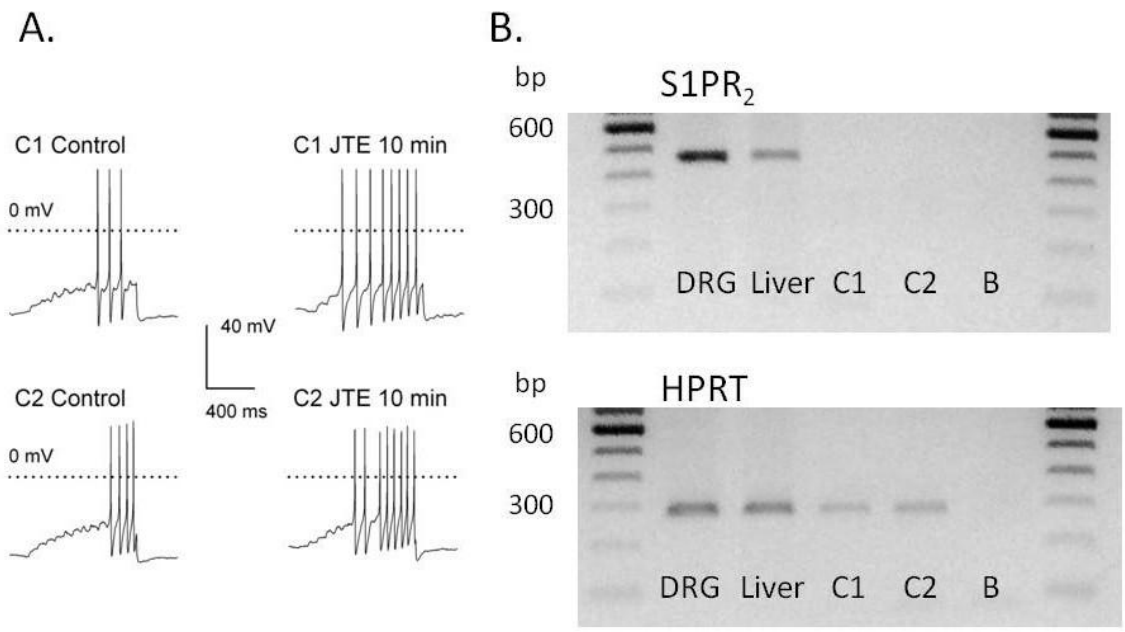

Figure 16. Two neurons sensitized by JTE-013 do not express S1PR2.

A shows the representative traces from two small-diameter (C1 and C2) sensory neurons in which 100 JTE-013 increased the number of APs in 10 min. These two neurons were collected and underwent single-cell RT-PCR. B shows that the gene expression profiles of C1 and C2 were detected and shown. DRG and liver tissues were used as positive control to show that they both expressed S1PR2 mRNA. The S1PR2 expression in C1 and C2 was undetectable. Water is used as template in the negative control (B) to rule out contamination. HPRT was used as house-keeping gene marker and its expression was detected in all samples but the blank control

3.7. JTE-013 prevents the S1P-induced inhibition of B16 migration

The unexpected result from JTE-013 increasing excitability raised our concern as to whether the JTE-013 used in our experiments was effective as an antagonist. A previous study demonstrated that JTE-013 could block the inhibitory effect of S1P on B16 melanoma cell migration via S1PR2 (Arikawa et al., 2003). Therefore, I performed a similar wound-healing assay as shown in that study to test the effectiveness of our JTE-013. B16 melanoma cells were cultured in 6-well plates until they reached 80% confluence, and a “wound” was created by scratching through the center of the well. Then cells cultured in each well were given three different treatments, including 100 nM S1P, 100 nM S1P + 1 μM JTE-013, and 1 μM JTE-013, and one well was not treated with any drug to serve as a control. Cultured cells were supplemented with migration media to help the healing process over the next 48 hr. Since S1PR2 is a dominant S1PR subtype in B16 cells, upon activation by S1P, the signaling pathway downstream of S1PR2 exhibits an inhibitory effect on cell migration (Okamoto et al., 2000). As shown in both Figure 17A and B, 100 nM S1P greatly inhibited the migration of B16 cells into the scratched center, compared with the control. In contrast, by adding JTE-013 to antagonize the binding of S1P to S1PR2, B16 cells migrated into the center of the wound, which was similar to what the control condition exhibited. Moreover, JTE-013 itself did not change the migration pattern of the cells compared to the control.

These results demonstrate that JTE-013 blocked the inhibitory effect of S1P via S1PR2 on cell migration, and confirmed that the JTE-013 used in our experiments was indeed effective in the same manner as previously reported (Arikawa et al., 2003). The unexpected agonistic effect of JTE-013 on enhancing neuronal excitability is probably due to the off-target effect of JTE-013 on another yet-to-be-identified receptor in sensory neurons. Because of this, JTE-013 is not an ideal tool to use in sensory neurons. Therefore, in order to study the role of S1PR2 in S1P-induced sensitization, I have to resort to other tools.

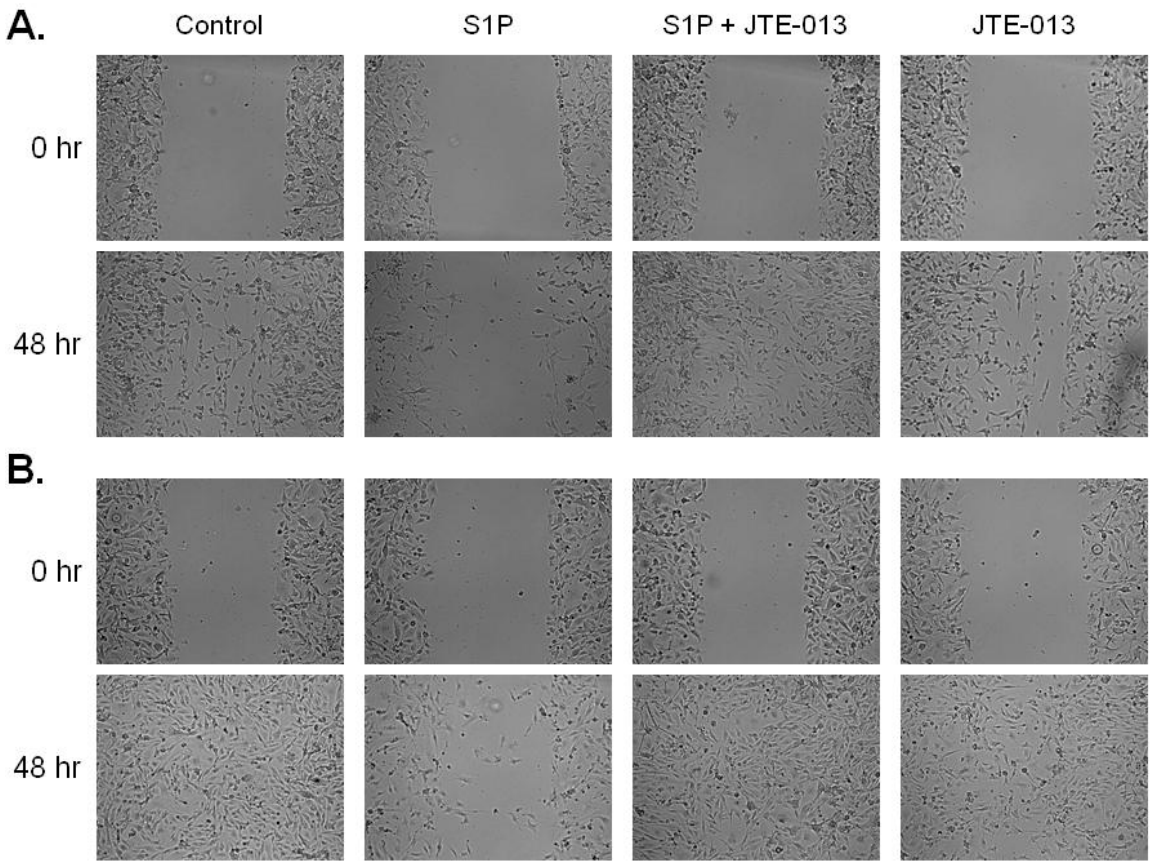

Figure 17. S1P inhibition on B16 cell migration is reserved by JTE-013.

A and B represent two separate experiments where B16 melanoma cells underwent would-healing process over a course of 48 hr. Pictures were taken at 0 hr when a scratch was artificially created in the center of cultured cells. Then cells were given different treatments: control, 100 nM S1P, 100 nM S1P + 1 μM JTE-013, and 1 μM JTE-013. After 48 hr, pictures were taken again from the same cell populations and different migration patterns were shown. S1P inhibited cell migration compared to control, and JTE-013 reversed the inhibitory effect of S1P on migration, but JTE-013 itself did not change the migration pattern of B16 cells.

3.8. FTY720, an agonist on S1PR1/R3/R4/R5 increases excitability in sensory neurons

The idea of examining the role of S1PR2 by using a putative S1PR2-specific antagonist JTE-013 did not come through because of the unexpected finding that JTE-013 increases the excitability of sensory neurons. Unfortunately, there are no other specific S1PR2 agonists or antagonists available, which makes it difficult for us to study the function of S1PR2 directly. However, it is possible that we can tackle this question indirectly by using FTY720 (fingolimod).

FTY720 is a structural analog of sphingosine. When phosphorylated by SphK2, it becomes FTY720 phosphate (FTY720P), which has high affinity for all S1PRs except S1PR2 (Mandala et al., 2002). Bioassays for receptor binding and function revealed that FTY720P functions as an agonist at S1PR1, S1PR4 and S1PR5 (EC_{50} ~0.3–0.6 nM) and at S1PR3 (EC_{50} ~3 nM) in vitro, but it had no activity at S1PR2 (Brinkmann et al., 2002; Brinkmann et al., 2010). Moreover, it has been shown that FTY720P causes internalization of S1PR1 (Mullershausen et al., 2009), a mechanism that enables FTY720 to be a potent immunomodulatory agent to treat multiple sclerosis (Brinkmann et al., 2010). Based on this evidence, I decided to treat sensory neurons with FTY720, and this treatment may potentially downregulate S1PR1, R3, R4 and R5, if any of them are expressed in the neurons. Such an approach will possibly lead to S1PR2

being the only S1PR subtype on the cell membrane, and allow me to study S1PR2 function in isolation.

The potential internalization of S1PRs (all but S1PR2) results from the phosphorylation of FTY720 and subsequent activation of S1PRs by FTY720P. Therefore, before using FTY720 as a tool to downregulate S1PRs, I confirmed the agonistic effect of FTY720. Sensory neurons were exposed to 10, 30 and 100 nM FTY720, and the numbers of evoked APs were recorded during the next 20 min as a measurement of FTY720's agonistic effect. As shown in Figure 18, 10 and 30 nM FTY720 did not significantly change the number of APs in sensory neurons during a 20-min recording period. However, treatment with 100 nM FTY720 significantly increases the excitability of 6 neurons out of a total of 11 recorded neurons (RM ANOVA followed by Holm-Sidak test). In these FTY720-sensitive neurons, the number of APs they generated at control was 3.5 ± 0.3, and the number increased to 11.7 ± 1.4 and 11.3 ± 1.5 at 15 and 20 min respectively, after treatment with 100 nM FTY720. On the other hand, there were 5 sensory neurons whose excitability was not significantly altered by 100 nM FTY720 (Figure 18, FTY insensitive).

The result indicates that FTY720 is able to function as an agonist to increase neuronal excitability. We also noticed that the significant increase in the number of elicited APs took place at 15 min after sensory neurons were exposed to FTY720, whereas for S1P, the significant increase happens within 6 min (Zhang

et al., 2006a). This slight delay in the enhancement of excitability may well reflect the fact that FTY720 must first be phosphorylated, such that FTY720P then activates S1PRs to increase neuronal excitability. In addition, about 50% of the sensory neurons were sensitized by FTY720, compared to about 90% by S1P. This may be due to the different properties of these two agonists, e.g. affinity, selectivity and efficacy, which may lead to biased activation of downstream signaling pathways.

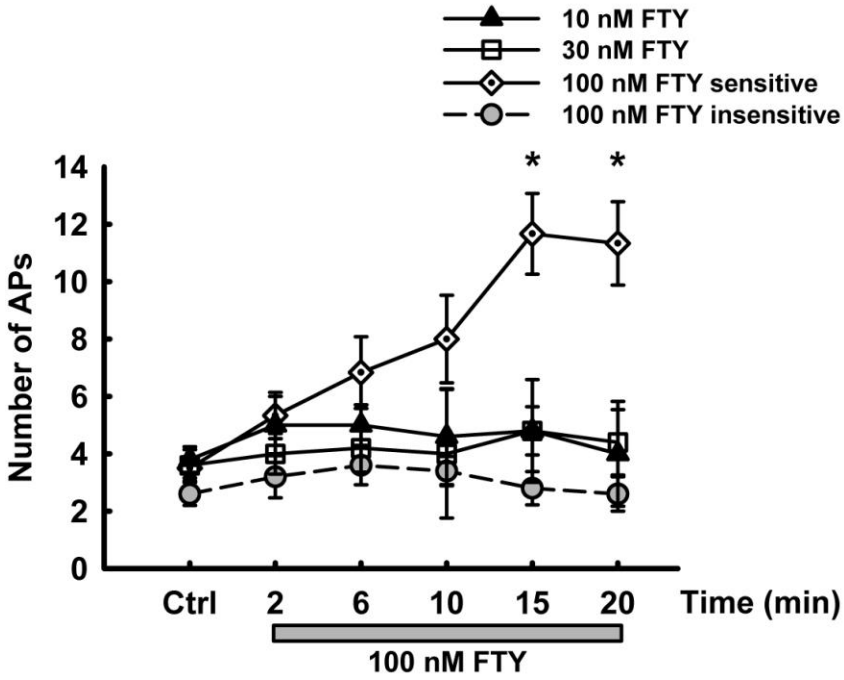

Figure 18. FTY720 increases neuronal excitability in a concentration- and time-dependent manner.

The data represent the mean value of the number of APs (means ± SEM) in small diameter sensory neurons generated under control conditions, 2, 6, 10, 15 and 20 min after 10, 30 and 100 nM FTY720 treatment. The numbers of neurons recorded under each concentration is: 10 nM 5 (4 at 20 min), 30 nM 5, 100 nM sensitive 6, and 100 nM insensitive 5. For 10 nM FTY720, there was no different in the number of APs among all time points before and after FTY720 exposure (ANOVA). For 30 nM FTY720, the number of APs did not significantly change after FTY720 treatment compared to control ($P = 0.228$, RM ANOVA on ranks). For 100 nM FTY720, there were two groups. 5 neurons in one group were not sensitized by FTY720, and the number of APs in those neurons was not significantly altered over time (RM ANOVA). 6 neurons in the other group were sensitive to 100 nM FTY720, the number of evoked APs was significantly

increases at 15 and 20 min compared to control (* $P < 0.05$, RM ANOVA followed by Holm-Sidak test).

3.9. The sphingosine kinase inhibitor, SKI-2, blocks the effect of FTY720 to increase neuronal excitability

The effect of FTY720 to increase neuronal excitability is based on the idea that FTY720 is taken up by the sensory neuron and phosphorylated by SphK2 in the cytoplasm to generate FTY720P (Zemann et al., 2006). FTY720P is then transported by transporters outside the neuron where it can function as an agonist at all S1PRs but S1PR2 (Hisano et al., 2011). To confirm that the agonistic effect of FTY720 is through this mechanism, I used a selective inhibitor of SphK2, SKI-2 (Orr Gandy and Obeid, 2013), to block the transformation of FTY720 to FTY720P. After a 30-min pretreatment with 5 μM SKI-2, 100 nM FTY720 failed to augment the number of APs in sensory neurons within 20 min (Figure 19, RM ANOVA on rank). This result demonstrates that SphK2 is essential in the FTY720-induced increase in neuronal excitability, and also confirms the mechanism that the function of FTY720 as an agonist is sphingosine kinase-dependent.

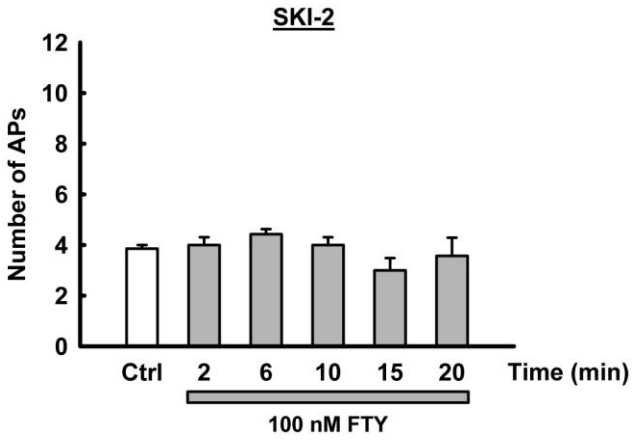

Figure 19. Sphingosine kinase inhibitor, SKI-2, prevents FTY720 from increasing neuronal excitability.

Data show that the number of APs in small-diameter sensory neurons at control and 20 min after 100 nM FTY720 exposure. These neurons were pretreated with 5 μM SKI-2 for 30 min prior to recording. The number of evoked APs was not significantly changed at any time point (2, 6, 10, 15 or 20 min) compared to control (P=0.228, RM ANOVA on rank, n = 7). Data are presented as means ± SEM.

3.10. S1PR2 is not sufficient in mediating S1P-induced enhancement of excitability

The agonistic function of FTY720 indicates FTY720 can be phosphorylated to FTY720P, activate all S1PRs except S1PR2, and possibly internalize the S1PRs that it binds to. Therefore, I pretreated sensory neurons with 1 μM FTY720 for 1 hr, and this treatment may conceivably lead to internalization of S1PR1, R3, R4 and R5, leaving R2 as the only S1PR subtype on the cell membrane. Following the pretreatment with FTY720, the baseline for sensory neuronal firing was established as the control, and the number of APs was recorded (2.8 ± 0.3). To test whether S1PR1 is internalized by FTY720 pretreatment, 100 nM SEW2871, a selective S1PR1 agonist which has been shown to increase neuronal excitability (Chi and Nicol, 2010), was applied to FTY720-pretreated neurons. After 10 min of SEW2871 exposure, the number of APs sensory neurons generated was 3.3 ± 0.6, and not significantly different from the control (RM ANOVA on ranks). The fact that SEW2871 failed to increase neuronal excitability suggests there was no functional S1PR1 or not enough S1PR1 to mediate S1P-induced sensitization on the cell membrane. Following SEW2871 exposure, the same sensory neurons were exposed to 1 μM S1P for 10 min. Over the course of the next 10-min recording period, the number of APs was not significantly changed at any time point compared to the control or to SEW2871 treatment (RM ANOVA on ranks), demonstrating that the 1 hr pretreatment with 1 μM FTY720 blocks S1P from increasing excitability in sensory neurons. With S1PR2 possibly

being the only functioning S1PR subtype in sensory neurons pretreated with FTY720, this result suggests that S1PR2 itself is not sufficient to mediate S1P enhancement of excitability. Therefore, FTY720 functionally antagonizes the effect of S1P to increase neuronal excitability.

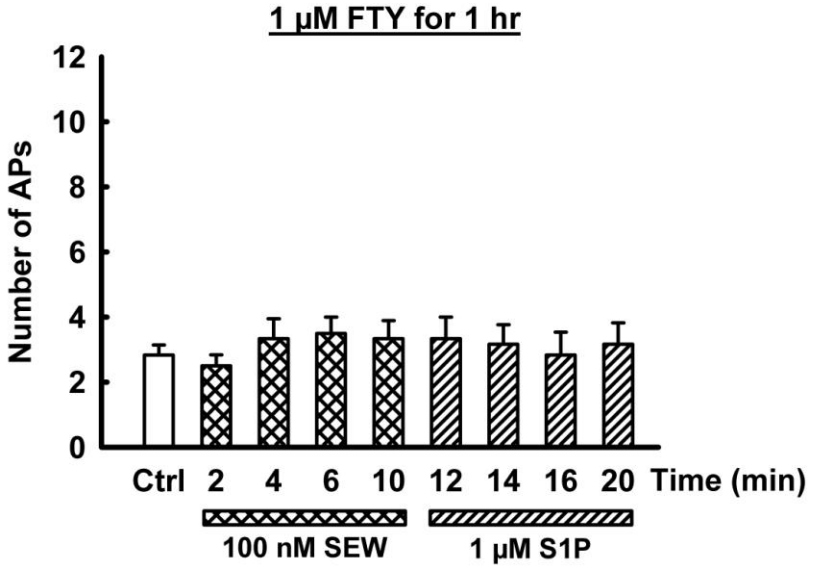

Figure 20. FTY720 pretreatment prevents SEW2871 and S1P from increasing excitability

Data represent the number of APs in small-diameter sensory neurons at control, within 10 min of 100 nM SEW2871 treatment and another 10 min of 1 μM S1P exposure. These neurons were pretreated with 1 μM FTY720 for 1 hr prior to recording. The number of evoked APs was not significantly changed at any time point (2, 4, 6, 10, 12, 14, 16 or 20 min) compared to control ($P = 0.386$, RM ANOVA on rank, $n = 6$). Data are presented as means ± SEM**.**

4. S1P increases excitability through S1PR1 and/or S1PR3 in small-diameter sensory neurons

So far, I have shown that the knockdown of S1PR1, R2 and R3 mRNAs prevents S1P from increasing excitability in sensory neurons, and that pretreatment with FTY720 abolishes the S1P enhancement of excitability. Taken together, these data indicate S1PR2, R4 and R5 are not sufficient to mediate the S1P-induced increase of excitability. S1PR1 was shown to play a predominant but not exclusive role in mediating S1P-induced enhancement in excitability, suggesting other S1PR subtypes also play a role. All the evidence leads me to hypothesize that S1PR3 contributes to the S1P-induced sensitization in sensory neurons.

4.1. Knockdown of S1PR1 and R3 by siRNAs blocks S1P from increasing neuronal excitability

To test the hypothesis that S1PR3 is the other S1PR, besides S1PR1, mediating the S1P-induced sensitization, I knocked down the expression of S1PR3 by siRNA technique, and then measured the response of sensory neurons to S1P. Also, to study S1PR3, it is critical to eliminate the role of S1PR1 as a predominant player in mediating S1P-induced increase in excitability. To do this, I treated sensory neurons with a combination of siRNAs targeted to S1PR1 and R3. Then those neurons were exposed to 1 μM S1P for 10 min and their excitability was measured in terms of the number of elicited APs. Following

treatment with S1P, the same neurons were further exposed to 1 μM PGE2, a potent agonist that increases neuronal excitability through the Gαs-cAMP-PKA signaling pathway (England et al., 1996), as a positive control to show that the excitability of sensory neurons can still be enhanced through a GPCR different from that of S1PRs.

Figure 21A shows representative traces of the elicited elicited APs from one sensory neuron under the control condition, 10 min after S1P treatment and another 10 min after PGE2 treatment. As the figure shows, under the control condition, the neuron generated 3 APs, 10 min after S1P exposure, the number went down to 2, but another 10 min exposure to PGE2 greatly increased the number of APs to 13. The summarized result (Figure 21B) from multiple neurons treated with a combination of siRNAs targeted to S1PR1 and R3 demonstrates, after the S1PR1 and R3 knockdown, 1 μM S1P did not significantly change the number of APs within 10 min. For those neurons, additional exposure to 1 μM PGE2 caused a significant increase in the AP firing at 14 and 20 min versus the control, suggesting the neurons insensitive to S1P were still capable of being sensitized by another GPCR-mediated agonist.

These data show that the knockdown of S1PR1 and R3 in sensory neurons prevents S1P from increasing neuronal excitability, suggesting S1PR1 and R3 are essential in S1P-induced sensitization. The increase of excitability produced by PGE2 indicates that the machinery of altering excitability in sensory neurons

that underwent siRNA treatment is still intact, and therefore, the insensitivity to S1P is not a false negative result. However, one limitation in interpreting this result is that S1PR1 and R3 were downregulated at mRNA level. The mRNA knockdown may not necessarily result in functional receptor downregulation. Therefore, it is critical that this experiment should be corroborated by using pharmacological tools to block receptor function directly.

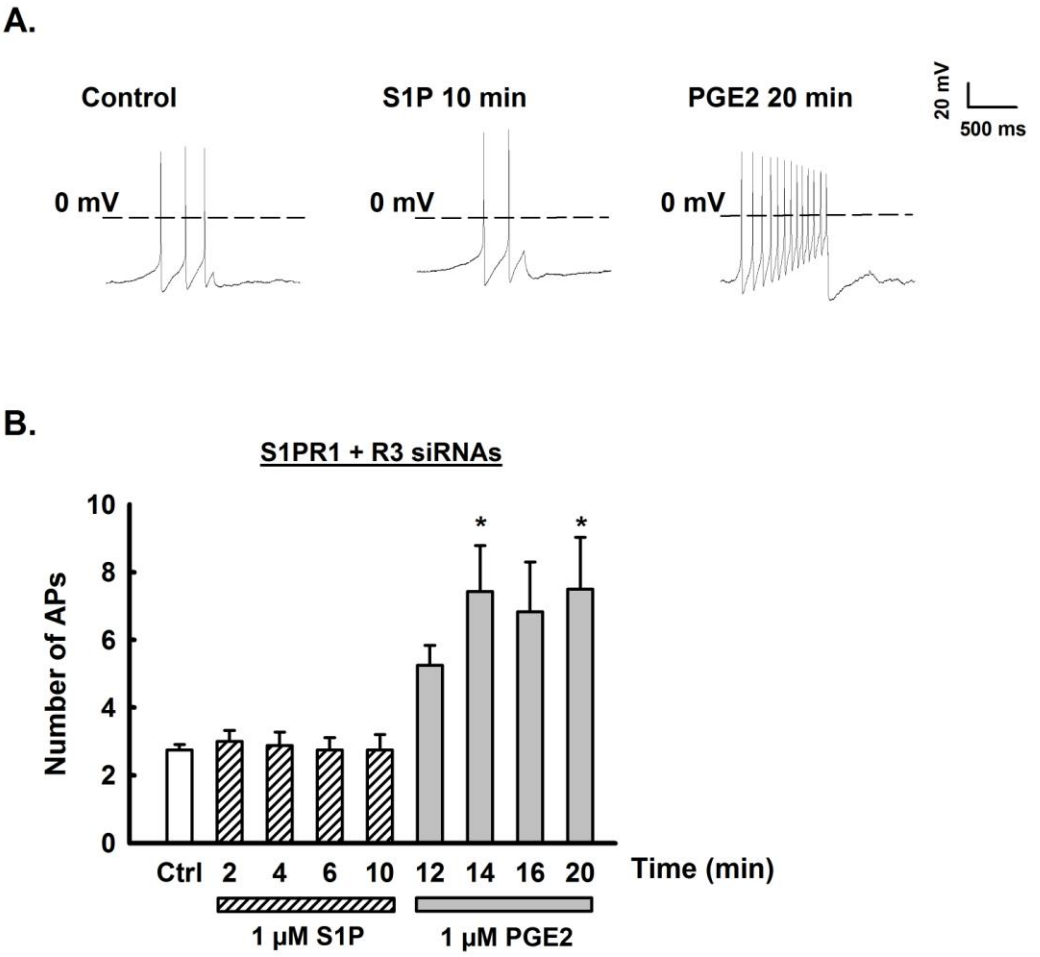

Figure 21. Neurons treated with S1PR1/R3 siRNAs did not respond to S1P, but were sensitized by PGE2.

A demonstrates representative traces from one sensory neuron treated with S1PR1 and R3 siRNAs, 100 nM each. Under control condition, the neuron generated 3 APs, 10 min after 1 μM S1P exposure, the number was 2, but another 10 min after 1 μM PGE2 incubation, the number went up to 13. B illustrates the averaged number of APs from 8 neurons (until 12 min, n = 7 at 14 min, n = 6 at 16 and 20 min). 1 μM S1P did not significantly alter the number of APs within 10 min (ANOVA on ranks); however, as a positive control to show

sensitivity, 1 μM PGE2 sensitized neurons (defined as > 2-fold increase in the number of APs) at 14 and 20 min (* $P < 0.05$ compared to control, ANOVA on ranks followed by Dunn's method).

4.2. Selective S1PR1 and R3 antagonists together abolish the effect of S1P

To corroborate the finding that siRNAs targeted to S1PR1 and R3 prevent S1P from increasing neuronal excitability, I took a pharmacological approach by using specific antagonists to block receptor function. To target S1PR1, a selective S1PR1 antagonist W146 was utilized (Sanna et al., 2006); meanwhile, a selective S1PR3 antagonist CAY10444 was used to target S1PR3 (Koide et al., 2002). I treated sensory neurons with a combination of these two antagonists, to block the availability of S1PR1 and R3 for S1P binding, and then tested the neuronal response to S1P. Sensory neurons were pretreated 1 μM W146 and 10 μM CAY10444 for 30 min, and the neuronal excitability was established as the control. Then, neurons were exposed to 1 μM S1P, and the number of elicited APs was recorded. Following treatment with S1P, 1 μM PGE2 was applied to the neurons and their excitability was measured as a positive control to indicate the capability of sensory neurons being sensitized despite the pretreatment of two antagonists.

Figure 22A is the representative traces showing elicited APs from one sensory neuron pretreated with W146 and CAY10444 under the control condition, 10 min after S1P treatment and another 10 min after PGE2 treatment. Under the control condition, the neuron fired 4 APs; 10 min after S1P exposure, the same neuron fired 6 APs under the same current stimulus. An extra 10-min exposure to PGE2 greatly increased AP firing to 9 APs, indicating PGE2 sensitized the neuron.

Averaged data from 9 neurons shows that S1P did not significantly alter the number of APs after sensory neurons underwent a combined treatment with S1PR1 and R3 antagonists (Figure 22B). W146 and CAY10444 together blocked the capacity of S1P to enhance neuronal excitability. On the other hand, the positive control shows PGE2 significantly increased the number of AP and sensitized neurons within 2 min, and the effect lasted until 10 min (Figure 22B), suggesting the reason that antagonists-pretreated sensory neurons failed to respond to S1P is due to the blockage of S1PR1 and R3, rather than the intrinsic condition of neurons.

This experiment demonstrates that blocking S1PR1 and R3 abolished the effect of S1P to enhance neuronal excitability, which is consistent with the previous S1PR1/R3 siRNAs experiment, where the knockdown of S1PR1 and R3 mRNAs prevented S1P from elevating neuronal excitability. Together, both results show that without S1PR1 and R3, S1P cannot increase neuronal excitability, suggesting S1P enhances excitability through S1PR1 and/or R3 in sensory neurons.

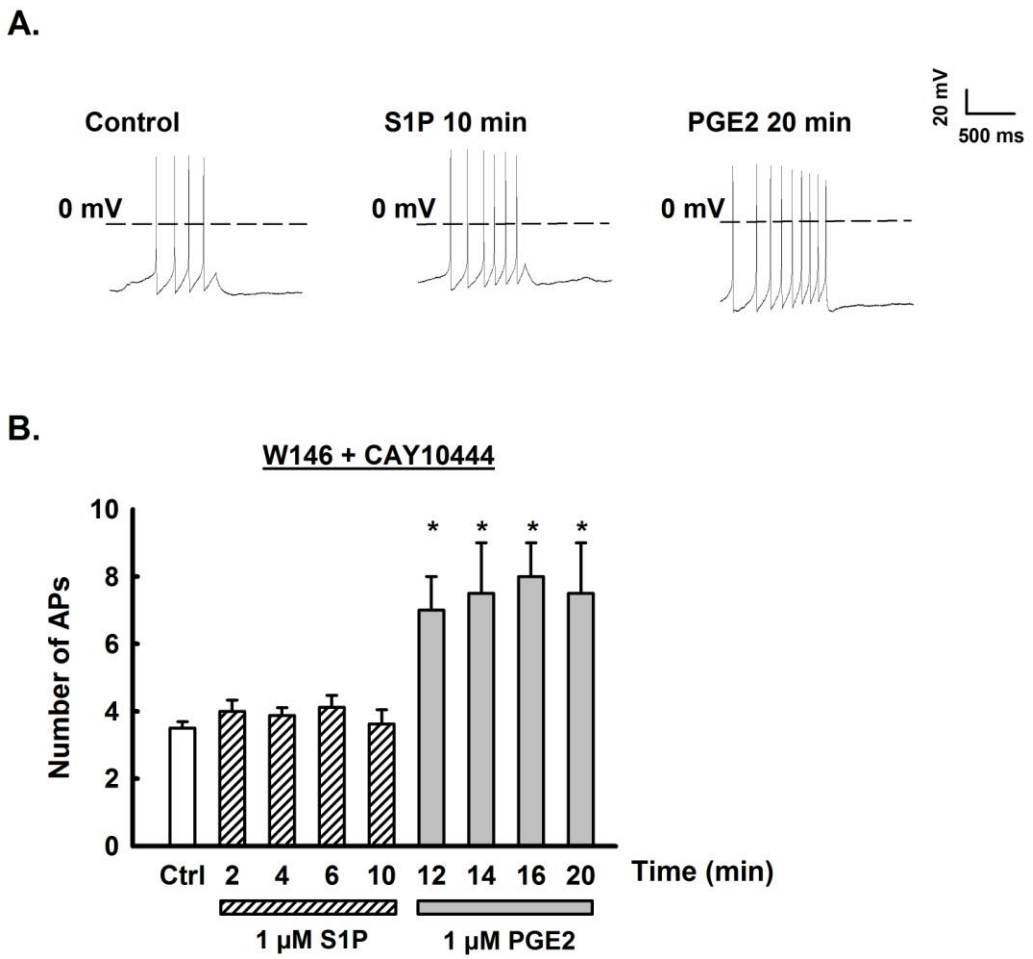

Figure 22. W146, a selective S1PR1 antagonist, and CAY10444, a selective S1PR3 antagonist, together abolished the effect of S1P, but not PGE2.

A demonstrates representative traces from one sensory neuron pretreated with 1 μM W146 and 10 μM CAY10444 for 30 min. Under control condition, the neuron generated 4 APs, and 10 min after 1 μM S1P exposure, it generated 6 APs, while 10 min after 1 μM PGE2 exposure, the number went up to 9. B illustrates the averaged number of APs from 9 neurons (until 10 min, n = 4 at 12 min, n = 3 at 14, 16 and 20 min). 1 μM S1P did not significantly alter the number of APs within

10 min (ANOVA); however, as a positive control to show sensitivity, 1 μM PGE2 sensitized neurons (* $P < 0.05$ compared to control, ANOVA followed by Holm-Sidak method).

4.3. VPC23019, a S1PR1/R3 antagonist, blocks S1P-induced sensitization

To further support the finding that S1PR1 and R3 are essential in mediating the S1P-induced increase in excitability, I used a compound VPC23019 to antagonize S1PR1 and R3 in sensory neurons, and then observed the neuronal response to S1P. VPC23019 is a competitive antagonist at S1PR1 and R3, inactive at S1PR2, an agonist at S1PR4 and a partial agonist at S1PR5 (Davis et al., 2005; Foss et al., 2007). First, to test if VPC23019 has any off-target effect to alter neuronal excitability, I performed a control experiment where I applied 1 μM VPC23019 to sensory neurons and measured the excitability of neurons over time. As shown in Figure 23A, 1 μM VPC23019 did not significantly change the number of APs within 15 min, demonstrating VPC23019 does not affect neuronal excitability. It is worth pointing out that VPC23019 is also an agonist on S1PR4 and a partial agonist on R5, and the fact that it did not alter neuronal excitability further indicates that S1PR4 and R5 do not mediate S1P increase in excitability.

Next, I pretreated neurons with 1 μM VPC23019 for 30 min, and then exposed them to 100 nM SEW2871, a selective S1PR1 agonist, to determine if VPC23019 blocks the agonistic effect of SEW2871 through S1PR1. The result shows that SEW2871 failed to increase the number of APs in sensory neurons pretreated with VPC23019 (Figure 23B), demonstrating that the function of S1PR1 was blocked by VPC23019, which serves as an indication of VPC23019 antagonism on S1PR1. Following SEW2871 exposure, sensory neurons were further

exposed to 1 μM S1P, and the data shows S1P did not significantly alter the number of elicited APs in sensory neurons pretreated with VPC23019 (Figure 23B, hatched bars). On the other hand, naive neurons from the same tissue harvest as the VPC23019-pretreated neurons were also exposed to SEW2871 and S1P. SEW2871 significantly increased the number of APs, and S1P did not further enhance the increased excitability (Figure 23B, open bars). The numbers of APs at the control from VPC23019-pretreated and naive neurons are not significantly different from each other (t-test). These results demonstrate that VPC23019 antagonizes the effect of S1P to increase neuronal excitability through S1PR1 and R3, suggesting S1P enhances neuronal excitability through S1PR1 and/or R3.

Lastly, as a positive control to show the machinery of regulating neuronal excitability is not affected by VPC23019 pretreatment, neurons pretreated with VPC23019 were first exposed to 1 μM S1P, and S1P did not significantly change the neuronal excitability. After S1P treatment, those neurons were further exposed to 1 μM PGE2, and PGE2 significantly increased the number of APs (Figure 23C), indicating the sensitivity of VPC23019-pretreated neurons was not impaired, and that the blockage of S1PR1 and R3 prevents S1P from increasing neuronal excitability.

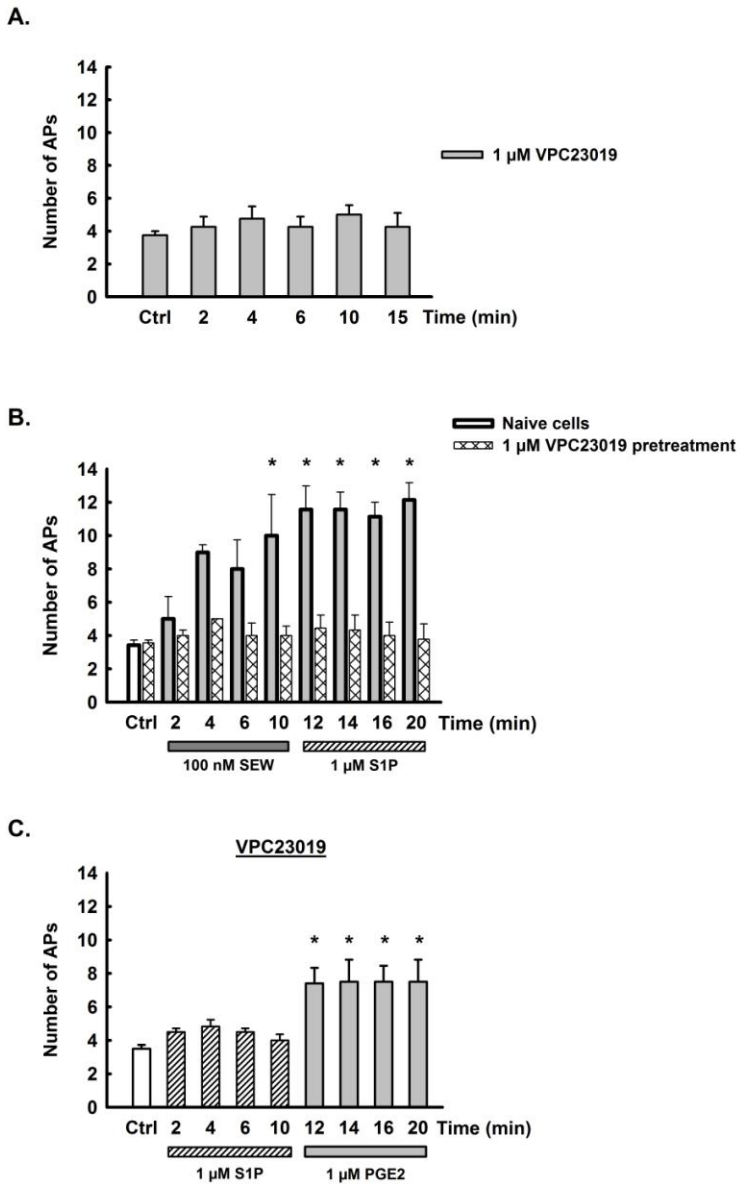

Figure 23. VPC23019, a S1PR1/R3 antagonist and S1PR4/R5 agonist, blocked S1P-induced sensitization.

A: 1 μM VPC23019 itself did not significantly alter the number of APs in sensory neurons within 15 min treatment (RM ANOVA, n = 4). B: in naive neurons, 100 nM SEW2871, a selective S1PR1 agonist, significantly increased the number of APs at 10 min; 1 μM S1P also significantly augmented the number of APs, but did not further sensitized neurons (* $P < 0.05$ compared to control, RM ANOVA

on ranks followed by Tukey test, n = 7). After pretreatment with 1 μM VPC23019 for 30 min, the AP firing of sensory neurons was not significantly increased by SEW2871 or S1P (RM ANOVA, n = 8). C shows the averaged number of APs from neurons that underwent 1 μM VPC23019 pretreatment for 30 min. The number of neurons recorded is as follows: 6 until 10 min, 5 at 12 min, n = 4 at 14, 16 and 20 min). 1 μM S1P did not significantly alter the number of APs within 10 min (RM ANOVA); however, as a positive control to show sensitivity, 1 μM PGE2 significantly increase the AP firing at 12, 14, 16 and 20 min (* $P < 0.05$ compared to control, ANOVA followed by Holm-Sidak method).

4.4. VPC44116, the phosphonate analog of VPC23019, also blocks S1P-induced sensitization

To further support my finding from the VPC23019 experiments that S1PR1 and/or R3 mediate, and that S1PR4 and R5 do not mediate S1P-induced increase in excitability, I used another compound VPC44116, which is the phosphonate analog of VPC23019. VPC44116 is also a potent antagonist at S1PR1 and R3, an agonist at S1PR4 and a partial agonist at S1PR5. VPC44116 and VPC23019 are nearly indistinguishable in their affinity for the S1PR1 and R3 (Foss et al., 2007). Based on a similar experimental design as with VPC23019, first I tested the effect of 1 μM VPC44116 to change excitability in sensory neurons. As shown in Figure 24A, 1 μM VPC44116 did not significantly change the number of APs within 15 min, demonstrating VPC44116 does not affect neuronal excitability. Of note, VPC44116 has agonistic effect on S1PR4 and R5, and the fact that it did not alter neuronal excitability suggests again S1PR4 and R5 do not mediate S1P enhancement of excitability.

Next, I pretreated neurons with 1 μM VPC44116 for 30 min, and then exposed them to 1 μM S1P. The data shows S1P did not significantly alter the number of elicited APs in sensory neurons pretreated with VPC44116 (Figure 24B, hatched bars). In contrast, naive neurons from the same harvest as VPC44116-pretreated neurons were sensitized by S1P (Figure 24B, open bars). The numbers of APs at the control from VPC44116-pretreated and naive neurons are not significantly

different from each other (t-test). These results demonstrate that VPC44116 blocks the effect of S1P to increase neuronal excitability through S1PR1 and R3, suggesting S1PR1 and/or R3 are essential in S1P-induced neuronal sensitization.

Following S1P treatment, the neurons were further exposed to 1 μM PGE2 (Figure 24B). In the naive neuron group, PGE2 maintained but did not further increase the elevated excitability caused by S1P. In the VPC44116-pretreated group, PGE2 significantly increased the number of APs, indicating the sensitivity of VPC44116-pretreated neurons was not impaired.

Taken together, the results demonstrate that the downregulation of S1PR1 and R3 by siRNAs and the blockage of S1PR1 and R3 by specific antagonists prevent S1P from increasing neuronal excitability. Also, the data shows that S1PR4 and R5 agonists are not able to enhance neuronal excitability. In conclusion, these results demonstrate the enhanced excitability in sensory neurons produced by S1P is mediated by the activation of S1PR1 and/or R3, but not R4 and R5. Considering the differential expression of different S1PR subtypes in different sensory neurons, and that so far we do not have enough data relating excitability to receptor expression, it is not easy to tease out which receptor (S1PR1 or R3) actually mediates S1P-induced sensitization in different neurons, or what is the percent of contribution each receptor (S1PR1 or R3) makes in mediating S1P-induced increase in excitability. It is possible that whichever receptor has the highest expression (S1PR1 or R3) plays the

dominant role in a certain sensory neuron, and also possible that there is a synergistic effect between S1PR1 and R3 in mediating S1P-induced increase of excitability.

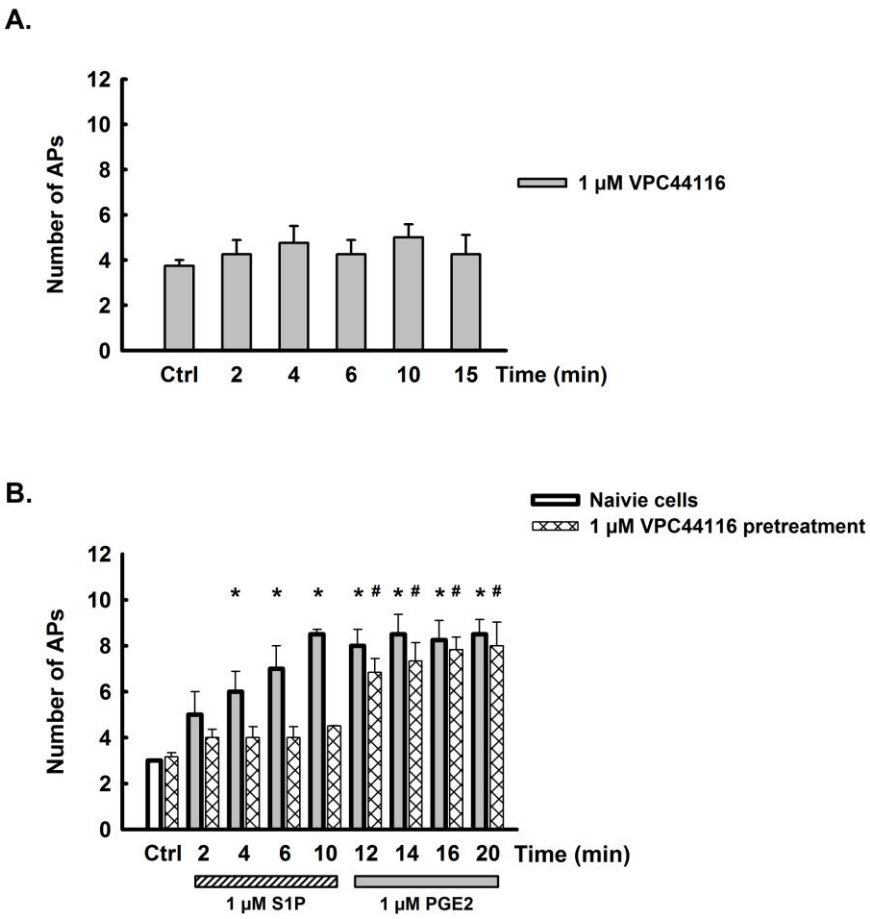

Figure 24. VPC44116, a potent antagonist on S1PR1/R3 and partial agonist on S1PR4/R5, blocked S1P-induced sensitization.

A: 1 μM VPC44116 itself did not significantly alter the number of APs in sensory neurons within 15 min treatment (RM ANOVA, n = 4). B: in naive neurons, 1 μM S1P significantly increased the number of APs at 4, 6 and 10 min; 1 μM PGE2 also significantly augmented the number of APs, but did not further sensitized neurons (* $P < 0.05$ compared to control, RM ANOVA followed by Holm-Sidak method, n = 4). In neurons that underwent 1 μM VPC44116 pretreatment for 30 min, 1 μM S1P did not significantly alter the number of APs within 10; however, as a positive control to show sensitivity, 1 μM PGE2 significantly increase the AP

firing at 12, 14, 16 and 20 min (# P <0.05 compared to control, RM ANOVA followed by Holm-Sidak method).

5. S1PR1 is possibly coupled to other types of heterotrimeric G proteins downstream signaling in sensory neurons

Having established that S1PR1 and R3 are the players in contributing to S1P-induced enhancement of excitability in small-diameter sensory neurons, I then asked the question, what signaling pathways downstream of S1PR1 and R3 transduce the effect of S1P to trigger the increase in excitability. Since S1PR1 is the predominate player in mediating excitability and there is a selective S1PR1 agonist, SEW2871, available to facilitate the isolation of S1PR1 signaling pathways, I decided to first look into the downstream signaling of S1PR1.

5.1. The selective S1PR1 agonist SEW2871 increases the excitability of sensory neurons

To study the signaling cascade of S1PR1, I needed a pharmacological tool to specifically activate this receptor. SEW2871, a selective S1PR1 agonist (Jo et al., 2005), is such a tool. A previous study shows that SEW2871 increases the number of evoked APs in some, but not all, sensory neurons, and that sensory neurons where S1PR1 was knocked down by siRNA were not able to be sensitized by SEW2871, indicating that SEW2871 specifically sensitizes neurons via S1PR1 (Chi and Nicol, 2010). To confirm the agonistic effect of SEW2871 in my study, sensory neurons were exposed to 100 nM SEW2871 for 15 min and the number of evoked APs was recorded. Figure 25 shows the number of fired

APs at different time points over a 15-min recording period. In a total of 14 neurons, 8 of them responded to SEW2871; their numbers of APs were significantly elevated 4 min after SEW2871 exposure compared with the control, and the effect of enhanced excitability was maintained until 15 min ($P < 0.05$, ANOVA followed by Holm-Sidak method). Meanwhile, 6 out of 14 neurons were insensitive to SEW2871, and the number of their APs were not significantly changed over time compared to the control ($P = 0.51$, ANOVA). The numbers of APs at the control from SEW2871-sensitive and insensitive neurons were not significantly different.

The data demonstrates that SEW2871 is capable of enhancing excitability of sensory neurons in about 60% of the total population, which agrees with our previous finding that 100 nM SEW2871 produced a significant increase in the number of APs in 5 of 9 neurons (Chi and Nicol, 2010). Therefore, in the following study, SEW2871 can be used as a selective agonist to examine S1PR1 signaling pathway.

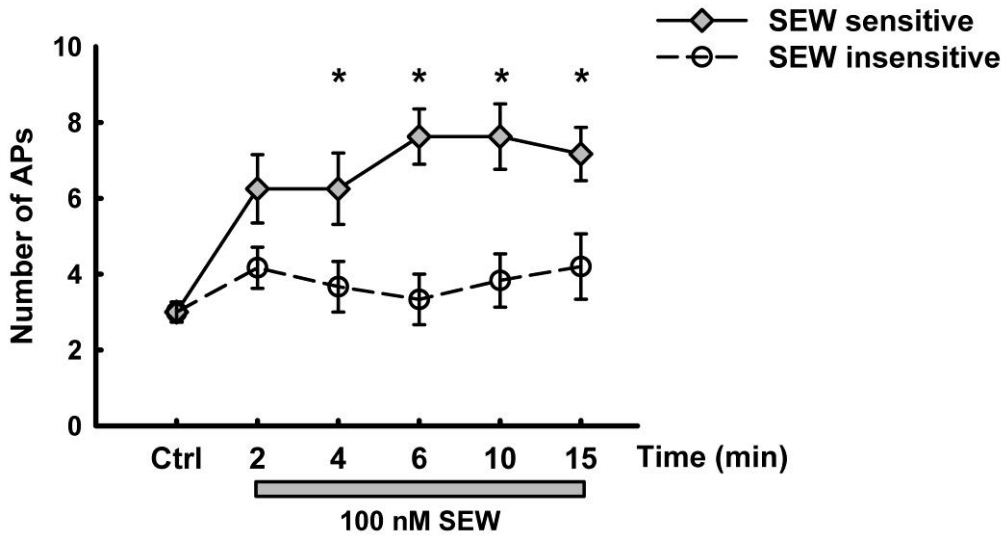

Figure 25. SEW2871 increases the excitability of some, but not all, sensory neurons.

The data show the number of APs sensory neurons fired during a 15-min recording period. The baseline firing was established at control, and then 100 nM SEW2871, selective S1PR1 agonist, was perfused continuously in the bath solution for the next 15 min. The numbers of APs were recorded at 2, 4, 6, 10 and 15 min. The number of APs in 8 out of 14 neurons was significantly altered by SEW2871, started from 4 min and maintained until 15 min (* $P < 0.05$ compared to control, ANOVA followed by Holm-Sidak method). The number of APs from the other 6 neurons remained unchanged over the 15-min period (ANOVA on rank, $P = 0.51$). The number of neurons in the SEW2871-sensitive population was 8 at all time points except 6 at 15 min; in the SEW2871-insensitive population, 6 except 5 at 15 min. Data were represented as means ± SEM.

5.2. PTX pretreatment does not block SEW2871 from increasing the excitability of sensory neurons

The initial study providing solid evidence that S1PR1 is exclusively coupled to the Gi/o family of G proteins was conducted in recombinant cell lines using the GTPγS binding assay (Windh et al., 1999). However, there has been no study to determine if this is the case in sensory neurons. Is S1PR1 exclusively coupled to the Gi/o-type trimeric G protein in sensory neurons as well? To address this issue, I decided to disrupt the activation of Gαi/o by using PTX, and then to observe whether the excitability of sensory neurons could still be increased via S1PR1 by using an S1PR1 selective agonist, SEW2871.

Sensory neurons were treated with 200 ng/ml PTX for 24 hr, after that, the neurons were exposed to 100 nM SEW2871. As Figure 26 shows, SEW2871 failed to significantly increase the excitability of 6 out of 10 neurons within 10 min (RM ANOVA), indicating that the S1PR1 signaling cascade might be disrupted by PTX, or those neurons might not be intrinsically responsive to SEW2871. Then, these 6 neurons were further exposed to 1 μM S1P for another 10 min. Among them (one neuron was lost during recording), 4 neurons did not respond to S1P treatment, suggesting that S1PR1 was the predominant player in mediating the S1P-induced enhancement in excitability. However, there was one neuron whose AP firing was increased from 3 APs at 10 min to 8 APs at 20 min, indicating that besides S1PR1, there were other S1PR(s) contributing to S1P increase in

excitability. This observation is consistent with our previous results where siRNA was used to knock down S1PR1 expression (Chi and Nicol, 2010). Based on our finding that S1P enhances neuronal excitability through S1PR1 and/or R3, it is very likely that S1PR3 mediated the increased excitability in that single neuron that was insensitive to SEW2871 but sensitive to S1P.

If S1PR1 couples exclusively to Gi/o in sensory neurons, after PTX treatment, the specific S1PR1 agonist SEW2871 should not be able to increase the neuronal excitability. Interestingly, I saw that there was another population of neurons pretreated with PTX (4 out of 10 in total) where SEW2871 significantly enhanced their excitability at 10 min compared to control (RM ANOVA followed by Holm-Sidak method). The percentage of SEW2871-sensitive neurons in PTX-treated neurons is not statistically different from that in naive neurons (Chi-sqaure test), indicating PTX did not block SEW2871 from increasing neuronal excitability. There could be several explantions for the results. First, according to the literuature, a treatment of 200 ng/ml PTX for 24 hr should be adequent to disrupt Gi/o signaling. However, it is possible that this treatment did not inhibit Gi/o activation in sensory neurons. It would be ideal to include a positive control experiment showing the effect of PTX to casue ADP-ribosylation of Gαi/o in sensory neurons. Second, if PTX did indeed effectively block Gi/o signaling in my experiment, S1PR1 may not only couple to Gαi/o, but also to another trimeric G protein like Gαq.

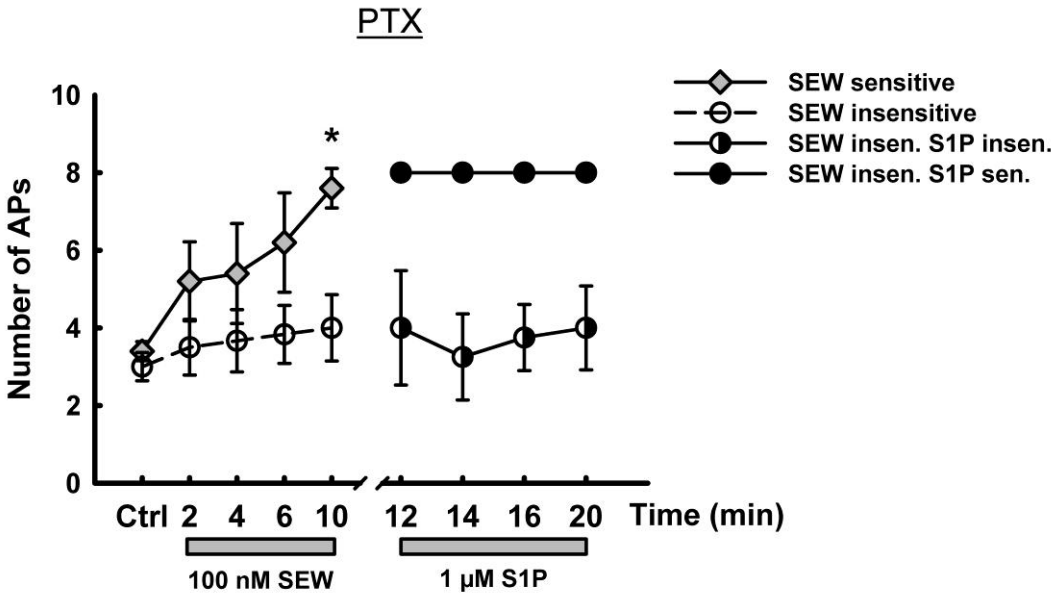

Figure 26. Sensory neurons pretreated with PTX exhibit diverged profiles upon exposed to SEW2871 and S1P.

Data show the number of APs firing in sensory neurons pretreated with 200 ng/ml PTX for 24 hr during a 20-min recording period. The baseline firing was established at control, and then 100 nM SEW2871, a selective S1PR1 agonist, was perfused continuously in the bath solution for the first 10 min. Then the bath solution was switched to 1 μM S1P and the perfusion continued until 20 min time point. The number of APs from 5 neurons was significantly increased with 10 min SEW2871 treatment (SEW sensitive), and the number at 10 min (7.6 ± 0.5 APs) was significantly different from control (3.4 ± 0.2 APs, * $P < 0.05$ compared to control, RM ANOVA followed by Holm-Sidak method). The numbers of APs from 6 sensory neurons remained unchanged after SEW2871 treatment for 10 min (SEW-insensitive, RM ANOVA). Following that, those neurons were given 1 μM S1P for another 10 min. Out of those 6 neurons, 1 neuron was lost during the

recording, the number of APs from 4 neurons (SEW insen. S1P insen.) was not significantly changed by S1P (RM ANOVA), but the number of AP firing from 1 neuron (SEW insen S1P sen.) was increased from 3 APs at 10 min to 8 APs at 12, 14, 16 and 20 min. Data were represented as means ± SEM.

5.3. SEW2871 increases the excitability in some, but not all, sensory neurons treated with phospholipase C (PLC) inhibitor U73122

The results from Figure 26 suggest that besides coupling to the Gαi/o-type G protein, S1PR1 may couple to another subtype of trimeric G protein to mediate S1P in enhancing excitability in sensory neurons. I wondered whether there is a possibility that S1PR1 couples to Gαq-type G protein. Upon activation, Gαq transduces the signal to activate PLC. PLC breaks down PIP_2 into IP_3 and DAG. IP_3 releases Ca^{2+} from intracellular storage, and the Ca^{2+}, together with DAG, activates downstream effector PKC. PKC has a variety of downstream targets, including mitogen-activated protein kinase kinase (MEK), to mediate cellular functions. To investigate the possibility that S1PR1 signaling activates PLC, I utilized a PLC inhibitor U73122 (Bleasdale et al., 1990; Shi et al., 2008) to disrupt signaling pathways that activate PLC in sensory neurons and then tested the effect of SEW2871 and S1P in increasing excitability.

Sensory neurons were first pretreated with 5 μM U73122 for 30 min and then exposed to 100 nM SEW2871. The excitability of the neurons exhibited two distinct profiles (Figure 27). In 3 out of 5 sensory neurons pretreated with U73122, the number of evoked APs was not significantly altered by SEW2871 (RM ANOVA). The three neurons that were insensitive to SEW2871 were further exposed to 1 μM S1P for 2 min, and S1P did not significantly change the number of APs (RM ANOVA). For the other 2 out of 5 neurons pretreated with U73122,

SEW2871 elevated the number of their AP firing, from 3.5 APs at control to 9.5 APs at 10 min, and the following S1P treatment led to 11 APs at 12 min.

The number of neurons recorded in this experiment is quite small. Thus, I hesitate to draw any conclusions from this preliminary data. Besides increasing the number of sensory neurons, it will be interesting to know the effect of SEW2871 on excitability in sensory neurons treated with both PTX and U73122.

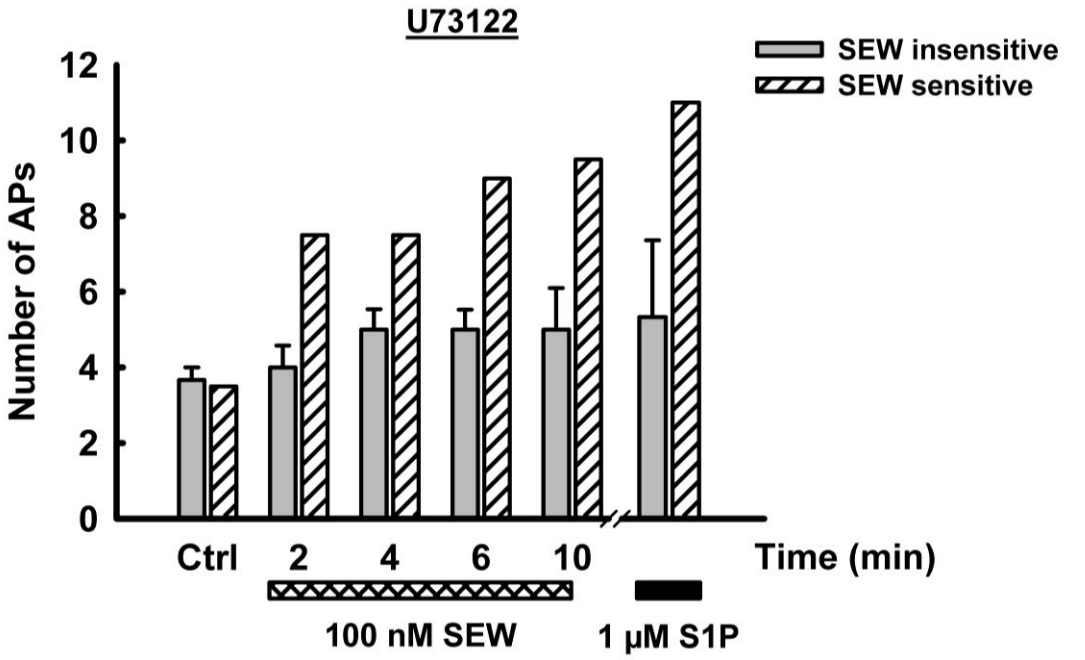

Figure 27. Sensory neurons pretreated with PLC inhibitor U73122 exhibit distinct profiles upon SEW2871 and S1P exposure.

Data show the number of APs firing in sensory neurons pretreated with 5 μM U73122 for 30 min during a 12-min recording period. The baseline firing was established at control, and then 100 nM SEW2871, a selective S1PR1 agonist, was perfused continuously in the bath solution for the first 10 min. Then the bath solution was replaced with 1 μM S1P for 2 min. The number of APs from 3 neurons (5 in total) was not changed by SEW2871 within 10 min (SEW-insensitive, RM ANOVA). Moreover, additional 1 μM S1P for another 2 min did not change the number of AP firing either. The other 2 neurons were sensitive to SEW2871, their average number of APs went up from 3.5 APs at control to 9.5 APs at 10 min, and S1P further increased the number to 11 APs at 12 min. Data were represented as means or means ± SEM.

5.4. Downstream signaling pathways of S1P/S1PR1 may converge on MEK

The previous two experiments suggest that S1PR1 may mediate S1P-induced increase in excitability through Gαi/o, Gβγ and/or Gαq G proteins signaling pathways. Gαi inhibits the activity of AC, leading to a reduction of the basal level of cAMP, which decreases PKA activity. Reduced PKA activity is not very likely to increase neuronal excitability. Therefore, it is not very likely that S1PR1 mediates S1P-induced increase in neuronal excitability through Gαi-cAMP-PKA pathway. On the other hand, the trimeric Gi/o-type protein complex contains Gαi/o and Gβγ subunits. Upon activation, the Gβγ subunits dissociate from the Gαi/o subunit. Studies have shown that Gβγ from Gαi/o can transduce signaling to change cellular functions in neurons, such as activating inward rectifier potassium channels (GIRKs) and PI3K (Berlin et al., 2010; Rosen and Goetzl, 2005). PI3K may function through the Ras-MEK-ERK pathway to increase neuronal excitability. Also, Gαq can activate downstream effector PLC which further activates PKC and MEK to modulate neuronal excitability.

Taken together, S1PR1 may mediate S1P-induced enhancement in excitability through both Gαi and Gαq pathways, and these two pathways may converge at MEK to increase neuronal excitability. To test this hypothesis, neurons were pretreated with 10 μM PD98059, an inhibitor of MEK (Alessi et al., 1995; Wiklund et al., 2002), and then the effect of S1P in increasing excitability was determined in those neurons. As shown in Figure 28, after pretreatment to inhibit MEK

activity, S1P did not significantly alter the number of APs neurons fired (RM ANOVA). At the control, the number was 3.5 ± 0.3 APs; 15 min after S1P treatment, the number was 2.0 ± 0.7 APs (n = 4). In regard to the small number of sensory neurons in this experiment, it is still too early to draw any conclusion at this point. Also, it is of interest to test the agonistic effect of SEW2871 on PD98059-pretreated neurons to provide us with a better picture of S1PR1 signaling. This preliminary data demonstrates the inhibition of MEK blocked S1P from increasing neuronal excitability, suggesting the downstream signaling pathways of S1PR1 and R3 to mediate S1P-induced excitability increase may converge on MEK.

The assumption of involvement of specific G proteins based on the changes in downstream effectors might be specious. Furthermore, it is worth mentioning that convergence of signaling pathways initiated by different G proteins, e.g. the activation of PLC by Gαq and Gβγ subunits released by Gαi/o, makes the identification of S1PRs and their coupled G proteins difficult. Therefore, more studies need to be performed before we have a good knowledge of S1PR1 signaling in sensory neurons.

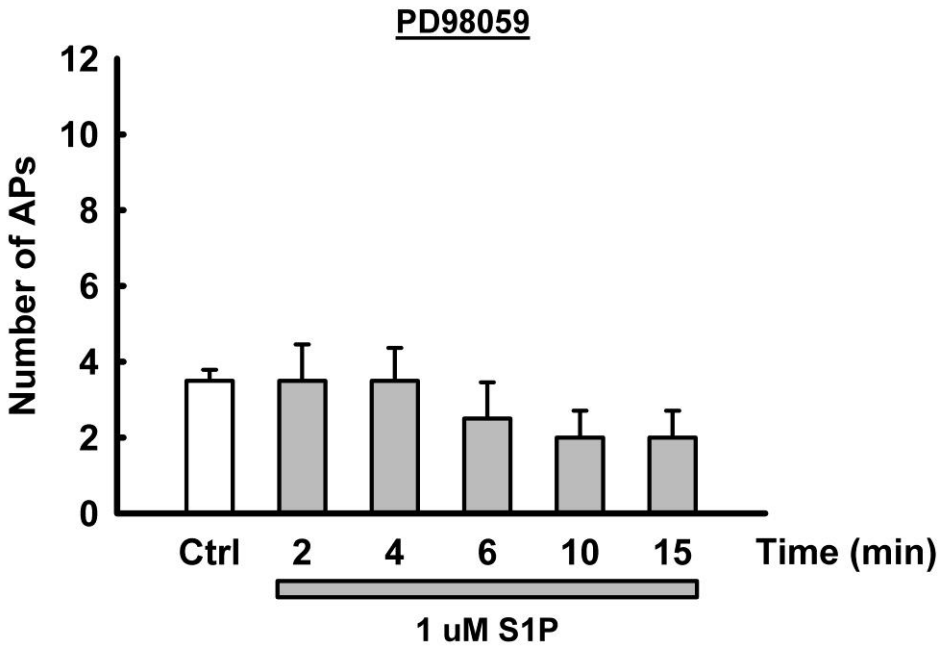

Figure 28. Neurons pretreated with PD98059, an MEK inhibitor, prevent S1P from increasing excitability.

Data show the number of APs firing in sensory neurons pretreated with 10 μM PD98059 for 30 min during a 15-min recording period. The baseline firing was established at control, and then 1 μM S1P was perfused continuously in the bath solution for 15 min. The number of APs in those neurons was not significantly changed by S1P (RM ANOVA, n = 4). Data were represented as means or means ± SEM.

6. Antagonism at S1PR1 prevents SEW2871 from increasing neuronal excitability, and S1PR1 is a potential target for pain therapeutics

Pain is an unpleasant feeling often caused by noxious stimuli. The sensation of pain warns us of real or impending injury, and diverts us away from potential damage. However, if the signals of pain remain active and unregulated, the pain may become chronic and debilitating. In the United States, approximately 30% of the population is living with chronic pain (Debono et al., 2013). Chronic pain may be classified into inflammatory nociceptive pain and/or neuropathic pain. Inflammatory nociceptive pain is associated with tissue damage and the inflammatory process, while neuropathic pain is caused by the damage to and the malfunction of the nervous system. People suffering from chronic pain often resort to medication. Current medication for chronic pain management, especially narcotic analgesics such as opioids, has the risk of drug addiction, tolerance and unpleasant side effects. Therefore, there is a need for renewed focus on novel targets that will be effective in treating chronic pain. Several studies in animal models have indicated that inflammatory and neuropathic pain is mediated through the S1P/S1PR1 axis (Doyle et al., 2011a; Doyle et al., 2011b; Mair et al., 2011; Patti et al., 2012). At the same time, our study demonstrates that S1PR1 plays a predominant role in modulating S1P-induced increase in excitability in sensory neurons. It is likely that at the systemic level, S1PR1 also plays a primary role in modulating nociceptive sensation and even pain syndrome.

Therefore, the successful antagonism at S1PR1 may possibly alleviate pain syndrome and provide potential pain therapeutics.

The idea of targeting S1PR1 as a novel pain therapeutic encompasses two aspects: first, to directly block S1PR1 activation, and second, to desensitize or internalize S1PR1 thus obstructing its effect. To test these two ideas in vitro, I performed the following two experiments.

6.1. NIBR-15, a potent and selective S1PR1 antagonist, prevents SEW2871 from enhancing neuronal excitability

The first idea of targeting S1PR1 is to directly block S1PR1 activation. To test this idea in vitro, sensory neurons without functional S1PR1 are going to be exposed to a S1PR1 specific agonist SEW2871, and their excitability will be observed. There are several ways to block S1PR1 activation, with a selective antagonist being the most straightforward approach. A novel, potent and selective S1PR1 antagonist NIBR-15 has been developed and shown to induce sustained peripheral blood lymphocyte reduction in rats when orally administered (Angst et al., 2012). Therefore, I decided to use NIBR-15 to answer the question as to whether NIBR-15 could block the excitatory effect of SEW2871.

First of all, since NIBR-15 as an antagonist was not well characterized in sensory neurons, I tested its possible off-target activity on sensory neurons by looking at

the change of neuronal excitability over time in the presence of NIBR-15. As shown in Figure 29A, sensory neurons were exposed to 10 nM NIBR-15 for 15 min. During that period, NIBR-15 did not significantly alter the number of APs (RM ANOVA). At control, the neurons generated an average of 3.7 ± 0.3 APs; at 15 min, the number was 3.7 ± 0.9 APs (n = 3). This result demonstrates NIBR-15 does not change neuronal excitability and excludes the possibility of an off-target effect on excitability from NIBR-15 in sensory neurons, and therefore, NIBR-15 will be used in the following experiment to block S1PR1 activation.

Next, sensory neurons were pretreated with 10 nM NIBR-15 for 30 min and then exposed to 100 nM SEW2871 for 15 min. As demonstrated in Figure 25, SEW2871 is capable of enhancing excitability in the majority of sensory neurons within 4 min. However, after the NIBR-15 pretreatment, SEW2871 failed to significantly elevate the number of APs within 15 min in a total of 17 neurons (Figure 29B, ANOVA on ranks). The average numbers of AP firing were 3.3 ± 0.1 APs at control and 3.5 ± 0.2 APs at 15 min. This result shows that NIBR-15 effectively blocked the function of S1PR1.

Following the 15-min recording, the SEW2871 was washed away and neurons were immediately exposed to 1 μM PGE2, a potent agonist to increase neuronal excitability through the Gαs pathway. This additional step serves as a positive control to confirm that the recorded neurons, though not responsive to SEW2871, were still capable of being sensitized by another compound acting through a

distinct GPCR. As shown in Figure 29B, PGE2 significantly increased the number of APs fired within 5 min (ANOVA on ranks followed by Dunn's method). The average number of AP was 9.8 ± 2.3 (n = 4), significantly different from the control (3.3 ± 0.1), and the 10-min (3.4 ± 0.2) and 15-min time points after SEW2871 treatment (3.5 ± 0.2), indicating the insensitivity to SEW2871 was not an artifact. This experiment suggests that in vitro, NIBR-15 is a functional antagonist on S1PR1 to block SEW2871-induced enhancement in excitability. It also provides support for NIBR-15 to be tested in vivo in terms of its potential analgesic effect to alleviate nociceptive behaviors.

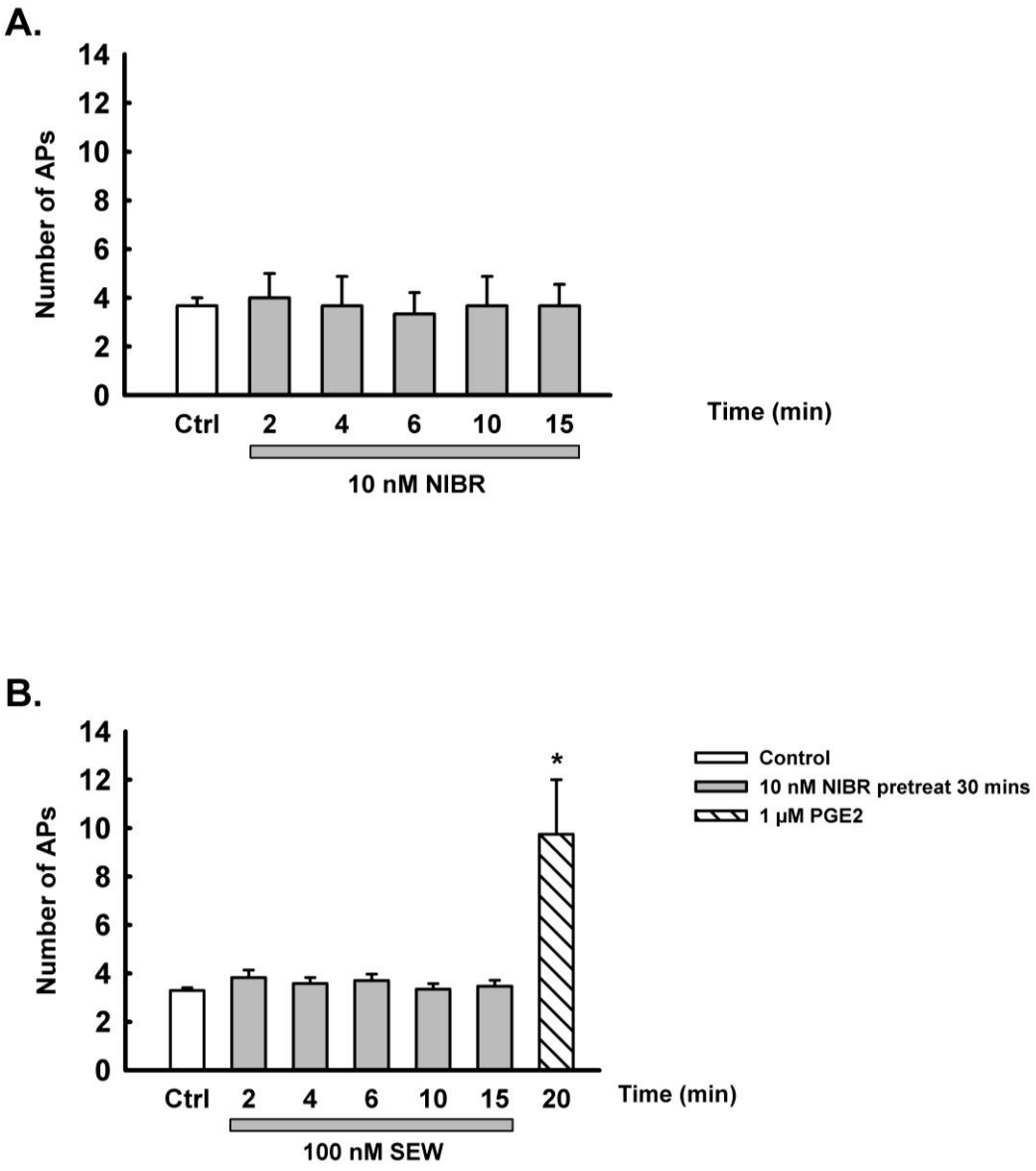

Figure 29. NIBR-15, a selective antagonist on S1PR1 blocked SEW2871 from increasing excitability in sensory neurons.

A shows the number of AP firing in 3 sensory neurons over a course of 15-min recoding with the presence of 10 nM NIBR-15. The baseline firing was established at control and then 10 nM NIBR-15 was perfused continuously in the bath solution for 15 min. The number of APs was not significantly altered by NIBR-15 within 15 min treatment (P = 0.87, RM ANOVA). B represents the

number of APs firing in sensory neurons pretreated with 10 nM NIBR-15 for 30 min during a 20-min recording period. The baseline firing was established at control, and then 100 nM SEW2871, a selective S1PR1 agonist, was perfused continuously in the bath solution for the first 15 min. Then the bath solution was replaced with 1 μM PGE2 for 5 min. The number of APs was not significantly changed by SEW2871 within 15 min (ANOVA on ranks compared to control). However, additional 1 μM PGE2 significantly increased the number of AP firing at 20-min point (* $P < 0.05$ compared to control, 10 min and 15 min, ANOVA on ranks followed by Dunn's method). The number of neurons were 17 within 15 min and 4 at 20 min. Data were represented as means ± SEM.

6.2. Pretreatment with S1PR1 agonist CYM-5442 prevents SEW2871 from increasing excitability, and thus CYM-5442 serves as a functional antagonist to block S1PR1 activation

The second idea of blocking S1PR1 function is achieved by an indirect approach. S1PR1 is a GPCR, and upon activation, S1PR1 gets desensitized and internalized, leaving cells void of S1PR1 on the membrane (Cahalan et al., 2011). Animals without functional S1PR1 in their sensory neurons may exhibit attenuated nociceptive behavior. To test this idea in vitro, sensory neurons are pretreated with CYM-5442, a selective agonist on S1PR1 and also a full agonist in vitro for S1PR1 internalization, phosphorylation, and ubiquitination (Gonzalez-Cabrera et al., 2008). The excitability of pretreated sensory neurons will be observed in the presence of SEW2871.

Much like FTY720, CYM-5442 is an agonist. Therefore, I decided to confirm its agonistic effect in sensory neurons. A total of 8 sensory neurons were exposed to 100 nM CYM-5442 for 15 min and the number of their AP firing was recorded over time. Interesting, similar to the other S1PR1 agonists, SEW2871 and FTY720, CYM-5442 increased the excitability of some, but not all, sensory neurons. There were two populations of neurons in the presence of CYM-5442 (Figure 30A): 4 neurons were sensitized and the other 4 were not sensitized by 100 nM CYM-5442. In the CYM-5442-sensitive group, the average number of AP firing at control was 3 ± 0.4 APs, and it significantly increased to 13.3 ± 3.7 APs

at 10 min and 14.3 ± 3.7 APs at 15 min (RM ANOVA on ranks followed by Tukey test). In the CYM-5442-insensitive group, the average number of AP firing was 3.5 ± 0.3 APs at control and 3.0 ± 1.1 APs at 15 min. This result confirms that CYM-5442 functions as an agonist in sensory neurons to increase excitability.

Having confirmed its agonist effect, 100 nM CYM-5442 was then used to pretreat sensory neurons. After a 1 hr pretreatment, the baseline AP firing of those neurons was established as the control (3.1 ± 0.1 APs). Then these neurons were exposed to 100 nM SEW2871 for 15 min. At 15 min, the number of AP firing was 4.0 ± 0.6, not significantly different from either the control or any time points over the 15-min recording period (Figure 30B, RM ANOVA). This result indicates that, as a selective S1PR1 agonist, CYM-5442 internalized the receptor and SEW2871 could no longer increase neuronal excitability through S1PR1. Therefore, CYM-5442, though an agonist, functions as an antagonist at S1PR1 in sensory neurons. This functional antagonism of CYM-5442 at S1PR1 is similar to that of FTY720 (Figure 20). The results from my FTY720 and CYM-5442 experiments both offer insight into the application of FTY720 and CYM-5442 in animal models where it may function as an antagonist to modulate nociceptive behaviors.

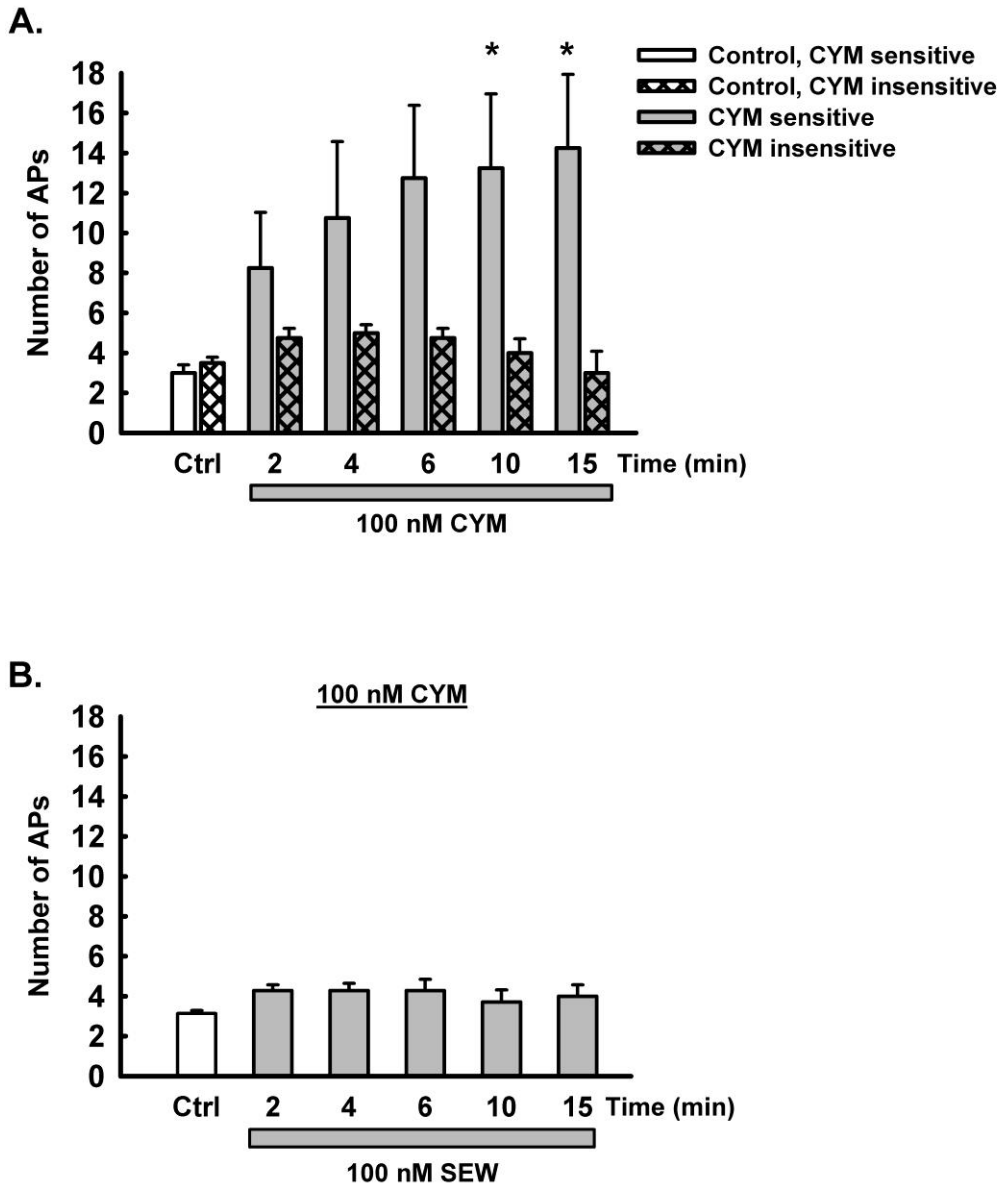

Figure 30. Pretreatment with CYM-5442, a selective S1PR1 agonist, prevents SEW2871 from increasing excitability in sensory neurons.

A shows the number of APs sensory neurons fired during a 15-min recording period with the presence of 100 nM CYM-5442, a selective S1PR1 agonist. The baseline firing was established at control, and then 100 nM CYM-5442 was perfused continuously in the bath solution for the next 15 min. The numbers of APs were recorded at 2, 4, 6, 10 and 15 min. There were two populations of

neurons after CYM-5442 treatment. The number of AP firing from 4 out of a total of 8 neurons was significantly altered by CYM-5442, at 10 and 15 min (* $P < 0.05$ compared to control, RM ANOVA on ranks followed by Tukey test). The number of APs from the other 4 neurons remained unchanged over the 15-min period (RM ANOVA on rank, $P = 0.09$). B represents the number of APs firing in sensory neurons pretreated with 100 nM CYM-5442 for 1 hr during a 15-min recording period. After the pretreatment, the baseline firing was established at control, and then 100 nM SEW2871, a selective S1PR1 agonist, was perfused continuously in the bath solution for 15 min. The number of APs was not significantly changed by SEW2871 between any two time points within a 15-min period (ANOVA, $n = 7$). Data were represented as means ± SEM.

DISCUSSION

1. S1PR expression patterns and receptor contribution to the S1P-induced increase in neuronal excitability

The expression patterns of S1P receptor subtypes vary spatially and temporally. In different tissues and cell types in the same animal, S1PRs exhibit unique expression profiles (Anliker and Chun, 2004). Likewise, at different stages of development, S1PRs have differential expressions (Meng and Lee, 2009). However, there are still some discrepancies across various studies on S1PR expression patterns, due to the fact that most early studies were conducted by using in situ hybridization, and there is a limitation to the sensitivity of this tool in detecting levels of mRNA expression.

In my thesis experiments, I utilized a sensitive and quantifiable technique, real-time reverse-transcription PCR, to investigate the expression of S1PR mRNA in small-diameter DRG neurons. Our results from real-time PCR experiments provide molecular evidence for the predominant role of S1PR1 in mediating the S1P-induced increase in excitability (Chi and Nicol, 2010), as we discovered that S1PR1 is the highest expresser among all five S1PR subtypes in about half of the small-diameter sensory neurons analyzed with single-cell qPCR (Figure 6), and that, in the same neuronal population, the overall expression of S1PR1 is the highest among all S1PRs (Figure 8).

Although at the mRNA level, the expression of S1PR3 in DRG neurons on average is similar to that of S1PR4 and R5, functionally, S1PR3 does, but S1PR4 and/or R5 do not mediate the S1P-induced enhancement of excitability in sensory neurons. The different contributions of S1PR3, R4 and R5 to excitability could be due to the different G proteins and the corresponding downstream signaling pathways with which S1PR3, R4 and R5 couple with. S1P may preferentially activate a certain signaling cascade that is unique to S1PR3, compared with S1PR4 and R5 (e.g. Gq-PLC pathway), to increase the excitability of small-diameter sensory neurons.

However, qPCR technique has its limitations. There are multiple steps from mRNA to mature and functional protein, including translation, posttranslational modification, and protein trafficking and sorting, as well as RNA interference. Each step adds to the complexity of protein expression. Therefore, mRNA expression is indicative of, but not necessarily equal to the amount of membrane protein expression, in our case, S1PRs expression. As shown in Figure 5, TRPV1 mRNA expression was detected by single-cell qPCR in about half of small-diameter sensory neurons, whereas about 90% of small-diameter sensory neurons showed capsaicin sensitivity by patch clamp recording. This discrepancy may be due to the limit of detection of single-cell qPCR technique, which reminds us that we should be cautious when interpreting negative results.

In my thesis, I have demonstrated that S1PR3 mediates S1P-induced enhancement of neuronal excitability. There has been no specific agonist at S1PR3, which makes directly studying the activation of S1PR3 to increase neuronal excitability difficult. However, selective knockdown of S1PR1 and R3 by siRNAs (Figure 21) and specific antagonism at S1PR1 and R3 (Figures 22, 23 and 24), abolished the excitatory effect of S1P, indicating the essential role of S1PR1 and R3 in S1P-induced sensitization. Taking our laboratory's previous finding into consideration that, besides S1PR1, other S1PRs also contribute to S1P-induced sensitization (Chi and Nicol, 2010), and based on the finding in my thesis that S1PR2, R4 and R5 do not mediate the S1P-induced sensitization, I conclude that S1PR3 is the other player in mediating S1P-induced enhancement of neuronal excitability. As shown in Figure 5, the S1PR expression profile is quite variable in different neurons, and therefore it is possible that in neurons where S1PR1 has the highest expression, S1PR1 mediates S1P-induced sensitization; whereas in neurons where S1PR3 is highly expressed, this receptor mediates S1P-induced sensitization; in some neurons where S1PR1 and R3 together have the highest expression, S1PR1 and/or R3 could mediate S1P-induced sensitization; and in some neurons where the expression of S1PR1 and/or R3 does not dominate, S1P does not increase excitability. This could be why a small number of sensory neurons were not sensitized by S1P. Therefore, S1P enhances neuronal excitability via S1PR1 and/or R3. It will be valuable to pair a neuron's sensitivity to S1P with its S1PR expression profile, and thus we

will have a better idea about the expression of each S1PR subtype in S1P-sensitive and S1P-insensitive neurons.

S1PR4 and R5 are less explored members of the S1PR family, and to date, there has been no report about their function in the peripheral nervous system (PNS). S1PR5 was reported to be expressed in the CNS, especially the brain, and in the spleen (Im et al., 2000; Terai et al., 2003). However, a recent study from our laboratory, together with my results (Figure 5), shows that S1PR5 is expressed in the rat lung, brain and DRG tissues, as well as single isolated DRG neurons (Kays et al., 2012). The mRNA expression profiles of S1PR4 and R5 in small-diameter sensory neurons from our study show that their average expression is lower than S1PR1, the most highly expressed subtype and the predominate player in modulating excitability. In some neurons, either S1PR4 or R5 is the highest expresser among all five S1PRs. This raises an interesting question whether S1PR4 and/or R5 are functionally critical to regulate S1P-induced sensitization. My results demonstrate that S1PR4 and R5 do not mediate S1P-induced increase in excitability (Figures 10, 23 and 24). Neurons treated with a pooled siRNAs targeted to S1PR1, R2 and R3, presumably leaving only S1PR4 and R5 subtypes on the membrane, failed to respond to S1P (Figure 10). Moreover, the excitability of naive neurons was not changed by either VPC23019 or VPC44116, both agonists at S1PR4 and partial agonists at R5 while being antagonists at S1PR1 and R3 (Figures 23 and 24). However, the results do not exclude the possibility that S1PR4 and/or R5 may play an auxiliary role in

mediating excitability, such as they could have structural and/or functional significance to dimerize with S1PR1 and/or R3 to assist the signaling transduction. Before we can definitively conclude that S1PR4 and R5 are not involved in S1P-induced enhancement in excitability, further experiments are needed, with one possible experiment being to measure neuronal excitability in response to S1P in sensory neurons with S1PR4 and R5 knockdown by siRNAs.

On average, S1PR2 has the lowest expression among all five S1PRs in small diameter sensory neurons. In accordance with its expression, S1PR2 by itself is not sufficient to mediate the ability of S1P to increase excitability. Pretreatment with FTY720, an agonist at all S1PRs but R2, presumably led to the internalization of activated S1PRs in sensory neurons, conceivably leaving S1PR2 the only functioning S1PR subtype on the membrane. In sensory neurons pretreated with FTY720, S1P failed to significantly elevate excitability (Figure 20), suggesting S1PR2 does not play a direct role in enhancing neuronal excitability. However, it is still possible that S1PR2 plays an indirect and synergistic role in enhancing excitability only with the presence of S1PR1 and/or R3. Further experiments ruled out this possibility (Figures 22, 23 and 24), because even in the presence of S1PR1 and R3 with their function being pharmacologically blocked by antagonists, S1P did not alter the neuronal excitability through S1PR2. These results suggest S1PR2 does not mediate the ability of S1P to increase neuronal excitability. Taken together, my results demonstrate that S1PR1 and/or

R3 are essential in S1P-induced elevation of excitability in sensory neurons, while S1PR2, R4 and R5 do not mediate such enhancement.

2. Tools for studying S1PR functions -- siRNAs

Because of the diverse expression profile of S1PRs across single DRG neurons, in order to study the function of each individual receptor subtype, genetic, molecular, and/or pharmacological tools are needed to isolate an individual receptor of interest while eliminating the influence of other receptor subtypes. Genetic perturbation of S1PRs to cause a loss of function or a gain of function of a certain receptor subtype is an effective way to study receptor function. An early study from our laboratory demonstrated that S1PR1 plays a prominent, but not exclusive role in mediating the S1P-induced increase in excitability (Chi and Nicol, 2010). Therefore, the goal of my thesis is to identify other S1P receptor(s) that are also involved in mediating excitability. This requires the elimination of S1PR1 in order to establish the contribution of other S1PRs to regulating the S1P-induced increase in excitability. An efficient approach to eliminate the predominate effect of S1PR1 in S1P-induced sensitization is to utilize S1PR1 knockout animals to study the function of other S1PRs in sensory neurons. Unfortunately, $S1PR1^{-/-}$ mice are embryonically lethal (Liu et al., 2000b). This prevents us from using knockout animals, and therefore we had to search for other tools to address the question.

RNA interference (RNAi) is a biological process in which RNA molecules inhibit gene expression at the post-transcriptional level by destabilizing or destroying specific mRNA molecules. In 1998, double-stranded RNAs (dsRNA) were first

discovered to silence gene function in *Caenorhabditis elegans* (Fire et al., 1998). Shortly after that, the same phenomenon was observed in plants (Hamilton and Baulcombe, 1999). The mechanism to cause gene silencing is to destroy specific mRNAs. The mediators of the sequence-specific mRNA degradation are 21- and 22-nucleotide small interfering RNAs (siRNAs) generated from dsRNAs (Hammond et al., 2000). In 2001, the first synthetic exogenous siRNA was shown to induce sequence–specific gene suppression in mammalian cells in vitro. Since then, siRNA has become a widely used tool to manipulate gene expression and function in both basic and translational research.

Previously, our laboratory has successfully utilized siRNA targeted to S1PR1 to specifically knock down receptor expression, and the consequences of S1PR1 knockdown were shown by Western blot and patch clamp techniques. siRNA targeted to S1PR1 significantly reduced the S1PR1 protein level by about 75%, and totally abolished the effect of the S1PR1 selective agonist SEW2871 to increase neuronal excitability (Chi and Nicol, 2010). I designed siRNAs targeted to S1PR2 and R3 to study their respective functions in sensory neurons. To validate the effectiveness of those siRNAs, I decided to use Western blotting to measure protein expression. However, due to the poor quality of the antibodies against S1PR2 and R3, it was impossible to accurately detect and quantify S1PR2 and R3 proteins. Therefore, I had to take an alternative approach to determine the effectiveness of siRNA knockdown by measuring the mRNA levels of S1PR2 and R3.

By using qPCR, the relative mRNA levels (normalized to the reference gene Arbp) of S1PR2 and R3 were determined. Compared with the untreated control, siRNA targeted to S1PR2 or R3 exhibited a significant effect on reducing its corresponding amount of mRNA. Interestingly, I also tested the effectiveness of siRNA targeted to S1PR1 by qPCR, the same siRNA used in the previous published experiments (Chi and Nicol, 2010). The result shows that the siRNA induced about a 65% reduction in S1PR1 mRNA expression compared with the control, which correlates well with 75% reduction in S1PR1 protein shown by Western blot. These data gave me confidence to believe that siRNAs targeted to S1PR2 and R3 might have a significant knockdown effect on S1PR2 and R3 proteins.

One advantage of using qPCR to characterize siRNAs is that the expression of multiple genes can be detected at the same time. Therefore, not only could I determine the effectiveness of one siRNA, but also I could examine the specificity of the same siRNA to other targets (off-target effects). Since S1P receptor subtypes share a high sequence similarity, siRNA targeted to one subtype may have off-target effects and influence the expression of other subtypes, which may confound the interpretation of our results. To rule out the possible off-targets of siRNAs, I examined the influence of one siRNA targeted to a specific S1PR on the mRNA expression of the other S1PR subtypes (Figure 9).

Additionally, in order to establish the function of multiple S1PRs subtypes, I combined siRNAs targeted to different S1PRs together to knock down receptor expression. Because of its easy manipulation, the siRNA technique allowed me to carry out various combinations of siRNAs targeted to different S1PRs to address my questions. One concern related to the pooled siRNAs approach is whether each individual neuron is transfected with all the pooled siRNAs. To address this concern, each siRNA was labeled with a different fluorescence tag. Before recording from a neuron, I first confirmed that the neuron had taken up the pooled siRNAs by detecting the presence of different fluorescence tags in the neurons using the fluorescence microscope. In the case of Figure 21 where sensory neurons were transfected with two types of siRNAs targeted to S1PR1 and R3 respectively, about 85% of neurons had both siRNAs. More importantly, almost all neurons taking up one type of siRNA also took up the other. The distribution of siRNAs showed no pattern, as they scattered within the cells randomly. In the experiment shown in Figure 10, I combined three types of siRNAs targeted to S1PR1, R2 and R3. Although I did not verify the transfection of neurons by the pooled siRNAs, based on the observation from transfection by two types of siRNAs, it was quite possible that the majority of neurons were transfected with all three types of siRNAs.

3. Tools for studying S1PR functions – agonists and antagonists

S1PRs were newly discovered and characterized in the late 1990s. So far, there have been a few ligands that function as selective agonists and/or antagonists on specific S1PRs (Im, 2010). Most of these newly developed agents are commercially available and increasingly used to study the function of S1PR subtypes in specific biological systems or cells. However, beyond characterization using receptor-transfected cell lines in vitro, most ligands have often not been screened for specificity against a wide array of targets, nor have they been studied systemically in vivo. Therefore, without knowing the possible off-target effect of a certain ligand, we should always be cautious when first utilizing that ligand to study S1PR(s) function in sensory neurons.

A good example of the above concern is our unexpected finding of JTE-013 increasing neuronal excitability. JTE-013 was developed by the Central Pharmaceutical Research Institute, Japan Tobacco Incorporation (Patent WO 01/98301; December 27, 2001). It was confirmed that JTE-013 inhibited the specific binding of radiolabeled S1P to membranes of CHO cells stably transfected with human S1PR2 and rat S1PR2, with IC_{50} values of 17 ± 6 nM and 22 ± 9 nM, respectively. Oppositely, at concentrations up to 10 μM, JTE-013 did not affect the S1P binding to membranes of CHO cells transfected with human S1PR1; 10 μM JTE-013 inhibited S1P binding to membranes of CHO cells expressing S1PR3 by only 4.2% (Ohmori et al., 2003).

JTE-013 was used in several studies where it showed an antagonistic effect to S1P through S1PR2. In vascular smooth muscle cells (SMCs), which abundantly expressed S1PR2 protein, JTE-013 reversed the effect of S1P on inhibition of migration through S1PR2 (Osada et al., 2002). S1P elicits coronary vasoconstriction in vivo and strongly induces contraction of human coronary artery smooth muscle cells (CASMCs), and this latter effect was inhibited by JTE-013 (Ohmori et al., 2003). S1P also induces inhibition of B16 melanoma cell migration and invasion through activation of RhoA and inhibition of Rac via S1PR2, which was abrogated by JTE-013 (Arikawa et al., 2003).

When we decided to use JTE-013 to study the contribution of S1PR2 to the S1P-induced increase in neuronal excitability, we realized that JTE-013 had not been used in sensory neurons. Therefore, to rule out the possible off-target effect of JTE-013, we first performed a control experiment to measure the effect of JTE-013 on sensory neurons to alter neuronal excitability over time. Surprisingly, we found that 100 nM JTE-013 significantly increased the neuronal excitability in a concentration- and time-dependent manner (Figure 12). The elevated excitability was observed even in neurons that did not express S1PR2 mRNA (Figure 16). Furthermore, the enhancement of excitability by JTE-013 was abolished by GDP-β-S, as well as PTX and W146, a S1PR1 specific antagonist, indicating the excitatory effect of JTE-013 was mediated by another GPCR, possibly S1PR1. However, without understanding the metabolism of JTE-013, it is too soon to

claim that the sensitizing effect of JTE-013 is due to its agonistic activity on S1PR1. In addition, the initial study on JTE-013 demonstrates that JTE-013 did not affect the S1P binding to S1PR1 at a concentration as high as 10 μM (Ohmori et al., 2003). Our study shows that as low as 10 nM JTE-013 is enough to elevate excitability. This evidence together lessens the possibility that JTE-013 acts as an agonist on S1PR1 to increase neuronal excitability, but raises the possibility that JTE-013 enhances excitability through another unidentified GPCR in sensory neurons.

One caveat with increased neuronal excitability by JTE-013 is related to the authenticity of the commercialized drug, e.g., the concern can be whether the JTE-013 we used is structurally identical to the one as was first developed. We had limited chemical tools to address this concern, albeit we eliminated this possibility by using JTE-013 from several different purchase orders indicating different batches and we acquired the same result. In addition, we repeated the same experiment done by Arikawa et al. (2003) to show that JTE-013 reversed S1P-induced inhibition of migration in B16 cells, as an indication that JTE-013 we used was indeed an antagonist at S1PR2.

This off-target effect of JTE-013 is not the only one reported in the literature where the selectivity and specificity of JTE-013, as well as some other S1P ligands, are put into question. S1P constricts basilar artery in rats, wild type mice and S1PR2-null mice, but barely affects vascular tone in S1PR3-null mice,

indicating vasoconstriction is mediated through S1PR3. Surprisingly, 10 μM JTE-013 inhibited S1P-induced vasoconstriction in the wild type mice, suggesting S1PR2 instead of, or in addition to, S1PR3 regulates the S1P effect. Further investigation showed that JTE-013 inhibition of vasoconstriction was not only restricted to S1P, but also to the prostanoid analog U46619 (acting on thromboxane A2 receptor), endothelin-1 (acting on ET_A and ET_B receptors), and high KCl (vasoconstriction induced by high KCl is not receptor-mediated but related to L-type calcium channels). More importantly, JTE-013 inhibited S1P-induced vasoconstriction in S1PR2-null mice (Salomone et al., 2008). These results demonstrate that JTE-013 is not a selective antagonist at micromolar range, with other non-specific targets besides S1PR2.

The effect of JTE-013 warned us of being cautious in the future when using putative S1PR specific ligands, as they may act on unidentified targets to complicate our experiment results. To minimize the risk of using a possible non-selective agent, firstly, I would perform a control experiment to show that the drug, if not well characterized in sensory neurons, does not alter the excitability of sensory neurons by itself over a certain period of time (usually 20 min). Secondly, I would utilize another technique to verify the findings I make from using a specific ligand. For example, I combined S1PR1 specific antagonist, W146, and S1PR3 specific antagonist, CAY10444 to examine the role of S1PR1 and R3 in mediating neuronal excitability. In addition to that, I used siRNA to knock down S1PR1 and R3 mRNA expression to confirm the finding that S1PR1 and R3 are

essential in S1P-induced sensitization (Figures 21 and 22). Thirdly, if the result obtained from using a pharmacological ligand is not confirmed by another approach, I would use a different ligand with similar pharmacological properties to corroborate the finding. For example, the experiment to determine the contribution of S1PR subtypes to S1P-induced increase in neuronal excitability by using VPC23019, an antagonist of S1PR1 and R3 while a full agonist of S1PR4 and a partial agonist of S1PR5, is further supported by using its phosphonate analog of VPC44016, which is also an antagonist of S1PR1 and R3, an agonist of S1PR4 and a partial agonist of R5 (Figures 23 and 24). Both experiments demonstrate that S1PR1 and R3 are, but S1PR2, R4 and R5 are not, essential in mediating S1P-induced sensitization in sensory neurons.

CAY10444 is a selective S1PR3 antagonist. It was developed based on the structural information for S1P. When tested at 10 μM, CAY10444 exhibited an inhibitory effect on the S1P-induced increase in intracellular Ca^{2+} in HeLa-S1PR cells, with 37% inhibition in S1PR3 expressing cells while only 7% in S1PR1 expressing cells (Koide et al., 2002). This study measured a single concentration of CAY10444 against two potential targets, S1PR1 and R3, in one assay. Based on this initial study, CAY10444 is then used as a selective S1PR3 antagonist in several other studies. One study showed that 10 μM CAY10444 inhibited the rise in intracellular Ca^{2+} concentration induced by S1PR3, as well as by S1PR2 and by purinergic P2 receptor or α(1A)-adrenoceptor stimulation. But CAY10444 did not affect the S1PR3-mediated decrease of forskolin-induced cAMP

accumulation (Jongsma et al., 2006). Another study using a cell-based receptor-specific expression assay discovered that S1PR3 cell line response to S1P was inhibited by 22% by 100 μM CAY10444 (Wetter et al., 2009).

In light of the inconsistency in the literature regarding the efficacy and specificity of CAY10444, I decided to first test the effect of 10 μM CAY10444 by itself on sensory neurons. I found that CAY10444 did not significantly alter the neuronal excitability. The average number of evoked APs by sensory neurons at the control, and 2, 4, 6 and 10 min after CAY10444 perfusion was 4.0, 4.7 ± 0.3, 5.0 ± 0.6, 5.7 ± 0.3, 5.0, 6.7 ± 1.2 (data presented as means ± SEM, n = 3, RM ANOVA). These data eliminate the possibility that the off-target effects of CAY1044 changes neuronal excitability, and therefore helps strengthen my finding using W146 and CAY10444 to show that S1PR1 and R3 are essential in S1P-induced increase in neuronal excitability.

VPC23019 was initially developed as an antagonist at S1PR1 and R3, also behaving as an agonist at S1PR4 and a partial agonist at R5. Further experiments showed that 10 nM VPC23019 blocked the migration of T24 cells expressing human S1PR1 induced by the selective S1PR1 agonist VPC22277. Cell-based receptor binding assays demonstrated that the antagonistic effect of VPC23091 at S1PR3 was about 10-fold less potent than at S1PR1(Davis et al., 2005). However, one studying using CHO cells expressing the human S1PR3 shows that VPC23019 behaved as a full agonist in the inhibition of cAMP

accumulation, and displayed partial agonist activity in the elevation of intracellular Ca^{2+} concentration (Jongsma et al., 2009).

These results remind me that I should be cautious when interpreting the data from using VPC23019. Meanwhile, I realized it was worthwhile to corroborate the finding by using another compound with similar agonistic and antagonistic activities as VPC23019. VPC44016, produced by modifying the phosphate head group of VPC23019 (Davis et al., 2005), is such a compound. It is an antagonist at S1PR1 and R3, an agonist at S1PR4 and a partial agonist at S1PR5. I obtained the same result using both compounds; VPC23019 or VPC44016 did not change the excitability of sensory neurons, indicating S1PR4 and R5 are not essential in mediating neuronal excitability. Also, pretreatment with VPC23019 or VPC44016 prevented S1P from increasing neuronal excitability, suggesting S1PR1 and/or R3 are crucial in mediating S1P-induced enhancement in excitability.

In contrast to other S1PR ligands, FTY720 (fingolimod) has more detailed and available pharmacological data documenting its properties and therapeutic effects. It is an FDA- and EMA-approved drug for treating multiple sclerosis. Once phosphorylated to FTY720 phosphate (FTY720P), FTY720P binds to and acts as an agonist on all S1PRs except S1PR2. In vivo, FTY720P inhibits the egress of lymphocytes by downregulating activated S1PR1 and disrupting the chemotaxis to S1P, and thus the lymphocytes are sequestered in the lymph

nodes and prevented from traveling to the CNS to initiate autoimmune responses seen in the multiple sclerosis patients (Kappos et al., 2010; Matloubian et al., 2004).

In CHO cells expressing S1PR1, 1 μM FTY720P led to internalization of S1PR1 within 60 min of treatment (Mullershausen et al., 2009). Based on these data, I decided to use 1 μM FTY720 to pretreat sensory neurons for 1 hr, supposedly enough time for the compound to be phosphorylated by intracellular SphK2, be exported outside the neuron, and activate all S1PRs but R2. The activation of S1PRs will result in the internalization of S1PRs, thus disrupting the S1PR signaling (except for S1PR2). The FTY720 pretreated sensory neurons would have S1PR2 as the only functional S1PR subtype on the membrane, and I could then study the contribution of S1PR2 in S1P-induced sensitization. I found that S1PR2 is not sufficient in mediating S1P-induced increase in neuronal excitability (Figure 20).

Of note, there were three control experiments I performed to support the above finding. First, I confirmed the agonistic activity of FTY720P by showing that acute application of 100 nM FTY720 significantly increased the excitability in about half of sensory neurons. However, there was a delay in the excitatory effect of FTY720: the increase in excitability did not become significant until 15 min after FTY720 exposure (Figure 18), while S1P can significantly augment neuronal excitability within 6 min (Zhang et al., 2006a). This phenomenon of delayed

FTY720 response likely reflects the process where FTY720 needs to be first phosphorylated to FTY720P and transported outside the neuron to exhibit its agonist activity. The idea that FTY720 must be phosphorylated by neuronal SphK2 was confirmed by a second control experiment where intracellular application of SKI-2, a selective SphK inhibitor (Orr Gandy and Obeid, 2013), prevented FTY720 from increasing neuronal excitability (Figure 19). Third, after sensory neurons were pretreated with FTY720 for 1 hr and before being exposed to S1P, they were treated with SEW2871, a selective S1PR1 agonist. I found that SEW2871 did not enhance the excitability of FTY720-pretreated neurons within 10 min (Figure 20), indicating FTY720 had desensitized and/or internalized S1PR1, presumably S1PR3, R4 and R5 as well.

Besides FTY720P, several other S1P ligands have been shown to cause receptor internalization, including S1P, SEW2871 and CYM-5442. Interestingly, the fate of internalized S1PRs, either recycling back to the membrane or undergoing degradation, depends on the ligand. S1P and SEW2871 internalize S1PR1 following agonist binding, but they induce negligible receptor polyubiquitination even at a concentration of 10 μM with saturated receptor occupancy. The internalized S1PRs are captured in large vesicles and recycled back to the plasma membrane, where signaling can be reinitiated (Gatfield et al., 2014). In contrast, FTY720P and CYM-5442 induce significant polyubiquitination of S1PR1 at nanomolar concentrations. The internalized S1PRs are rapidly sorted to the lysosomes for degradation (Rosen et al., 2009). The distinct

properties of various S1PR agonists to dictate the fate of activated S1PRs provide insight into the therapeutic application of certain agonists in modulating nociceptive behavior or even alleviate pain. FTY720P and CYM-5442 are ideal drug candidates because their activation of S1PRs leads to receptor internalization and degradation, eventually terminating the S1PR signaling. The termination of the signaling obstructs the ability of S1P to increase neuronal excitability, which may result in reduced nociception.

4. Possible oligomerization of S1PRs

Our results demonstrate that S1PR1 and/or S1PR3 mediate the S1P-induced increase of excitability in small-diameter sensory neurons. Data from our previous study shows that S1PR1 contributes to S1P-induced enhancement of excitability in about 50% of small-diameter sensory neurons, while other S1PR subtype(s) contribute in about 30% of neurons (Chi and Nicol, 2010). Taken together, it is reasonable to conclude that S1PR3 is the other S1PR subtype besides S1PR1 that contributes to the increased excitability caused by S1P in about 30% of sensory neurons. However, S1PR2, R4 and R5 do not mediate S1P-induce increase of neuronal excitability.

It would be ideal if I could directly examine the contribution from S1PR3 to the increased excitability in sensory neurons. This would require me to show the direct activation of S1PR3 to augment neuronal excitability, and to determine if the percentage of sensory neurons sensitized exclusively via S1PR3 fits with the previously finding, about 30%. Unfortunately, there is no selective S1PR3 agonist available, which makes examining the role of S1PR3 in mediating excitability difficult. It is possible that I can confirm the percentage of S1PR3 contribution to increased excitability indirectly by either blocking the receptor activation pharmacologically (CAY10444) or knocking down S1PR3 expression by siRNA. However, these experiments cannot eliminate the predominant effect of S1PR1 to mediate S1P-induced sensitization.

On the other hand, if I focus on the percentage of contribution from each S1PR subtype in S1P-induced sensitization, I will run into the problem that I assume each S1PR subtype functions independently, without structural interaction or functional overlapping. Yet, GPCRs may structurally dimerize and functionally share some common downstream signaling pathways. Therefore, I cannot neglect discussing the possibility that S1PR1 and R3 dimerize and function synergistically to mediate S1P-induced enhancement of excitability. That said, it is more important to recognize that S1P increases neuronal excitability through S1PR1 and/or R3, than to determine the percentage of contribution each receptor subtype makes in regulating excitability.

S1PRs are members of the class A GPCR family, which may form homodimers, heterodimers, or even higher-order oligomers to mediate neuronal excitability. The oligomers can function in multiple forms, like S1PR1 homodimers, S1PR3 homodimers, S1PR1/R3 heteromers, and S1PR1 or R3 forming heteromers with other S1PR subtypes. The formation of oligomers may play an important role in modulating GPCR signaling, influencing ligand binding, receptor structure and/or intracellular signaling.

Allosterism can be defined as the process in which the binding and interaction of a compound at one location (the allosteric site) of an enzyme or a macromolecular complex affects the binding and function of the same or another

compound at a topographically distinct location (possibly the orthosteric site). This definition provides a framework to understand the properties of GPCR oligomers from three interacting components: the “modulator,” a ligand that binds to the “conduit” (a receptor or receptor complex), which transfers the allosteric effect to the “guest,” the target of the modulation. As for GPCRs, there are three different kinds of allosteric modulations depending on the targets of the modulation. If the target of the modulation is another ligand cobinding with the allosteric modulator, this is referred to as classic allosterism. If the target of the modulation is in the cytosol, it is called cytosolic allosterism. If the target of the modulation interacts with other receptors in close vicinity on the membrane, this is referred to as lateral allosterism (Kenakin and Miller, 2010).

One example for the classic allosterism is the case of a ligand that allosterically modulates the effect of an orthosteric ligand to change its affinity for the receptor and its intrinsic efficacy. In the cytosolic allosterism, modulated targets are cytosolic signaling effectors, such as G proteins, GRKs and β-arrestins. This type of allosterism can lead to functional selectivity, the ability of a ligand to preferentially activate a specific signaling pathway. One model for the lateral allosterism is the dimer-cooperativity model (Ferre et al., 2014). The model defines that there is a positive and a negative cooperativity, in which the binding of a ligand to the first receptor increases or decreases the affinity and efficacy of a ligand for the second receptor, respectively. In our case, for example, this model can be described as that S1PR1 or R3 form homodimers or S1PR1 and

R3 form heterodimers. The binding of S1P to one receptor subtype positively increases S1P binding affinity and functional efficacy to another subtype; therefore, S1PR1 and R3 cooperatively modulate S1P-induced enhancement of neuronal excitability. However, it is very difficult to measure this positive cooperativity between S1PR1 and R3 in sensory neurons. To achieve that, we have to first identify three sensory neuron populations, one expressing only S1PR1, one expressing S1PR3 only, and one expressing both S1PR1 and R3, and then compare the excitatory effect induced by S1P in each population to determine the extra enhancement of excitability through S1PR1/R3 cooperativity. However, it is almost impossible to categorize sensory neurons based on the above criteria because each individual neuron displays a distinct expression profile of S1PRs. Furthermore, our end point of measuring neuronal excitability is the number of evoked APs, which may encounter a ceiling effect, meaning that the excitatory effect of S1P mediated through one S1PR (S1PR1 or S1PR3) already maximizes the number of APs a sensory neuron can generate. Therefore, the added effect of increased neuronal excitability mediated by the cooperativity between S1PR1 and R3 may not be reflected by the number of APs. However, if we can identify another parameter to measure increased neuronal excitability, like the Na^+ or Ca^{2+} current, the degree of which has a linear correlation with excitability, we may be able to measure the functional positive cooperativity between S1PR1 and R3.

Besides functional measurements, there are several biochemical and biophysical methods to measure dimerization as well, with the limitation being that structural binding does not always translate into a functional outcome. Such methods include fluorescence/bioluminescence resonance energy transfer (FRET and BRET) techniques (Kaczor and Selent, 2011). These techniques are able to sensitively monitor the distances between two labels (fluorescence or bioluminescence) at the nanometer scale, which provides a tool to detect the conformational change within a receptor and protein-protein interactions (between GPCRs and G proteins, or between homo-/hetero-GPCR dimers).

5. Functional selectivity of S1P at S1PRs

S1P has a similar affinity on the five receptor subtypes (several nM for S1PR1, R2, R3 and R5, and tens of nM for S1PR4), but our data clearly shows that S1P has biased efficacies to increase excitability of small-diameter sensory neurons through S1PR1 and/or R3, but not S1PR2, R4 and R5. From a GPCR functional selectivity standpoint, this phenomenon can be explained from three aspects, the ligand binding, the receptor interaction and the intracellular effectors. I have discussed the second point, the receptor interaction/oligomerization, in the previous section, and here I am going to use the theory of functional selectivity to elaborate the other two points.

Functional selectivity is defined as the intrinsic efficacy of a ligand for different signaling pathways. Individual ligands, either orthosteric or allosteric, stabilize a particular active conformation of the GPCR and thereby render the difference in GPCR conformation to the activation of different downstream signaling pathways coupled to the same receptor. This phenomenon is also called “stimulus bias.” The rationale behind functional selectivity is that one ligand can change the structure of a GPCR to a confirmation that favors or impairs the binding of a particular signaling protein, leading to biased agonism or antagonism (Reiter et al., 2012). Importantly, functional selectivity can be induced directly by an orthosteric ligand, or indirectly by an allosteric ligand that affects orthosteric binding. Functional selectivity reflects the allosteric nature of GPCRs: the binding

of an extracellular ligand stimulates a conformational change that is transmitted to distinct intracellular sites, which then selectively engage different signaling effectors (Lane et al., 2013).

In our case of S1P, it elevates neuronal excitability through S1PR1 and/or R3. S1PR1 couples with the Gi/o-type G protein, while S1PR3 couples with Gi/o-, Gq-, G12/13-type G proteins. Each signaling pathway downstream of a G protein has different molecular targets, and elicits various cellular activities. It is probable that not all signaling pathways downstream of S1PR1 and/or R3 are activated to the same extent and thus contribute equally to the enhanced excitability induced by S1P. Therefore, it is important to understand the functional selectivity of S1P on S1PR1 and R3, meaning which intracellular signaling cascade S1P activates through S1PR1 and/or R3 to increase neuronal excitability.

Although there are hundreds of distinct GPCRs, signal transduction initiated by the receptors is dependent on the intracellular heterotrimeric G proteins. There are 23 Gα units, 5 Gβ units and 12 Gγ units, and thus about 1000 combinations of Gα and Gβγ are possible. Gα subunits play a major role in defining the specificity of a certain GPCR-G protein signling cascade; the contributions of βγ subunits are less clear (Gibson and Gilman, 2006). With regard to S1PR1, it couples to Gi/o-type G protein, and upon receptor activation, GDP-bound Gαi subunit exchanges the GDP molecule for GTP, leading to Gαi activation. The active form of Gαi inhibits AC to reduce intracellular cAMP levels, which leads to

the reduction of PKA activity. Increased PKA activity leads to increased neuronal excitability (England et al., 1996). It seems unlikely that decreased PKA activity would eventually increase neuronal excitability, and therefore, it is unlikely that S1PR1 mediates S1P-induced excitability through Gαi signaling pathway. Several studies have shown that activated heterotrimeric Gi/o protein dissociates GαGTP and Gβγ subunits. The Gβγ dimer can also modulate a series of cellular activities, such as the activation of phospholipase A_2 (PLA_2), PLC, PI3K, ERK, the opening of G protein-coupled inwardly-rectifying potassium channels (GIRKs), and the inhibition of Ca^{2+} channels (Bondar and Lazar, 2014; Bromberg et al., 2008; Leurs et al., 2005). Taking the functional selectivity of S1P into consideration, it is likely that S1P has an agonistic bias towards activating Gβγ subunit through S1PR1 to enhance neuronal excitability.

S1PR1 is reported to exclusively couple to the Gi/o-family of G proteins (Windh et al., 1999). PTX ADP-ribosylates the Gαi/o subunit to disrupt the activation of Gi/o. Therefore, PTX should block the signaling downstream of S1PR1. Surprisingly, I found that PTX did not block the signaling of S1PR1 activated by SEW2871 to increase excitability in all sensory neurons (Figure 26). Besides the possibilities that PTX treatment in my experiment was insufficient to completely block Gi/o signaling, another explanation is that S1PR1 may couple to another G protein. Interestingly, there is one study that also puts the S1PR1 coupling of G protein into question. In S1PR1-expressing HEK293 cells, S1P induced the formation of a network of cell-cell aggregates. This network formation resembles

that of differentiated endothelial cells, and is termed morphogenesis. Further investigation established that S1PR1 mediated S1P-induced morphogenesis through the ERK signaling pathway. However, PTX, which inhibits the Gi/o signaling pathway, did not inhibit S1P-induced morphogenesis through S1PR1 (Lee et al., 1998). This result, together with mine, raises the question as to whether S1PR1 couples to another G protein besides Gi/o.

If pretreatment of sensory neurons with PTX is sufficient to cause ADP-ribosylation of all the Gαi/o subunits and to prevent G proteins from interacting with S1PR1, there should be dissociated Gβγ subunits in the cytosol, possibly in close proximity with S1PR1. The binding of SEW2871 to S1PR1 triggers a receptor conformational change, which may directly activate Gβγ subunits to initiate downstream signaling to increase neuronal excitability. One effector downstream of active Gβγ subunits is PLC. If SEW2871 activates S1PR1 and only initiates Gβγ-PLC signaling cascade to enhance neuronal excitability, the inhibition of PLC activity should be able to totally prevent sensitization. However, I found that PLC inhibitor U73122 did not completely block SEW2871 from increasing neuronal excitability (Figure 27), suggesting that Gβγ subunits may activate other downstream targets (e.g., ERK, GIRKs) to enhance neuronal excitability. However, considering the small sample size (n = 2), more experiments are needed before any conclusion is drawn.

In the case of S1PR3, since it couples with Gi/o-, Gq- and G12/13-type G proteins, S1P may selectively activate one or several G proteins and its corresponding signaling pathways. My preliminary data on S1PR signaling is not strong enough to draw any definitive conclusion, but the result that the MEK inhibitor PD98059 prevented S1P from increasing excitability (Figure 28) indicates that S1P may favor the activation of Ras-MEK-ERK pathway through S1PR1 and/or R3 to modulate neuronal excitability.

The theory of functional selectivity also helps explain why S1PR2, R4 and R5 do not mediate S1P-induced enhancement in excitability. Firstly, S1P may still be an orthosteric ligand on all three receptor subtypes. However, S1P may exhibit a biased agonism to preferentially activate certain signaling pathways downstream of those S1PRs that do not affect neuronal excitability. Secondly, the binding of a ligand to a GPCR may not always initiate an intracellular response, as is determined by intracellular GPCR occupancy by G proteins and scaffold proteins. This may be the case for S1PR2, R4 and R5 that, constitutively, S1PR1 and R3 have an intrinsic advantage to couple with G proteins in sensory neurons that initiate signaling cascade(s) which increase neuronal excitability whereas the G proteins coupled to S1PR2, R4 and R5 do not.

6. Physiological characteristics of S1P and transactivation of S1PRs

S1P concentrations in blood and lymph plasmas are high, within the high nanomolar to low micromolar range, whereas S1P concentrations in tissues are low. This S1P gradient creates the basis of chemotaxis to S1P for cells expressing S1PRs, which is critical especially for cell migration and trafficking (Schwab et al., 2005). S1P is identified as a normal constituent of human plasma and serum. The study from Yatomi et al. detected that the S1P levels were ~200 nM in plasma and ~500 nM in serum (Yatomi et al., 1997). The study also identified that platelets could convert sphingosine into S1P, abundantly store S1P and extracellularly release part of the storage upon stimulation. S1P was found to be stable and not metabolized in plasma, indicating that plasma does not contain the enzymes for S1P dephosphorylation or degradation.

Another study measured the S1P concentrations in human plasma and serum at ~350 nM and ~800 nM, respectively. However, the active S1P contents in human plasma and serum were estimated at 7.3 nM and 11.2 nM (Murata et al., 2000). Data from this study also indicates that S1P is the only ligand to stimulate S1PRs in normal human serum samples. The huge difference between the total S1P content and the active S1P content suggests that there must be certain components in the plasma or serum to trap exogenous S1P and prevent it from binding to its receptors.

Once outside of the cell, S1P can bind to two known carriers, lipoprotein or albumin, to be transported in the blood stream. Approximately 60% of plasma S1P is bound to lipoproteins, including low-density lipoprotein (LDL) and high-density lipoprotein (HDL), with HDL trapping about 53% of plasma S1P, while LDL 7% (Aoki et al., 2005; Argraves and Argraves, 2007); plasma albumin binds the other 40% of S1P. The molecular nature of S1P binding to HDL and interacting with S1P receptors has been characterized recently (Christoffersen et al., 2011). Apolipoprotein M (ApoM) is a lipocalin residing mainly in the HDL fraction of the plasma (Xu and Dahlback, 1999). A study from Christoffersen et al. shows that HDL-associated S1P is bound specifically to human ApoM. Human $ApoM^+$ HDL induced internalization of S1PR1, activation of ERK and Akt, migration of endothelial cells and formation of endothelial adherens junctions, whereas HDL lacking ApoM did not. The result demonstrates that HDL-associated ApoM captures S1P and delivers S1P to S1PR1 on endothelial cells.

The circulation of S1P in the blood stream provides a picture of where S1P functions as a communicator between various cell types across different systems. S1P can regulate diverse cellular functions in a cell-specific manner, via S1PRs on the cell membrane. This phenomenon shows that S1PRs can be activated by S1P in a paracrine manner. However, this is not the only pattern where S1P exerts its function. S1P can also regulate cellular activities in an autocrine manner through transactivation.

One study from Toman et al. discovered that, in PC12 cells, nerve growth factor (NGF) induced the translocation of SphK1 to the plasma membrane through the neurotrophic tyrosine kinase receptor type 1, or TrkA receptor. This NGF-induced effect of SphK1 translocation further activated S1PR1 on PC12 cells to stimulate neurite extension in an SphK1-dependent manner. By contrast, overexpression of either S1PR2 or S1PR5 strongly inhibited NGF-induced neurite extension, suggesting that the differential transactivation of S1PRs by NGF leads to the antagonistic signaling pathways that negatively regulate neurite extension (Toman et al., 2004). This study provides the evidence that NGF can transactivate S1PRs and the transactivation depends on the activation of SphK.

Similarly, the study from our laboratory demonstrates that NGF increases the neuronal excitability through elevating intracellular S1P via SphK (Zhang et al., 2006b). At that time, the transporters of S1P on the cell membrane had not been well characterized, and therefore the possibility that the intracellularly produced S1P was actually transported outside the neurons and acted on S1PRs to increase the neuronal excitability was not tested. This assumption can be examined by using siRNAs targeted to S1P transporters, SPNS2 and the ABC family of transporters, to determine the excitatory effect of NGF in siRNA-treated neurons. If this assumption is proved, it will add additional evidence to show the autocrine activation of S1PRs by the transactivation of NGF.

Another example of transactivation has been shown in mast cells. Mast cells express a high-affinity receptor (FcεRI) for the Fc region of Immunoglobulin E (IgE). A study from Jolly et al. shows that cross-linking of IgE and FcεRI induces S1P formation and release from the bone marrow-derived mast cells (BMMCs). Sequentially, S1P activates S1PR1 and S1PR2 that are present in the BMMCs. The transactivation of S1PR1 mediates the chemotaxis of mast cells towards the antigen (Ag), while the transactivation of S1PR2 is critical for the IgE/Ag-induced degranulation (Jolly et al., 2004). This study demonstrates that the activation of FcεRI by cross-linked IgE leads to S1P production and eventually the transactivation of S1PRs.

The examples of NGF and FcεRI transactivating S1PRs fits into the paradigm of an S1P autocrine loop. The mechanism of this paradigm is that intracellular SphKs are activated by non-S1PR-related pathways, S1P is generated inside the cell and then released into the extracellular space, and stimulates membrane S1PRs (Kim et al., 2009). However, there might be a second paradigm of transactivation, with the mechanism being that S1P or other S1PR ligands initiate intracellular signaling cascades through S1PRs, and the downstream effectors activate other membrane receptors to trigger a distinct signaling pathway that alters cellular activities, e.g., the phosphorylation and transactivation of a certain type of RTKs via S1PR activation. The transactivation between S1PRs and RTKs add another layer of complexity to the already complex S1PR signaling pathways. This makes elucidating the exact mechanism on how S1P increases sensory

neuron excitability more difficult, and leaves many intriguing questions about S1PRs signaling for future studies.

7. S1P/S1PRs function in the nervous system

S1P and S1PRs not only play a key role in the development of the nervous system, but also in the regulation of various cell-specific functions in the developed nervous system, including the CNS and the PNS. Neural progenitor cells (NPCs) from the embryonic rat hippocampus express S1PR1, R2, R3 and R5, and S1P activates these receptor subtypes to regulate neurogenesis by inducing proliferation and morphological changes in the NPCs (Harada et al., 2004). The S1P/S1PR1 axis contributes to the migration of transplanted NPCs toward areas of spinal cord injury, and antagonism of S1PR2 enhances the endogenous NPC migration toward the area of ischemia (Kimura et al., 2007; Kimura et al., 2008). S1PR1-null mice show severe defects in the neural development, where massive cell loss in the forebrain was revealed at E12.5. In the earlier E11.5 embryo, mitotic cell numbers were significantly decreased in the telencephalon regions. These results indicate that S1P promotes neurogenesis, at least in part, through signaling from S1PR1 (Mizugishi et al., 2005).

Our study investigated the mRNA expression of all S1PR subtypes in single isolated DRG neurons. We found that, on average, S1PR1 is the most highly expressed subtype in isolated neurons regardless of the neuronal subtype (small-, medium- or large-diameter neurons) (Kays et al., 2012). The S1PR expression profile corresponds to the functional outcome wherein S1PR1 plays a predominant role in mediating S1P-induced enhancement of excitability in small-

diameter sensory neurons. The S1PR1 contribution to the increased neuronal excitability indicates its possible involvement in the elevated nociception, and this idea is supported up by several studies about the role of S1PR1 in regulating nociception in vivo.

One study showed that in mice, S1P and the S1PR1 specific agonist SEW2871 both induced the hypersensitivity to noxious thermal stimulation in vivo. This hypersensitivity was significantly attenuated in mice where the S1PR1 was conditionally knocked out in neurons expressing the nociceptor-specific $Na_V1.8$, indicating S1P plays a critical role in regulating sensitivity through S1PR1 (Mair et al., 2011). Another study showed that the intraplantar injection of ceramide in rats led to the development of thermal hyperalgesia, and this effect was reduced by a SphK inhibitor (SK-I) or an anti-S1P antibody (LT1002), suggesting that the hyperalgesia is driven by the formation of S1P from ceramide. Furthermore, this study demonstrated that S1P induced the development of hyperalgesia through S1PR1, as the S1PR1 specific antagonist W146 blocked the effect of S1P to induce hyperalgesia (Doyle et al., 2011a). Therefore, S1P acting via S1PR1 is the downstream signaling pathway in the ceramide-induced hyperalgesia.

The S1P/S1PRs axis has also been shown to mediate sensitivity in inflammatory pain and neuropathic pain animal models. The afferent fibers from rat hind paws project predominantly to lamina I and II at L4/5 spinal segments (Abbadie and Besson, 1992; Sharma et al., 2009). One study shows that in vivo perfusion of

S1P at rat L5 DRG increased the mechanical sensitivity at the hind paw. When the L5 ganglion was locally inflamed, a procedure that leads to mechanical hypersensitivity, the S1PR1 siRNA injected rats showed significantly less hypersensitivity than the scrambled siRNA injected rats, indicating that the S1PR1 in DRGs plays an important role in regulating behavioral sensitivity during inflammation (Xie et al., 2012).

Paclitaxel is a widely used chemotherapeutic agent. However, this anticancer drug may cause peripheral neuropathy accompanied by chronic neuropathic pain (CIPN). A recent study using the paclitaxel-induced neuropathic pain model shows that spinal administration of S1PR1 antagonist W146 blocks the development of mechano-hypersensitivity in rats. Moreover, systemic administration of FTY720, NIBR-15 and CYM-5442 also attenuated mechano-hypersensitivity, suggesting that S1PR1 is critical in modulating neuropathic pain behavior (Janes et al., 2014). These findings identified the S1P/S1PR1 axis as a promising therapeutic target for CIPN.

To the contrary, another study using mice with a nociceptor-specific deletion of S1PR1 (SNS-S1PR1-/-) showed that S1P-induced spontaneous pain behavior (flinches, licks and licking time) was not significantly different between S1PR1 floxed mice and SNS-S1PR1$^{-/-}$ mice. However, the S1P-induced spontaneous pain behavior was substantially reduced in S1PR3-null mice compared to the

wild type mice, indicating S1P-induced pain behavior is largely mediated by S1PR3 (Camprubi-Robles et al., 2013).

The discrepancies among the studies can have several explanations. First, in the Camprubi-Robles study, they used S1PR3 knockout mice, instead of a nociceptor-specific knockout, or local S1PR3 knockdown by siRNA, and therefore, the change in the pain behavior exhibited by S1PR3-null mice may not be a nociceptive neuron-specific or an S1PR-directly-mediated event. Second, in both the Camprubi-Robles et al. and the Mair et al. studies, they injected S1P intracutaneously, but the doses were different, being 15 µl of a 500 µM solution and 5 µl of a 100 µM solution, respectively. It is possible that the high dose of S1P in the Camprubi-Robles study desensitized S1PR1 rapidly and therefore the pronociceptive effect of S1PR1 became unable to measure. Thirdly, Camprubi-Robles shows that heat and mechanical sensitivity of S1PR3-null mice after plantar incision was reduced compared with the wild type mice. However, they did not investigae the heat and mechanical sensitivity in the SNS-S1PR1$^{-/-}$ mice compared with the wild type. Thus, their conclusion that S1PR3, but not S1PR1, mediates S1P-induced enhancement of nociceptive behaviors is questionable. Based on my results, S1PR1 and/or R3 mediate S1P-induced increase in excitability in sensory neurons, and therefore, it is likely that both S1PR1 and R3 play important roles in mediating nociceptive behavior as well. However, more studies are needed to determine the exclusive or complimentary role of S1PR1 and/or R3 in mediating S1P-induced increase of nociception.

So far, studies have indicated that S1P is pronociceptive in the PNS. In contrast, it seems not to be the case in the CNS. One study shows that intrathecal administration of S1P reduced nociceptive behavior in the formalin assay in rat. This effect was mediated by S1P inhibiting cAMP synthesis in the excitatory dorsal horn neurons (Coste et al., 2008a). Another sutdy shows that the intracerebroventricular S1P administration produced in vivo effects resembling the actions of cannabinoids, including thermal antinociception, hypoactivity, catalepsy and hypothermia. However, only the antinociception (tail-flick test in mice) was reversed by the S1PR1 and R3 antagonist VPC44116. Moreover, the S1PR1 selective agonist SEW2871 also produced thermal antinociception and the effect was blocked by VPC44116 (Sim-Selley et al., 2009), suggesting that S1PR1 and/or R3 mediates the S1P-induced decrease in nociception in the CNS. Although these studies support a role of central S1PRs in antinociception, S1PR functions in the CNS are still not well-studied. The fact that S1P and S1PRs are pronociceptive in the PNS while antinociceptive in the CNS is not surprsing, if we consider that neurons in the PNS and CNS may have distinct S1PR expression profiles, that S1P may activate certain signaling pathways downstream of S1PR1 and/or R3 to decrease neuronal excitability in the CNS, that maybe different S1PR subtypes mediate S1P-induced change in nociception through different signaling cascades, and that the supporting cells (oligodendrocytes, microglia, etc.) may also contribute to regulation of nociceptive behavior.

8. Conclusions and future directions

In conclusion, my results demonstrate that S1P enhances excitability of small-diameter sensory neurons through S1PR1 and/or R3. S1PR1 is the most highly expressed subtype among all five S1PR subtypes in about half of small-diameter sensory neurons. This finding supports the predominant role of S1PR1 in mediating S1P-induced increase in excitability. Moreover, my data provide insight into the signaling pathways downstream of S1PR1 contributing to the elevated excitability in sensory neurons. Finally, the evidence from the antagonism at S1PR1 to regulate neuronal excitability at the cellular level emphasizes the importance of targeting S1P/S1PR1 axis in pain research. Considering the role of S1P in regulating immune cell trafficking, it is also important to improve our knowledge of S1P and S1PRs especially in the potential interactions between immune cells and sensory neurons.

With regard to S1PR function and signaling in sensory neurons, there are still some interesting questions to be addressed in the future with additional and thorough experiments, such as:

1. Beyond differential expression patterns of S1PR subtypes, how is the cell-specific regulation of function carried out through different receptors or different combinations of receptors? Why is it that in some cells one receptor plays a prominent role, while in other cells, a different subtype plays a major role? Are

other S1PR subtypes not directly involved in the regulation of neuronal activities really redundant or do they function in ways we do not know yet or we are not capable of measuring due to the technical limitations?

2. How is the cell-specific preference of signaling to be characterized? What determines S1PR coupling and signaling transduction in different cells such as lymphocytes, endothelial cells, neurons and so forth? What are the physiological similarities and differences between the effects of extracellular S1P and intracellular S1P in mediating neuronal excitability? Does S1P exert different functions in different cellular compartments?

3. Structurally speaking, how does the proximity of one S1PR with other S1PRs, GPCRs or RTKs affect its own ligand binding and signaling initiation? How can we make more selective, subtype-specific ligands for S1PRs, and how can we better measure the specificity of different ligands? How effectively and safely can we translate our understanding and findings about S1PRs at the cellular level into therapeutics for human diseases such as multiple sclerosis and chronic pain?

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CURRICULUM VITAE

Chao Li

EDUCATION

2008-2014

Indiana University, Indianapolis, IN

PhD, Medical Neuroscience; Minor, Life Science

2003-2007

Sichuan University, Chengdu, China

BS, Biotechnology; BA, English Language & Literature

RESEARCH AND WORK EXPERIENCE

2009-2014

Department of Pharmacology & Toxicology, Indiana University, IN

Doctoral Dissertation Research

2007-2008

National Laboratory of Functional Genomics, Sichuan University, China

Research Assistant

2006-2007

Institute of Biology, Chinese Academy of Sciences, Chengdu, China

Student Research Assistant

PUBLICATIONS

Li C and Nicol GD. Sphingosine 1-phosphate receptors 1 and/or 3 mediate neuronal sensitivity. (In preparation)

Janes K, Little JW, Li C, et al. The development and maintenance of paclitaxel-induced neuropathic pain requires activation of the sphingosine 1-phosphate receptor subtype 1. J Biol Chem. 2014 May 29. [Epub ahead of print]

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CONFERENCE ABSTRACTS AND PRESENTATIONS

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Chao Li and Grant Nicol. Sphingosine 1-phosphate enhances excitability of sensory neurons through sphingosine 1-phosphate receptors 1 and/or 3. Poster presentation. Society for Neuroscience Annual Meeting, San Diego, CA, 2013.

Chao Li, Wenrui Xie, et al. JTE-013, a putative selective antagonist of sphingosine 1-phosphate receptor 2, increases the excitability of rat sensory neurons. Poster presentation. Society for Neuroscience Annual Meeting, DC, 2011.

Grant Nicol, Chao Li and Joanne Kays. Expression levels of sphingosine 1-phosphate receptors in single isolated rat sensory neurons. Poster. Society for Neuroscience Annual Meeting, DC, 2011.

AWARDS AND HONORS

Educational Enhancement Grant, Indiana University, 2011

Stark Scholar/Paul & Carole Stark Fellowship, Stark Neurosciences Research Institute, 2009-2010

Outstanding Graduate Award, Sichuan University, 2007