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Lysophosphatidic acid (LPA) signaling in neuropathic pain development and Schwann cell biology

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biomedical Sciences

by

Mu-En Lin

Committee in charge:

Professor Jerold Chun, Chair
Professor Joan Heller Brown, Co-Chair
Professor Katerina Akassoglou
Professor Henry Powell
Professor Linda Sorkin

2012

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This Dissertation of Mu-En Lin is approved, and it is acceptable

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University of California, San Diego

2012

DEDICATION

To my parents,

Feng-Sheng Lin and Li-Huei Huang,

for their unconditional love and support through the process.

To my sister,

Yi-Mei Lin,

for the encouragement and prayer.

Without them, this Dissertation would not be possible.

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ABSTRACT OF THE DISSERTATION

Lysophosphatidic acid (LPA) signaling in neuropathic pain development and Schwann cell biology

by

Mu-En Lin

Doctor of Philosophy in Biomedical Science

University of California, San Diego, 2012

Professor Jerold Chun, Chair

Professor Joan Heller Brown, Co-Chair

Neuropathic pain is a chonic pain state caused by lesions or diseases in the nervous system. Unlike acute pain, neuropathic pain persists without obvious injury or stimuli and can servely interefere with normal daily life for those who suffer from it. Despite numerous efforts on studying its mechanism and possible

treatments, there is no effective treatment currently available to remove or alleviate this symptom. This dissertation aims to provide further understanding into the relationship between lysophosphatidic acid (LPA) signaling and neuropathic pain development.

LPA is one of the lysophospholipids that has wide range of signaling abilities through its G protein-coupled receptors, LPA_1 to LPA_6. LPA is involved in multiple systems in both normal and pathological conditions, such as development, cardiovascular function, the reproductive system, cancer, and neuropsychiatric diseases. The first evidence of LPA involvement in neuropathic pain was shown in LPA_1 deficient animals where injury-induced neuropathic pain is prevented. Here we demonstrate for the first time, that a second LPA receptor, LPA_5, is also involved in neuropathic pain through pCREB activation in the spinal dorsal horn neurons via a mechanism, which is distinct from LPA_1. This suggests an alternative LPA signaling pathway in neuropathic pain development and LPA_5 could serve as potential therapeutic target.

We also analyzed the effect of LPA on Schwann cell physiology, since LPA_1 deficient mice also showed decreased demyelination in response to nerve injury. With LPA_1 null mice, we demonstrated that LPA_1 signaling is important for proper myelination during development and also regulates Schwann migration along dorsal root ganglion (DRG) neurons. These data suggest an important regulatory role of LPA on Schwann cell biology. Furthermore, we have shown that sphingosine 1-phosphate (S1P), another lysophospholipid closely related to LPA, is

also involved in Schwann cell biology and can modify LPA signaling. Through the balance between $S1P_2$ and $S1P_3$, S1P can fine-tune LPA_1 signaling in both Schwann cell migration and myelination gene regulation. Through these studies, we have extended our understanding of LPA signaling in neuropathic pain and Schwann cell biology. Hopefully these findings will bring us a step closer to potential therapeutic treatments.

I. INTRODUCTION

1.1 Lysophosphatidic acid (LPA)

Lysophosphatidic acid (LPA), once thought to be an inert metabolite in the biosynthesis of membrane phospholipids, is now recognized as an important signaling molecule. Acting through G protein-coupled receptors (GPCRs), LPA alters many different cellular responses, such as proliferation, survival, cytoskeletal changes, calcium influx, and much more (Fukushima, Ishii et al. 2001; Ishii, Fukushima et al. 2004). The cell stimulating action of LPA was first recognized in the 1960s for its ability to elicit calcium responses in smooth muscle cells (Vogt 1963). In the ensuing decades, numerous studies confirmed and expanded on the role that LPA plays as a signaling molecule. The primary molecular signaling mechanism was identified in 1996 with the cloning of the first cognate receptor for LPA (Hecht, Weiner et al. 1996). The receptor, now called LPA_1, is a GPCR that couples to G_i, G_q, $G_{12/13}$, and elicits multiple cellular responses upon LPA binding (Fukushima, Kimura et al. 1998; Fukushima, Ishii et al. 2001).

Based on sequence similarity, two other closely related LPA receptors were soon identified: LPA_2 and LPA_3 (An, Dickens et al. 1997; Bandoh, Aoki et al. 1999). Recently, two more distantly related GPCRs have also been shown to respond specifically to LPA, LPA_4/P2Y9/GPR23 and LPA_5/GPR92 (Noguchi, Ishii et al. 2003; Lee, Rivera et al. 2006). LPA_4 is more closely related to purinergic receptors and shares only 20-24% amino acid identity with LPA_{1-3} (Noguchi, Ishii et al. 2003). LPA_5 was identified using reverse transfection screening and shares approximately 35% identity with LPA_4 (Kotarsky, Boketoft et al. 2006; Lee, Rivera

et al. 2006). These receptors are encoded by distinct genes that are known as *LPAR1-5* (in humans) and *Lpar1-5* (in mouse) (HUGO ; MGI). Two additional receptors, GPR87 and P2Y5, have been proposed to be new LPA receptors (Tabata, Baba et al. 2007; Pasternack, von Kugelgen et al. 2008). While P2Y5 has been recognized as a cognate LPA receptor, LPA_6, GPR87 is still waiting for further validation (Lee, Choi et al. 2009; Kimura, Mogi et al. 2011). A summary of known LPA receptors, with associated downstream signaling molecules, is shown below (Figure 1.1).

Sphingosine 1-phosphate (S1P), a lysophospholipid structurally similar to and often compared to LPA, also has a wide range of signaling properties. Just like LPA, S1P was also identified as an extracellular signaling lipid when its first cognate receptor, $S1P_1$, was discovered (Lee, Van Brocklyn et al. 1998). Currently, five GPCRs are identified as cognate S1P receptors, named $S1P_{1-5}$ (Spiegel and Milstien 2003; Rosen, Gonzalez-Cabrera et al. 2009). Like LPA, S1P is present in many tissues at variable concentrations and affects a variety of organ systems and plays a role in many diseases by signaling through its receptors and associated downstream G-protein signaling molecules. An increasing amount of attention has been given to these two lipid molecules and their biological roles.

Here we describe the involvement of LPA and S1P in the development of neuropathic pain, a chronic pain state resulting from injury or damage to the nervous system. Previous studies showed that LPA_1, the first LPA receptor, was required in the initiation of neuropathic pain induced by both nerve injury and LPA

administration. This discovery led us to investigate the role of other LPA receptors in neuropathic pain development. In addition, LPA_1 is involved in peripheral nerve demyelination following injury and the role LPA_1 plays in affecting Schwann cell physiology will also be discussed. Finally, during this study, an unexpected possible interaction between LPA and its close relative, S1P was also found and these results will also be presented.

1.2 LPA metabolism

LPA is present in all mammalian cells and tissues, including blood, where concentrations in plasma range from 0.1 to 1μM, while concentrations in serum can exceed 10μM. Different detection methods are in current use, including enzymatic assays, TLC-gas chromatography and high performance liquid chromatography (HPLC)-coupled mass spectrometry (MS). A detailed comparison of the techniques used to measure LPA was recently reviewed (Smyth, Cheng et al. 2008). Biologically relevant LPA levels (well above apparent K_d and/or EC50 values for the six known LPA receptors) implicate their importance in physiological function.

There are at least two major pathways of LPA production. The first one involves hydrolysis of phosphatidic acids (PAs) by phospholipase A1 and A2 (PLA_1 and PLA_2) (Figure 1.2). This pathway is thought to be mainly intracellular or on the cell membrane since the substrate PAs are located in cell membranes (Aoki, Inoue et al. 2008). The second pathway is via cleavage of lysophospholipids (LPLs), such as lysophosphatidylcholine (LPC) and lysophosphatidylserine (LPS), by lysophospholipase D/autotaxin (LysoPLD/ATX). There are two additional pathways that can produce LPA: acylation of glycerol 3-phosphate by glycerophosphate acyltransferase (GPAT) and phosphorylation of monoacylglycerol by monoacylglycerol kinase (MAG-kinase). However, LPA produced by these two pathways appears to serve as precursors for glycerolipid synthesis rather than a source of extracellular signaling molecules (Pages, Simon et al. 2001). ATX was first identified as a cell motility-stimulating factor that possessed nucleotide

phosphodiesterase activity (Murata, Lee et al. 1994), but was subsequently identified as a major LPA producing enzyme (Aoki, Inoue et al. 2008). ATX activity is present in blood and strongly correlates with LPA concentration (Watanabe, Ikeda et al. 2007). While homozygous ATX knockout mice die at mid-gestation (see below) heterozygotes have an LPA concentration in the blood that is roughly 50% of that in wildtype mice. This suggests that ATX activity accounts for the majority of LPA production in blood (Tanaka, Okudaira et al. 2006). The degradation of LPA involves several different categories of enzymes, including LPA-acyltransferase (LPAAT), lipid phosphate phosphatase (LPP), and lysophospholipase (Pages, Simon et al. 2001). LPA may be converted back to PA by LPAAT, hydrolyzed by LPP-1, 2 and 3, or converted into glycerol-3-phosphate by lysophospholipases (Pages, Simon et al. 2001; Brauer, Savaskan et al. 2003). A subclass of the LPP family, lipid phosphatase-related proteins or plasticity related genes (LRPs/PRGs), were also shown to modulate LPA signaling. However, whether the LRPs/PRGs directly hydrolyze LPA remains to be determined (Brauer and Nitsch 2008).

1.3 LPA and sphingosine 1-phosphate (S1P) receptors

The biological activities of LPA and S1P are mediated largely through the activation of the their receptors, LPA_1 to LPA_6 and $S1P_1$ to $S1P_5$. All are Type 1, rhodopsin-like GPCRs with seven-transmembrane alpha helices. Distinct associations with heterotrimeric G protein subtypes and different expression patterns allow these lysophospholipids to produce various effects on different cellular and organ systems. A detailed review of these receptors can be found elsewhere and not every receptor will be addressed here (Rosen, Gonzalez-Cabrera et al. 2009; Choi, Herr et al. 2010). Only four receptors, LPA_1, LPA_5, $S1P_2$, and $S1P_3$, which were involved in our study, will be introduced here.

1.3.1 LPA_1

LPA_1 was the first LPA receptor identified through cloning and overexpression experiments (Hecht, Weiner et al. 1996). In human, the *LPAR1* gene encodes a 41kDa protein that consists of 364 amino acids and 7 transmembrane domains. In mouse, *Lpar1* also encodes a protein that has 95% identity with its human counterpart. LPA_1 couples to and activates three different G-proteins, $G_{\alpha i/o}$, $G_{\alpha q/11}$, and $G_{\alpha 12/13}$ (Fukushima, Kimura et al. 1998; Ishii, Contos et al. 2000). The activation of LPA_1 can lead to various cellular responses including proliferation, survival, migration, and cytoskeleton changes (Anliker and Chun 2004; Choi, Herr et al. 2010).

Targeted deletion of *Lpar1* in mice results in an approximately 50% decrease in survival rate, and impaired suckling in neonatal pups. Survivors are smaller, show craniofacial dysmorphism, and have increased apoptosis in sciatic nerve Schwann cells (Contos, Fukushima et al. 2000). Embryonic cerebral cortical neuroblasts from LPA_1 deficient mice also show a loss of LPA responsivity (Contos, Fukushima et al. 2000). A substrain derived from the original LPA_1 null mice was shown to have increased brain development abnormalities (Estivill-Torrus, Llebrez-Zayas et al. 2008).

LPA_1 is broadly expressed and transcripts are present in brain, lung, small intestine, heart, spleen, thymus, and skeletal muscles (Contos, Ishii et al. 2000; Choi, Herr et al. 2010). An enriched expression of LPA_1 in the developing nervous system indicates its importance during embryogenesis (Ohuchi, Hamada et al. 2008). In fact, the orignial name of LPA_1, vzg-1, is indicative of its high level of expression in the ventricular zone (VZ), where neural progenitor cells (NPCs) reside (Hecht, Weiner et al. 1996).

In the presence of exogenous LPA, *ex vivo* cultured mouse cortices increase in width and produce folds resembling gyri due to reduced cell death and increased mitosis. This effect is absent in cortices derived from LPA_1 and LPA_2 double knockout mice suggesting a role for LPA signaling in normal neurogenesis (Kingsbury, Rehen et al. 2003).

Recently in mice, LPA signaling through LPA_1 has been shown to induce fetal hydrocephalus (FH), which supports a significant role for LPA signaling in normal brain development (Yung, Mutoh et al. 2011).

LPA_1 is also expressed in oligodendrocytes and Schwann cells, which myelinate the CNS and PNS neurons respectively (Weiner, Hecht et al. 1998; Weiner and Chun 1999). In oligodendrocytes, LPA induces calcium responses and extracellular signal-regulated kinase (ERK) phosphorylation (Yu, Lariosa-Willingham et al. 2004). It is also important in maintaining network area and MBP expression in differentiating oligodendrocytes, suggesting a role in myelination and oligodendrocyte maturation (Nogaroli, Yuelling et al. 2008). In Schwann cells, LPA increases survival, cytoskeleton rearrangement, and adhesion through LPA_1 signaling (Weiner and Chun 1999; Weiner, Fukushima et al. 2001). In a neuropathic pain model, LPA_1 has been shown to mediate demyelination in the dorsal root after nerve injury and LPA injection (Inoue, Rashid et al. 2004). Other experiments with *ex vivo* dorsal root cultures also demonstrated a similar effect (Fujita, Kiguchi et al. 2007). Thus, LPA signaling through LPA_1 plays important roles in the nervous system ranging from development and normal myelination to neuropathic pain and demyelination. A detailed review of LPA_1 in other systems and diseases can be found in our previous reviews (Choi, Herr et al. 2010; Lin, Herr et al. 2010).

1.3.2 LPA_5

LPA_5, previously known as GPR92, was identified as a fifth LPA receptor in 2006. *LPAR5* encodes a 42kDa protein consisting of 372 amino acids and shares

81% identity with its mouse counterpart, *Lpar5*. It couples to G_q to increase intracellular calcium levels, and $G_{12/13}$ to induce neurite retraction (Lee, Rivera et al. 2006). Activation of LPA_5 also induces cAMP accumulation and phosphoinositide hydrolysis. The G-proteins responsible for these latter effects remain unclear, however, the phosphoinositide hydrolysis was insensitive to pertussis toxin treatment, indicating that G_i is not involved (Kotarsky, Boketoft et al. 2006; Lee, Rivera et al. 2006). Also, the cAMP response is not suppressed by a $G_{\alpha s}$ minigene and thus suggests an alternative pathway is utilized to increase cAMP levels (Lee, Rivera et al. 2006). Two other lipid-derived molecules, farnesyl pyrophosphate (FPP) and N-arachidonylglycin (NAG), have been proposed as LPA_5 ligands in a previous report (Oh, Yoon et al. 2008). However, follow up experiments have indicated that LPA binds to LPA_5 with a much higher affinity and is thus the likely major ligand for LPA_5 (Williams, Khandoga et al. 2009; Yin, Chu et al. 2009).

In early development, *Lpar5* expression was first seen at the anterior neural fold and then the forebrain, midbrain, and hindbrain area. Later, ubiquitous expression was observed from E9.5 to E12.5 (Ohuchi, Hamada et al. 2008). In adults, LPA_5 is expressed in various tissues including spleen, heart, small intestine, dorsal root ganglion (DRG), brain, and bone marrow (Kotarsky, Boketoft et al. 2006; Lee, Rivera et al. 2006). The high expression seen in spleen and lymphocytes suggests a function in the immune system. Indeed, the first functional role for LPA_5 was in platelets, where a non-lipid LPA_5 antagonist can inhibit platelet activation (Williams, Khandoga et al. 2009). Recently, LPA_5 has been identified as the

predominant LPA receptor in human mast cells. By using shRNA, it was demonstrated that LPA_5 is responsible for the LPA induced calcium influx and macrophage inflammatory protein-1β (MIP-1β) release in mast cells (Lundequist and Boyce 2011). Other reports have also suggested that LPA_5 is involved in tumor cell growth and fluid absorption in the intestine (Okabe, Hayashi et al. 2011; Yoo, He et al. 2011). However, the role LPA_5 signaling plays in the nervous system remains unclear despite its high expression in DRGs. Therefore, we generated LPA_5 null mice and tested its involvement in nerve injury induced neuropathic pain (see below).

1.3.3 $S1P_2$

S1PR2 encodes a protein with 353 amino acids where *S1pr2,* its analog in mouse, encodes 352 amino acids and shares 91% identity. $S1P_2$ is expressed in brain, heart, lung, kidney, liver, thymus, and testis (Zhang, Contos et al. 1999). Previously known as EDG5/AGR16/H218, $S1P_2$ couples to multiple different G-proteins, including G_i, G_q, and $G_{12/13}$ (Ancellin and Hla 1999; Windh, Lee et al. 1999). $S1P_2$ activates the MAPK, JNK, and PI3K pathways that are sensitive to pertussis toxin (PTX), indicating G_i involvement (Gonda, Okamoto et al. 1999). However, despite the coupling of G_i, $S1P_2$ activates adenylyl cyclase and thus increases cAMP levels when activated, possibly through G_{13} (Jiang, Collins et al. 2007). It also enhances calcium mobilization and stress fiber formation through the activation of the G_q pathway (Gonda, Okamoto et al. 1999). $S1P_2$-induced $G_{12/13}$ activation has been shown by the serum response element (SRE) driven reporter

gene activation and is sensitive to the C3 exoenzyme (An, Zheng et al. 2000). In addition, $S1P_2$ activation also promotes cell proliferation and suppresses apoptosis (An, Zheng et al. 2000).

Contrasting with the other two closely related S1P receptors, $S1P_1$ and $S1P_3$, $S1P_2$ decreases cell mobility through inhibition of Rac activation (Okamoto, Takuwa et al. 2000). Although it couples to and activates G_i signaling, $S1P_2$ seems to suppress Rac activity through activation of Rac-GAP, downstream of the $G_{12/13}$-Rho pathway (Okamoto, Takuwa et al. 2000). This provides a very interesting interaction among receptors sharing the same ligand. While both $S1P_2$ and $S1P_3$ couple to the G_i-Rac and $G_{12/13}$-Rho pathways, the "coupling strength" seems to be different and the outcome is completely the opposite of what is expected. $S1P_2$ strongly activates the $G_{12/13}$-Rho pathway, leads to the inhibition of Rac, and suppresses cell migration while $S1P_3$ does exactly the opposite. This type of interaction is important in fine-tuning S1P signaling and proved to be critical in our studies (See below).

The first $S1P_2$ knockout animal was generated in 2001 and $S1P_2$ null mice were born at the expected Mendelian frequency with no apparent anatomical or physiological defects (MacLennan, Carney et al. 2001). However, between 3 and 7 weeks of age, spontaneous seizures were observed in $S1P_2$ null mutant animals that occasionally led to death (MacLennan, Carney et al. 2001). Further investigation revealed that the loss of $S1P_2$ increases both the frequency and amplitude of spontaneous postsynaptic currents in neocortical pyramidal neurons, demonstrating

the importance of $S1P_2$ in neuronal excitability (MacLennan, Carney et al. 2001). In other studies, $S1P_2$ was also shown to be important for auditory and vestibular function. *S1pr2* null mice become deaf around 4 weeks of age due to hair cell loss and degeneration of inner ear structures; this effect is further exacerbated by the loss of $S1P_3$, showing a certain degree of redundancy and/or synergistic effects (MacLennan, Benner et al. 2006; Herr, Grillet et al. 2007).

1.3.4 $S1P_3$

Previously known as EDG3, $S1P_3$ shares 64% sequence identity with $S1P_2$, has a similar expression pattern, and couples to a similar set of G-proteins. $S1P_3$ is expressed in brain, heart, lung, kidney, spleen, and testis as assessed by Northern blotting (Zhang, Contos et al. 1999). PTX-sensitive ERK1/2, PI3K, Ras and Rac activation shows that G_i is involved in the regulation of these downstream pathways (Okamoto, Takuwa et al. 1999; Sugimoto, Takuwa et al. 2003). Similar to $S1P_2$, $S1P_3$ also promotes cell proliferation and survival (An, Zheng et al. 2000). $S1P_3$ also activates phospholipase C (PLC) and induces calcium mobilization through G_q coupling (Okamoto, Takuwa et al. 1999; Sugimoto, Takuwa et al. 2003). In addition, $S1P_3$ activated $G_{12/13}$ as measured by Rho activation and the following SRE activation (An, Zheng et al. 2000).

As discussed above, $S1P_3$ increases cell mobility while $S1P_2$ inhibits it (Kon, Sato et al. 1999). Along with receptor specificity, endothelial cells exhibit increased migration in the presence of S1P that is likely due to high $S1P_1$ and $S1P_3$ expression (Kimura, Watanabe et al. 2000). In contrast, smooth muscle cells that

predominantly express $S1P_2$, are negatively regulated by S1P (Tamama, Kon et al. 2001). The $S1P_3$ enhancement of cell migration occurs by activating Rac signaling through the G_i-PI3K-RacGEF pathway (Okamoto, Takuwa et al. 2000; Taha, Argraves et al. 2004). Although $S1P_3$ couples to the $G_{12/13}$-Rho pathway as well, the strength of coupling to G_i-Rac seems to be greater and thus promotes cell migration when activated by S1P.

$S1P_3$ knockout mice were first generated in 2001 (Ishii, Friedman et al. 2001). No significant abnormalities were observed and *S1pr3* null mice were born at expected Mendelien ratios. However, the average litter size from *S1pr3*$^{(-/-)}$ intercrosses was significantly smaller then those from heterozygotes, and reasons for this observation remain unclear. To date, no significant phenotype has been reported in $S1P_3$ single knockout animals. As described above, $S1P_2$ and $S1P_3$ double knockout mice exhibit auditory loss and vestibular function abnormalities, suggesting a functional overlap between these two receptors (Herr, Grillet et al. 2007). The double null animals also showed increased embryonic lethality and vascular abnormalities (Kono, Mi et al. 2004).

1.4 Neuropathic pain

Neuropathic pain, defined as "Pain caused by a lesion or disease of the somatosensory nervous system" by the International Association for the Study of Pain (IASP), is a serious challenge in health care. Approximately 7 to 8% of the population of developed countries is affected by neuropathic pain, with 5% of these representing severe cases (Torrance, Smith et al. 2006; Bouhassira, Lanteri-Minet et al. 2008). Unfortunately, there are no effective therapeutic drug treatments for neuropathic pain. Current treatments generally involve the use of analgesic or narcotic pain relievers, which often have limited efficacy. The cause of neuropathic pain is largely unknown; however, spontaneous signaling from nociceptive C-fibers or cross talk with low threshold A-delta sensory fibers may be contributing factors (Amir and Devor 2000; Wu, Ringkamp et al. 2001).

Unlike acute pain, which alerts us to damage or problems in our body and is usually diminished after the condition is relieved, neuropathic pain is chronic and usually not associated with obvious injury. Phenotypes include spontaneous pain (pain experienced in the absence of any obvious peripheral stimulus), hyperalgesia (an increased responsiveness to noxious stimuli), and allodynia (pain in response to normally innocuous stimuli). Alterations to the nervous system, both peripheral and central, are the main reasons for neuropathic pain while other factors, including immune changes, may also contribute. Multiple events are needed in order for the body to feel pain. Pain starts at the nociceptors in the periphery, which are free nerve endings that will respond to different stimuli that damage or threaten to

damage the tissue (Kruger, Kavookjian et al. 2003; Voscopoulos and Lema 2010). Activation of these receptors is then translated into an electric signal on A-δ and C-fibers that corresponds to the strength of the stimuli.

A-δ fibers are thinly myelinated nerves with faster conducting velocities that are activated by mechanothermal receptors and also by high-threshold mechanoreceptors. C-fibers are unmyelinated nerves that are slow conducting and high threshold that transfer peripheral nociceptors responses. These fibers mainly innervate the spinal dorsal horn in laminae I-II, whereas A-β fibers predominantly innervate lower laminae III-V and convey sensation of touch and muscle movement. The interneurons in these layers can receive input from A-δ and C-fibers, A-β fibers, or from all of them. In addition to receiving input from these primary neurons, they are also influenced by excitatory/inhibitory interneurons and glia cells like astrocytes and microglia. Input signals are then transmitted to the thalamus and then the cerebral cortex in order for the body to recognize pain. A modulatory decending system originating from the cortex and hypothalamus also plays an important role in pain sensation, this system modulates the secondary interneuron output that is also suggested to be involved in central sensitization and was discussed above (Millan 2002; Pertovaara and Almeida 2006; D'Mello and Dickenson 2008; Voscopoulos and Lema 2010).

Any alteration in the system discussed above could lead to aberrant pain sensation and therefore contribute to the development of neuropathic pain. Primary hyperalgesia comes from the periphery and is induced by molecules released from

damaged tissue and immune cells, such as adenosine triphosphate (ATP), tumor necrosis factor-α (TNFα), interleukin-1 β (IL-1β), nerve growth factor (NGF), and proton (H^+). These molecules then act on the nerve endings, resulting in increased channel activities and abnormal firing. Increased channel activity may result from increased channel protein expression or modification, trafficking, activation, and distribution. In contrast to primary hyperalgesia, secondary hyperalgesia requires alterations in the CNS and more long-term central sensitization. Nociceptive neurons in the dorsal horn are sensitized by peripheral stimuli which results in increased responsiveness to sensory input, decreased threshold, and an enlarged receptive field (Cook, Woolf et al. 1987). Also, reorganization of synaptic connections can lead to abnormal activation of nociceptive neurons by low threshold larger diameter neurons that normally does not convey pain sensation and thus contributes to hyperalgesia and allodynia (Ji, Kohno et al. 2003).

Ectopic discharges and ephaptic conduction are also important mechanisms in nerve-injury induced neuropathic pain. Ectopic discharges, the spontaneous firing from neurons without stimuli, are often seen in neurons associated with an injury site. Increased membrane potential oscillation levels after nerve injury could lead to a higher chance of spontaneous firing (Kajander and Bennett 1992; Amir, Michaelis et al. 1999). Ephaptic conduction, or cross talk between neurons, can be caused by either physical contact or chemical release. Loss of insulation by demyelination could lead to contact between axons and cross-excitation from low-threshold A- fibers to high-threshold C- fibers contributing to aberrant pain

sensation (Rasminsky 1980; Targ and Kocsis 1986). Chemically mediated cross-excitation has also been demonstrated in DRGs where insulation is still intact (Amir and Devor 2000). The combination of increased membrane potential oscillation and cross talk between axons, can lead to increased signal output and ultimately contributes to neuropathic pain.

Neuropathic pain is a complicated syndrome associated with multiple potential mechanisms. The heterogeneous nature of neuropathic pain is perhaps the biggest challenge in research for a therapeutic treatment. Further investigation into the multiple mechanisms behind this syndrome is needed for better patient diagnosis and treatment.

1.5 LPA signaling in neuropathic pain

In mouse models of neuropathy, local administration of LPA to the hind paw elicits pain. This occurs through the activation of LPA_1 and subsequent release of the pro-nociceptive factor substance P (Renback, Inoue et al. 1999; Renback, Inoue et al. 2000). The role of LPA in neuropathic pain was demonstrated with the use of LPA_1 knockout mice. Intrathecal injection of LPA produced allodynia and hyperalgesia in wildtype mice, which is commonly seen in neuropathic pain (Inoue, Rashid et al. 2004). This effect is completely blocked by deletion of LPA_1 or by inhibition of Rho signaling, thus demonstrating the involvement of the LPA_1/Rho/ROCK pathway in the development of neuropathic pain (Inoue, Rashid et al. 2004). Furthermore, *Lpar1*$^{-/-}$ mice are also resistant to hyperalgesia induced by partial nerve ligation (Inoue, Rashid et al. 2004).

Recently, Inoue et al. published a series of papers providing evidence that autotaxin induces neuropathic pain through the conversion of LPC to LPA (Inoue, Ma et al. 2008; Inoue, Ma et al. 2008; Inoue, Xie et al. 2008). This is supported by the observation that heterozygous autotaxin knockout mice ($ATX^{+/-}$) have a 50% decrease in ATX activity and a corresponding 50% protection from neuropathic pain induced by partial sciatic nerve ligation (PSNL) (Inoue, Ma et al. 2008). Furthermore, they showed that the local increase in LPA production depends on another LPA receptor, LPA_3 (Ma, Uchida et al. 2009). All the data above implicate LPA signaling in the initiation and alteration of neuronal responses in neuropathic pain.

Neuropathic pain due to demyelination is also influenced by LPA signaling. The two axon-myelinating cell types, Schwann cells in the periphery and oligodendrocytes in the CNS, are responsive to LPA. It has been shown that LPA increases Schwann cell survival against serum withdraw through the LPA_1/G_i/PI3K pathway (Weiner and Chun 1999). LPA also causes Schwann cells to form wreath-like structures, increase cell-cell adhesion, and reassemble focal adhesions (Weiner, Fukushima et al. 2001). LPA_1 mRNA expression levels are also increased after sciatic nerve transection (Weiner, Fukushima et al. 2001). *In vivo* intrathecal injection of LPA or application of LPA to *ex vivo* dorsal root cultures induces demyelination in the dorsal root area and these effects are abolished in *Lpar1* null mice, indicating the involvement of LPA_1 signaling (Inoue, Rashid et al. 2004; Fujita, Kiguchi et al. 2007). Recently, intrathecal injection of LPA has also been shown to produce an LPA_1 mediated calpain-regulated down-regulation of myelin-asscoiated glycoprotein (MAG) in the dorsal root area (Xie, Uchida et al. 2010).

Oligodendrocytes also respond to LPA exposure. Phospholipase C (PLC) activity, intracellular calcium levels, protein kinase C (PKC) activity, and mitogen-activated protein kinase (MAPK) activity are all activated through LPA signaling in oligodendrocytes (Yu, Lariosa-Willingham et al. 2004; Nogaroli, Yuelling et al. 2008). Recently, it was shown that exogenous administration of LPA leads to an increased protrusion network in oligodendrocytes, an important step in the maturation process. LPA also induces myelin basic protein (MBP) mRNA expression and increases the number of MBP positive oligodendrocytes (Nogaroli,

Yuelling et al. 2008). This effect of LPA requires ATX downregulation, while ATX also regulates focal adhesion formation in oligodendrocytes (Dennis, White et al. 2008; Nogaroli, Yuelling et al. 2008). Combined with the previous observations that *Lpar1* null mice are resistant to injury induced demyelination, and that LPA induces *ex vivo* DRG neuron demyelination (Inoue, Rashid et al. 2004; Fujita, Kiguchi et al. 2007), it has become clear that LPA signaling influences the histopathological events associated with demyelinating lesions. Further studies are needed to characterize the relationship between LPA, myelination, and neuropathic pain.

In addition to its role in demyelination and myelination, direct effects of LPA on neurons can also contribute to neuropathic pain. It has long been known that LPA mediates multiple cellular responses in neurons, including survival, differentiation, neurite retraction, and growth (Anliker and Chun 2004; Choi, Herr et al. 2010). In a bone cancer model, LPA was also shown to sensitize C-fibers and enhance pain sensation through potentiation of the transient receptor potential vanilloid 1 (TRPV1) receptor in the DRG (Pan, Zhang et al. 2010; Zhao, Pan et al. 2010). Another study also showed that LPA can modulate K(2P)2.1 (KCNK2, TREK-1) channel activity through the G_q pathway (Cohen, Sagron et al. 2009). Both findings show that LPA can directly act on neurons by altering their sensitivities and responses leading to an alteration of pain sensation.

1.6 LPA and S1P signaling in Schwann cells

As discussed above, demyelination is an important event in LPA induced neuropathic pain. The myelinating cell types in the CNS and PNS are oligodendrocytes and Schwann cells respectively, and both cell types express LPA and S1P receptors (Weiner, Hecht et al. 1998; Weiner, Fukushima et al. 2001; Yu, Lariosa-Willingham et al. 2004). In the dorsal root, where demyelination differences were observed in LPA_1 null mice, Schwann cells are the cell type responsible for myelination and thus LPA signaling in Schwann cells is one of our primary interests. LPA promotes Schwann cell survival through LPA_1 and the downstream PTX-sensitive G_i pathway, which then activates PI3K and Akt to protect Schwann cells from apoptosis (Weiner and Chun 1999). In addition, LPA also regulates Schwann cell migration, adhesion, and cytoskeleton rearrangement through an LPA_1 dependent pathway (Weiner, Fukushima et al. 2001; Barber, Mellor et al. 2004). Schwann cell morphology changes are not only important for migration, but also for proper myelin sheath formation that requires very precise morphological rearrangements (Fernandez-Valle, Gorman et al. 1997). Last but not least, LPA also promotes Schwann cell differentiation, an important step in both development and remyelination (Li, Gonzalez et al. 2003; Fukushima, Shano et al. 2007).

Single cell RT-PCR showed that numerous S1P receptors are expressed in Schwann cells, including $S1P_2$, $S1P_3$, and $S1P_4$ (Kobashi, Yaoi et al. 2006). Although very few studies have been done on the function of S1P in Schwann cells,

S1P does affect Schwann cell migration and morphological changes (Barber, Mellor et al. 2004). The expression of multiple S1P receptors in Schwann cells with signaling properties similar to LPA receptors, led us to examine the role of S1P and S1P receptors in regulating Schwann cell biology. We are also interested in identifying interactions between LPA and S1P and their functional significance in Schwann cells (see below).

1.7 Conclusion

Lysophosphatidic acid has received a lot of attention in the past two decades for its signaling properties and its involvement in a wide range of organ systems and diseases. However, little has been done to understand its role in neuropathic pain development. It is quite surprising that deletion of a single receptor, LPA_1, can fully inhibit the development of injury induced neuropathic pain, but this demonstrates the important role of LPA signaling in this complicated syndrome. We are interested in understanding how LPA contributes to neuropathic pain, including identifying the involvment of other LPA receptors and the possible interaction with another closely related lysophospholipid, S1P. Using a genetic knockout animal, we have shown that LPA_5 is also required for the initiation of neuropathic pain through a mechanism distinct from that of LPA_1. We also demonstrated that LPA_1 signaling is important in regulating Schwann cell myelination and migration, and provide additional evidence that LPA is involved in proper myelination. Lastly, we demonstrate for the first time, that an interaction between LPA and S1P affects myelination, gene expression, and migration, and thus can serve as a mechanism that fine tunes neuropathic pain and Schwann cell biology.

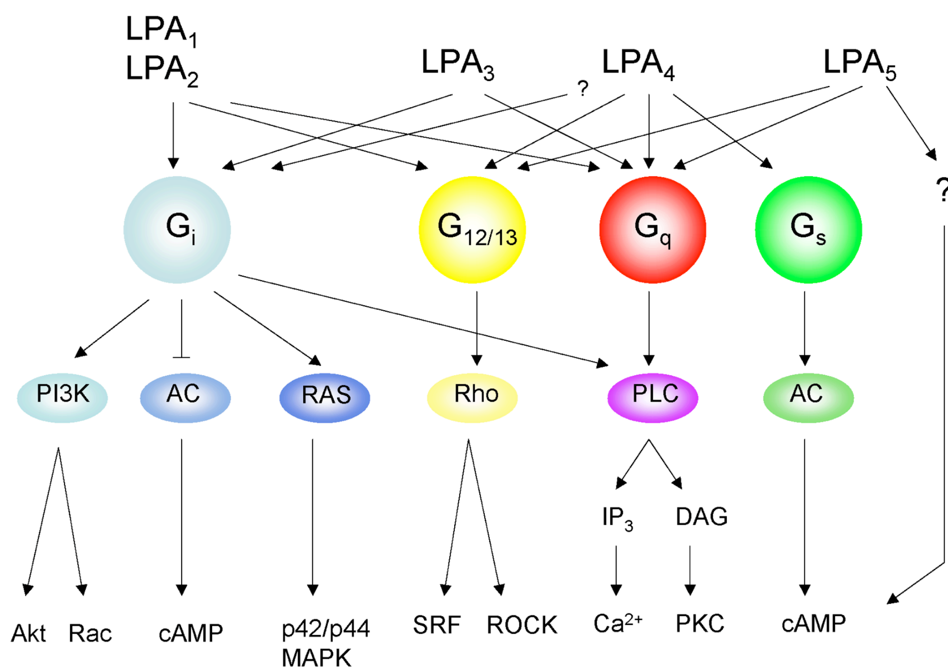

Figure 1.1 Summary of the downstream signaling pathways activated by known lysophosphatidic acid (LPA) receptors.

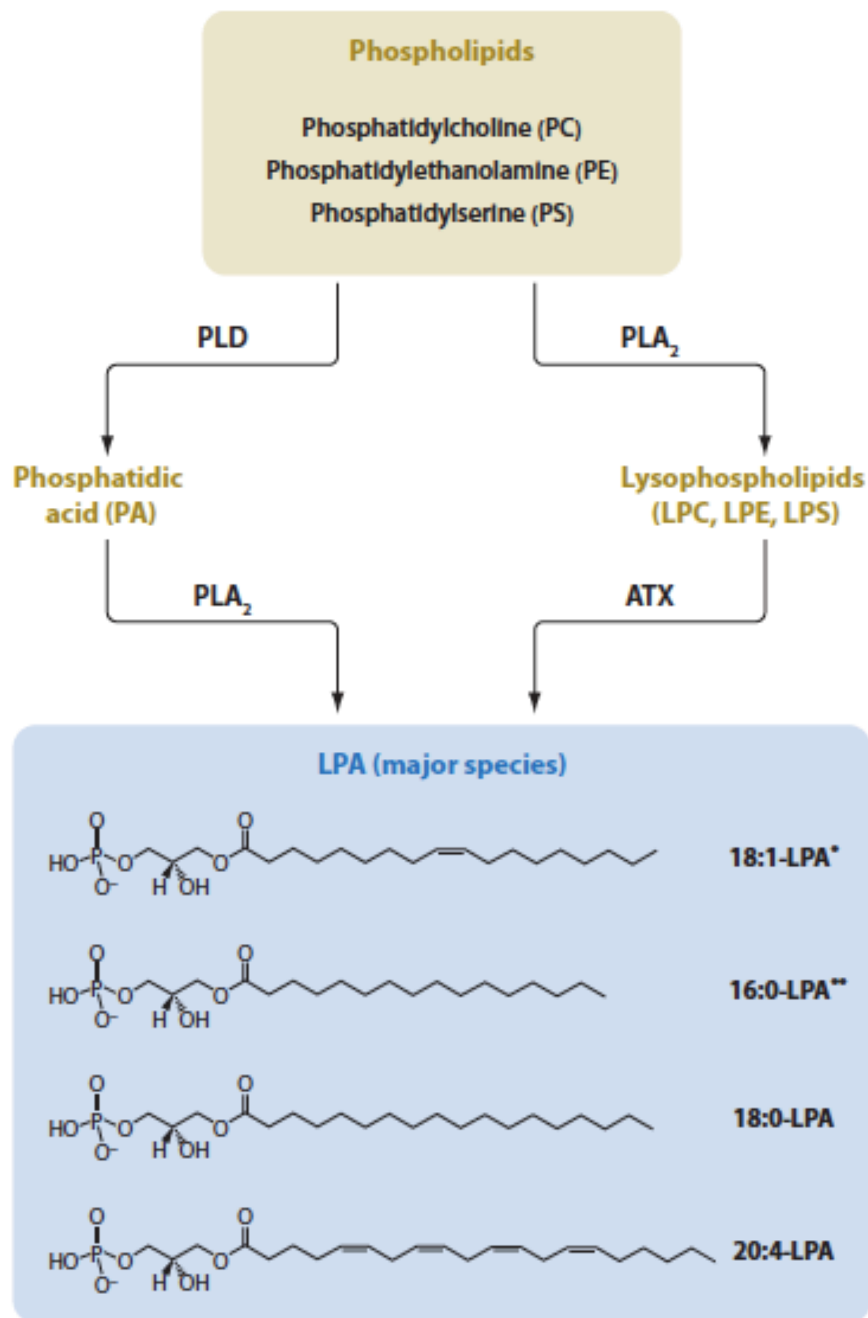

Figure 1.2 Summary of the two major LPA producing pathways.

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II.

Lysophosphatidic acid measurement using Mass Spectrometry

2.1 Introduction

Lysophosphatidic acid acts as a signaling molecule in many different systems and its distribution and regulation are key to understand its function in all physiological processes. Although many attempts have been made to measure LPA levels, reported concentrations vary widely and most of them have focused on LPA levels in serum/plasma, where LPA is present at high levels (Tokumura, Harada et al. 1986; Sasagawa, Okita et al. 1999; Xiao, Chen et al. 2000; Baker, Desiderio et al. 2001). Many different techniques have been used to detect LPA, including enzymatic cycling assay, thin layer chromatography, cell rounding assays and mass spectrometry (Eder, Sasagawa et al. 2000; Kishimoto, Matsuoka et al. 2003; Hosogaya, Yatomi et al. 2008; Shan, Jaffe et al. 2008). Amongst these techniques, mass spectrometry has the best sensitivity and ability to detect different LPA species. However, it requires a clean extraction and it is problematic due to lipid degradation and production during the process.

We used high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS), which includes two quadrupole mass analyzers connected with a collision chamber. Quadrupole mass analyzers are particular useful in detecting specific species through their ability to allow only molecules with a specific mass to charge ratio (m/z) to pass. Samples are injected into the HPLC column and separated based on their affinity for the stationary phase, which consists of silica-based particles with hydrophobic alkyl chains of C8 (octyl group). After eluting into the first quadrupole using a reverse gradient, samples are further

separate by m/z ratio, which is set to the target LPA species of interest. In order to further validate the identity, molecules collided with inert gas in the collision chamber and sent to the second quadrupole. Each molecule will break into specific pieces and the second quadrupole will then specifically select the LPA tracer and thus confirm the identity.

Mass spectrometry has excellent sensitivity and is able to detect LPA at the femtomole level. This is particularly important in biological samples where LPA levels are low or present at a limited amount in tissue. The ability to distinguish different chain lengths and even one double-bond differences is also a great advantage to other detection methods. However, the accuracy of measurement relies not only on detection but also on a refined extraction protocol. Many extraction protocols with various recovery rates have been reported and there is also the potential LPA production *in vitro* (Bjerve, Daae et al. 1974; Tokumura, Harada et al. 1986; Das and Hajra 1989; Xiao, Chen et al. 2000; Baker, Desiderio et al. 2001; Scherer, Schmitz et al. 2009). Here we modified a protocol from Baker *et al. (Baker, Desiderio et al. 2001)* extracting LPA from tissue using 1-butanol to increase the recovery rate from animal tissues and prevent LPC to LPA conversion *in vitro*.

Using this technique, we determined the amount of 18:1 LPA present in different tissues associated with pain processing, including sciatic nerve, spinal cord and cerebral cortex. We have proven the ability to detect different species of LPA using mass spectrometry and focused on the specific measurement of 18:1 LPA, which is one of the most abundant species and commonly used in the study of LPA

function. We found that the concentration of LPA present in the spinal cord is much higher than two other tissues examined and suggests an important role for LPA in the spinal cord.

2.2 Material and Methods

Sample preparation

For all procedures proper animal use and care guidelines approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute were followed. Animal tissues were harvested from 2 – 4 month old Balb/cByJ mice, weighed, quick frozen in liquid nitrogen and stored in low retention eppendorf tubes (Fisher Scientific) at -80°C until analysis. Samples (2 mg) were homogenized in 400 µl of 30 mM citric acid and 40 mM disodium phosphate, pH 2 with a hand-held homogenizer (Biospec Inc.). 100 fmole of C17 LPA was than added into the homogenate as an internal standard. Lipids were then extracted with 800 µl of 1-butanol and 400 µl of water-saturated 1-butanol. The pooled organic phase was dried down using a speedvac concentrator (Savant) and resuspended in 100 µl of methanol for mass spectrometry analysis.

Mass Spectrometry

LPA measurements were done using an Agilent 6410 triple quad mass spectrometer coupled to an Agilent 1200lc stack. Compounds were eluted with a mobile phase of H20/ACN 90:10 with 10mM NH_4OAC (A) and ACN/H_2O with 10mM NH_4OAc (B) at 0.2 ml/min. The column used was an Agilent 300SB-C8, 2.1mm x 100mm, 3.5um. Gradient was t=0, 80:20 (A:B), t=5 50:50, t=7 25:75, t=15 0:100, t=20 off. There was a 5 min re-equilibration time before the start of the next run.

The source was maintained at 350°C with a drying gas flow of 10 liters/h, and data were collected in the negative ion mode. The following transition states were monitored: LPA m/z 435 -> 153, C17 m/z 423 ->153. Calibration curves from 10 to 10000 fmol/injection of LPA standard (18:1) were prepared with LPA (17:0) as an internal standard. Peak areas of $[M-H]^-$ for LPA (18:1) form were normalized to that of the internal standard, and the normalized areas were plotted versus concentration.

2.3 Results

Standard curves are generated using pure 18:1 LPA with LC/MS/MS (Figure 2.1). The lowest detectable concentration is between 1 and 10 nM in the final sample and remains accurate with the coefficient of determination equal to 0.9997. We also wanted to see determine if the method is able to differentiate different forms of LPA with various chain lengths. A summary of our ability to detect different species of LPA is shown in Figure 2.2, where 16:0, 17:0 and 18:1 LPA are separately detected in a single scan with different m/z ratio (Figure 2.2).

Tissues from wildtype Balb/cByJ mice were analyzed for the presence and concentration of 18:1 LPA, one of the most abundant forms of LPA that is widely used among laboratories studying LPA function. Significantly high amounts of 18:1 LPA were observed in the spinal cord (101.75 ± 12.12 pmole/mg) compared to that present in the cortex (26.29 ± 0.44 pmole/mg) and sciatic nerve (35.56 ± 1.93 pmole/mg) (Figure 2.3). The overall recovery rate was 95.98% as calculated using an internal standard and an LPC to LPA conversion of only 0.19%, suggesting a reliable measurement.

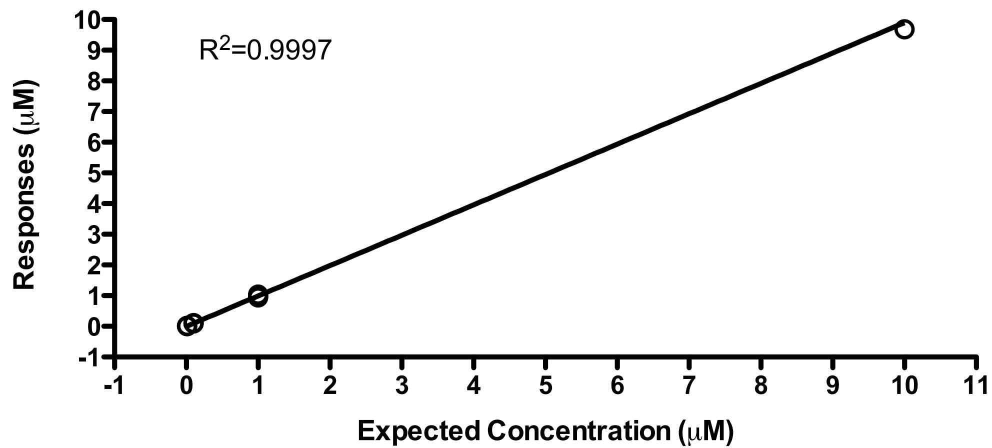

Figure 2.1 Standard curve of LPA responses using tandem mass spectrometry.

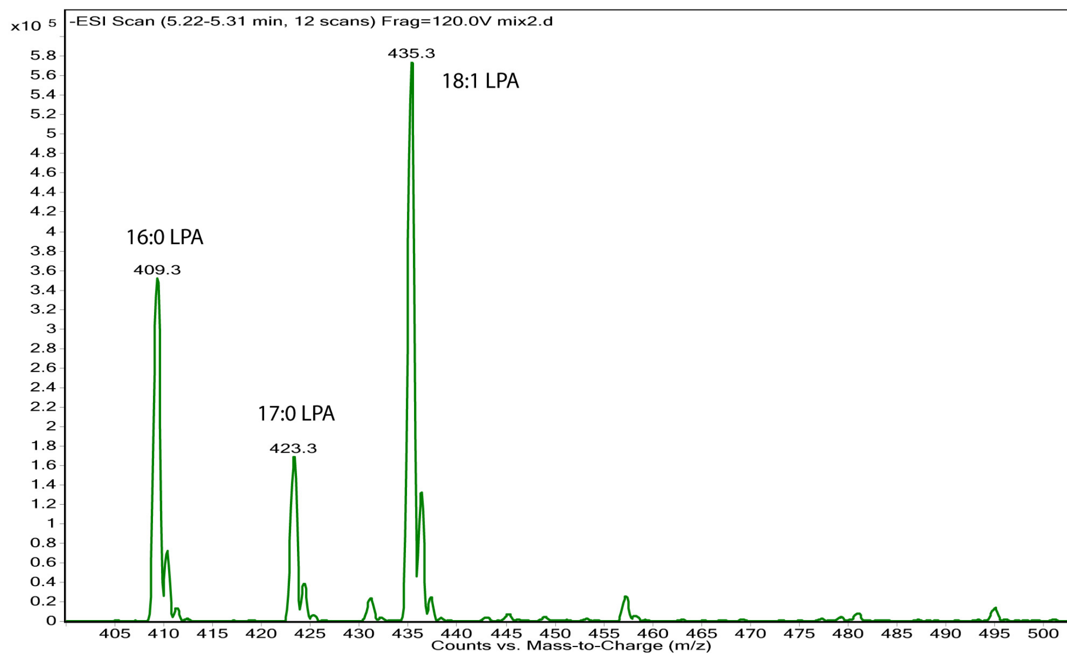

Figure 2.2 Separation of LPA with different carbon chain lengths using mass spectrometry.

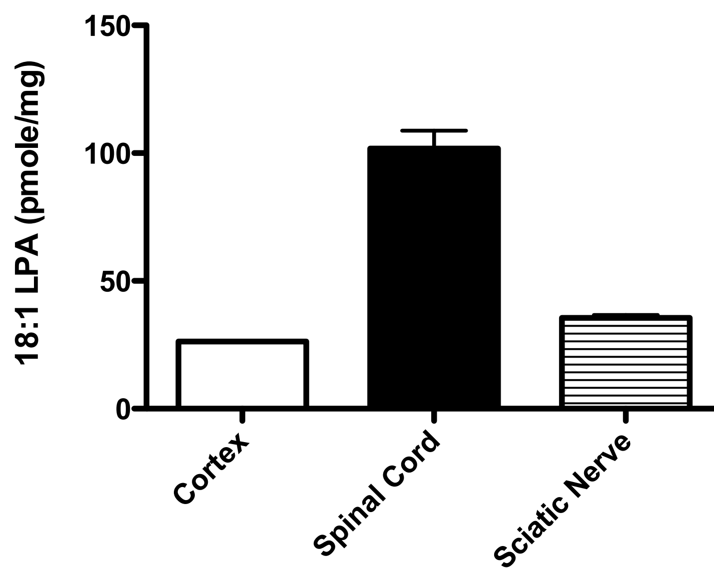

Figure 2.3 18:1 LPA concentration in wildtype mouse cortex, spinal cord (L4-6), and sciatic nerve. Bars represent mean ± SD of 18:1 LPA detected (pmole/mg), N = 3.

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III.

Development of neuropathic pain through lysophosphatidic acid (LPA) and its receptor subtype, LPA_5

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3.1 Abstract

Lysophosphatidic acid (LPA) is a bioactive lipid that serves as an extracellular signaling molecule acting through cognate G protein-coupled receptors (GPCRs) designated $LPA_{1\text{-}6}$, mediating a wide range of both normal and pathological effects. Previously, LPA_1, a $G_{\alpha i}$-coupled receptor (which also couples to other G_α proteins) that reduces cAMP, was shown to be essential for the initiation of neuropathic pain in the partial sciatic nerve ligation (PSNL) mouse model. Gene expression studies identified LPA_5, a $G_{\alpha 12/13}$ and G_q-coupled receptor that increases cAMP, in a subset of dorsal root ganglion (DRG) neurons and also within neurons of the spinal cord dorsal horn in a pattern complementing, yet distinct, from LPA_1. To determine the possible involvement of LPA_5 in aspects of neuropathic pain, we generated a null mutant by targeted deletion and challenged it with PSNL. Homozygous null mutants did not show obvious phenotypic defects. However, when challenged with PSNL, LPA_5 null mice failed to develop neuropathic pain through a mechanism distinct from LPA_1 null mutants that are protected from peripheral demyelination in response to PSNL. PSNL-challenged LPA_5 null mice also showed reduced pCREB expression within neurons of the dorsal horn and further showed continued upregulation of characteristic pain-related markers $Ca\alpha_2\delta_1$ and GFAP; these results contrasted with those previously observed for LPA_1 deletion. These analyses expand the involvement of LPA signaling in neuropathic pain through the identification of a second LPA receptor subtype, LPA_5 that is mechanistically distinct from LPA_1.

3.2 Introduction

Neuropathic pain is a chronic pain state initiated by injury or perturbation of the peripheral nervous system (PNS) or central nervous system (CNS). In developed countries, around 7-8% of the population is affected by neuropathic pain and the limited treatment options are often ineffectual (Torrance, Smith et al. 2006; Bouhassira, Lanteri-Minet et al. 2008). One of the animal models developed to study neuropathic pain is "partial sciatic nerve ligation (PSNL)" which mimics at least some of the major endpoints observed in human neuropathic pain (Seltzer, Dubner et al. 1990). These models led to the identification of a range of different stimuli that induces neuropathic pain (Miletic, Pankratz et al. 2002; Gao, Kim et al. 2005; Sorkin and Yaksh 2009), one of which is an extracellular signaling lipid known as lysophosphatidic acid (LPA), a lysophospholipid which normally participates in the regulation of diverse cellular activities (Inoue, Rashid et al. 2004).

LPA is involved in various pathological conditions including cardiovascular diseases (Karliner 2004), fibrosis (Swaney, Chapman et al. 2010), cancer (Panupinthu, Lee et al. 2010), infertility (Ye, Hama et al. 2005), and CNS disorders such as hydrocephalus (Yung, Mutoh et al. 2011), and neuropathic pain (Inoue, Rashid et al. 2004). These effects are produced through one or more of six confirmed G protein-coupled receptors, LPA_{1-6} (gene names *Lpar1*-*Lpar6*), that couple to different combinations of heterotrimeric G proteins (Noguchi, Herr et al. 2009; Lin, Herr et al. 2010). Insights into the biological roles for LPA signaling have come from LPA receptor null mutant mice studies (Contos, Ishii et al. 2000;

Contos, Ishii et al. 2002; Sumida, Noguchi et al. 2010), however, mice deficient for *Lpar5* have not been reported (Choi, Herr et al. 2010).

Using *Lpar1* null mutants, prior studies identified LPA_1 as an essential receptor for the initiation of neuropathic pain induced by both intrathecal injection of LPA and by PSNL (Inoue, Rashid et al. 2004). Surrogate markers of neuropathic pain such as demyelination of the dorsal root, Protein kinase C-γ (PKCγ), and calcium channel $Ca\alpha_2\delta_1$ expression were all significantly reduced in *Lpar1* null mice challenged with PSNL (Inoue, Rashid et al. 2004). Related studies have also supported the involvement of LPA_1 in neuropathic pain (Inoue, Rashid et al. 2004; Xie, Matsumoto et al. 2008; Cohen, Sagron et al. 2009). These reports also raised the possibility that other LPA receptors might contribute to aspects of neuropathic pain.

LPA_5, previously known as orphan receptor GPR92, was identified as a fifth LPA receptor in 2006 (Lee, Rivera et al. 2006). LPA_5 activates $G_{\alpha 12/13}$ and $G_{\alpha q}$ signaling responses, and can also increase intracellular cAMP levels, however, this response is not altered in the presence of a $G_{\alpha s}$ minigene, suggesting alternative G protein involvement (Lee, Rivera et al. 2006). *Lpar5* is expressed in spleen, heart, platelets, gastrointestinal lymphocytes, dorsal root ganglia (DRG), and in the developing brain (Kotarsky, Boketoft et al. 2006; Lee, Rivera et al. 2006; Amisten, Braun et al. 2008; Ohuchi, Hamada et al. 2008). The high expression level in DRG neurons suggested the possible involvement of LPA_5 in pain signaling. Here we

report the generation of an *Lpar5* deficient mouse and its evaluation in the PSNL model towards determining its contribution to neuropathic pain.

3.3 Material and Methods

Lpar5 gene targeting

Portions of the *Lpar5* genomic locus were amplified from a BAC clone obtained from Children's Hospital Oakland Research Institute (CHORI) using a high fidelity Pfx50 DNA polymerase (Invitrogen). Most of the coding exon (from the ATG to the BamHI site) was replaced in frame with EGFP using overlap PCR. A neomycin cassette flanked by loxP sites and an introduced HindIII restriction enzyme site was inserted into the BamHI site of the coding exon. All amplified genomic fragments and modified fragments were assembled in pBluescript and the entire targeting construct was sequenced at The Scripps Research Institute (TSRI) Center for Protein and Nucleic Acid Research.

The targeting construct was linearized and electroporated into R1 ES cells (purchased from the lab of Andras Nagy). Genomic DNA was extracted from neomycin resistant clones, digested with HindIII, and screened for homologous recombination by Southern blotting with the indicated probe using standard techniques. Positive clones were rescreened, tested for pathogens and two clones were injected into blastocysts at the TSRI Mouse Genetics Core. Resultant chimeras were crossed to C57BL/6J female mice to assay for germline transmission. Heterozygous mice were then bred together to generate null mutant animals. Genotypes of all heterozygous cross offspring were confirmed by Southern blotting and PCR genotyping with the following primers, GFP Int Rev: 5'-GTGGTGCAGATGAACTTCAGG-3', 92GTFor: 5'-

CAGAGTCTGTATTGCCACCAG-3', and 92GT Rev: 5'-GTCCACGTTGATGAGCATCAG-3'. Wildtype and mutant PCR product sizes are approximately 450 and 220 base pairs respectively.

Reverse transcription PCR

To confirm loss of *Lpar5* gene transcripts, bone marrow, spleen, and thymus were dissected from wildtype and null mutant mice. Single cell suspensions were made from all tissues and cells were pelleted at 1400 rpm in a centrifuge. Cell pellets were resuspended in TRIzol reagent (Invitrogen) and total RNA was isolated following the manufacturer’s protocol. RNA was DNAse treated and cDNA synthesized using the SuperScript II First Strand cDNA Synthesis System (Invitrogen). Reverse transcription PCR was used to amplify beta actin and *Lpar5* transcripts from the cDNA with the following primer pairs; beta actin For 5’-TGGAATCCTGTGGCATCCATGAAC-3’, beta actin Rev 5’-TAAAACGCAGCTCAGTAACAGTCCG-3’, 92RTFor 5’-ACTCCACGCTGGCTGTATATG-3’, 92RTRev 5’-GTAGCCAAAGGCCTGGTATTC-3’.

***In situ* hybridization**

For *in situ* hybridization analysis, a probe corresponding to the *Lpar5* deleted region was cloned into pBluescript, linearized with restriction enzymes on either side of the insert, and sense and antisense runoff probes were labeled with digoxigenin (DIG) labeling mix (Roche) using T7 and T3 RNA polymerases

(Roche), respectively. *In situ* hybridization was performed according to Braissant *et al*. with modifications (Braissant and Wahli 1998; Plenz, Weissen et al. 2003). Briefly, fresh-frozen blocks of DRG and spinal cord were cut at 16-20 μm and sections fixed with 4% PFA, permeablized, dehydrated, and stored at -80°C until use. Pre-hybridization was performed at 70°C for three hours and probes were incubated at 68°C overnight. Signal was detected with anti-DIG/AP Fab fragments (Roche) at a 1:200 dilution overnight and visualized in NBT/BCIP solution (Millipore). Double staining was then performed on the same slide using anti-NeuN antibodies (Millipore) at a 1:200 dilution.

Partial sciatic nerve ligation (PSNL)

The partial sciatic nerve ligation procedure was modified from Seltzer *et al* (Seltzer, Dubner et al. 1990). Briefly, 2-4 month old *Lpar5* null mutants and wildtype mice in a C57 background were deeply anesthetized using an isoflurane vaporizer with a nose cone throughout PSNL surgery. The right thigh of the mouse was opened and the sciatic nerve exposed, about 1/2 to 1/3 of the sciatic nerve was tightly ligated using 10-0 fine silk sutures. The wound was then closed and skin stitched for recovery. Paw withdrawal threshold responses were monitored before and after surgery.

Behavioral testing

Paw withdrawal threshold was performed with an automated von Frey apparatus (Ugo Basile, Italy). Mice were acclimated in plastic cages with metal

mesh bottoms for an hour prior to testing in a temperature and humidity controlled testing room. Paw withdrawal threshold (gram) against gradually increasing mechanical stimuli (0-50g in 20 seconds) was tested four separate times with at least a one-minute interval. The average response was normalized with pre-surgery control and presented in mean ± S.D.

Detection of demyelination

Dorsal root (DR) was collected and fixed in 1% PFA, 3% Glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) with 5 mM $CaCl_2$ overnight at 4°C. The fixed DR fiber was then osmicated in 1% OsO_4/0.12M cacodylate buffer/3.5% sucrose on ice for 2 hours. Samples were then dehydrated in a graded alcohol series followed by acetone treatment and embedded in Epon resins. Sections of 1 μm were cut and stained with 1% Toluidine blue-O and examined under a light microscope.

Immunohistochemistry

Spinal cord and DRGs from L4-L6 regions were collected and fresh frozen in OCT compound (Sakura Finetek). In a cryostat, 16 μm sections were cut and fixed with 4% paraformaldehyde, blocked with 3% normal goat serum, and 0.1% Triton-X in PBS. Primary antibodies against $Ca\alpha_2\delta_1$ (1:200, Sigma) and GFAP (1:500, Sigma) were diluted in blocking buffer and incubated overnight at 4°C. After washing three times in PBS, corresponding secondary antibodies were diluted 1:1,000 in the same buffer and incubated for one hour at room temperature. pCREB staining was performed using rabbit anti-pCREB (1:500, Cell Signaling) under the

same conditions and visualized using ABC and DAB kit (Vector Labs) following standard protocols.

3.4 Results

Generation of LPA_5 deficient mice

Initial gene expression studies (Lee, Rivera et al. 2006) identified LPA_5 in DRG neurons, suggesting functional consequences of this receptor that might be assessed through an *Lpar5* null mutant mouse that was not yet available. Gene targeting was therefore used to eliminate and replace most of the *Lpar5* coding region in frame with EGFP allowing for the production of *Lpar5* deficient mice (Figure 3.1A). Mice heterozygous for the *Lpar5* knock-in allele were crossed to each other and wildtype (+/+), heterozygous (+/-), and null mutant offspring (-/-) were produced, at near Mendelian ratios (data not shown), indicating that *Lpar5* is dispensable for embryonic viability. Genotypes of mice generated from crosses of heterozygous mice were determined by PCR and confirmed by Southern blotting (Figure 3.1B and C).

Lpar5 is expressed in lymphoid tissues including the thymus and spleen (Lee, Rivera et al. 2006). In order to confirm deletion of *Lpar5*, total RNA was isolated from wildtype and null mutant thymus, spleen, and bone marrow and the loss of *Lpar5* mRNA was assessed by reverse transcription (RT) PCR. An absence of an *Lpar5* specific RT-PCR product in null mutant tissues confirmed proper targeting of the *Lpar5* genomic locus (Figure 3.1D). Expression of *Lpar5* in these lymphoid organs suggests that *Lpar5* plays a functional role in these tissues, however thymus and spleen lymphocyte proportions and numbers were similar in both wildtype and

knockout mice (data not shown). The actual functions of LPA_5 in the immune system remain to be determined.

Using *in situ* hybridization, *Lpar5* mRNA was detected in a subset of dorsal root ganglion (DRG) neurons, and this expression pattern was completely absent from knockout animal tissue (Figure 3.2A and B). *Lpar5* was also expressed in the dorsal horn area of the lumbar spinal cord, and as expected, the specific signal is absent in tissues from *Lpar5* deficient mice (Figure 3.2C and D). Immunolabeling with an antibody against the neuronal specific marker NeuN combined with *Lpar5* *in situ* hybridization showed that the LPA_5 expressing cells are neurons, including dorsal horn sensory neurons, as anticipated, and in addition, CNS motor neurons of the spinal cord ventral horn (Figure 3.2E-I). The expression of LPA_5 in both DRG and spinal dorsal horn neurons is consistent with a role for LPA_5 in pain processing.

***Lpar5* null mice are protected from injury-induced neuropathic pain**

It has been shown that LPA_1 is required for the initiation of injury-induced neuropathic pain (Inoue, Rashid et al. 2004). However, other LPA receptors have not been evaluated in this context. To examine the role of LPA_5 in the development of neuropathic pain, we utilized partial sciatic nerve ligation (PSNL), as described by Seltzer *et al.* (Seltzer, Dubner et al. 1990) to induce neuropathic pain in *Lpar5* null mice. Neuropathic pain status was assessed with an automated Von Frey apparatus to record paw withdrawal threshold against mechanical stimulus. After PSNL, wildtype mice developed neuropathic pain that was evident by a decreased paw withdrawal threshold, however the threshold of *Lpar5* null mice remained at

baseline levels (Figure 3.3A). This phenotype was similar to that observed in *Lpar1* null mice and raised the question of whether the pain response occurs via common or distinct LPA-dependent pathway(s).

Following PSNL injury, affected neurons are often demyelinated, which can lead to cross talk between axons that can then contribute to the development of neuropathic pain (Rasminsky 1978; Smith and McDonald 1982). In wildtype animals subject to PSNL, both the sciatic nerve (data not shown) and dorsal root from L4-L6 showed an increased percentage of myelin sheath aberrations resembling demyelination (Inoue, Rashid et al. 2004). However, in *Lpar1* null mice, these myelin sheath aberrations in the dorsal root were decreased compared to control mice, which correlated with reduced pain responses (Inoue, Rashid et al. 2004). Since *Lpar5* null mutant mice showed a similar attenuated neuropathic pain response, this led us to examine the myelination status in the dorsal root area of *Lpar5* null mice after PSNL. Similar myelin sheath splitting was observed and was significantly increased in the PSNL animals (Figure. 3B-D, asterisk). Surprisingly, at post-surgery day 6 there were no significant difference observed between heterozygous control and null mutant mice (Figure. 3E). In addition, classic characteristics of demyelination including naked axons and macrophage infiltration were not seen in any dorsal root sections regardless of genotype or treatment. This result indicates that in contrast to LPA_1, LPA_5 is not significantly involved in a demyelination process induced by nerve injury.

Two markers of neuropathic pain, the $\alpha_2\delta_1$ subunit of voltage-gated calcium channels ($Ca\alpha_2\delta_1$) and the γ-isoform of protein kinase C (PKCγ), have been used in identifying LPA_1 involvement in neuropathic pain. $Ca\alpha_2\delta_1$ has been shown to be elevated in the DRG following nerve injury. This correlates with allodynia status and PKCγ levels in the spinal cord dorsal horn, which participates in neuropathic pain development (Malmberg, Chen et al. 1997; Luo, Chaplan et al. 2001; Luo, Calcutt et al. 2002). Both markers were significantly elevated in control mice subjected to PSNL, but not in mice deficient for LPA_1 (Inoue, Rashid et al. 2004). We compared the expression of $Ca\alpha_2\delta_1$ in DRG and PKCγ in the spinal cord dorsal horn of control vs. *Lpar5* deficient mice that had been subjected to PSNL. Surprisingly, $Ca\alpha_2\delta_1$ (Figure 3.4A-C) expression levels were not reduced in *Lpar5* null mutants. And the PKCγ level were not changing regardless of treatment and genotypes (data not shown). It has been reported that astrogliosis increases after PSNL and correlates with the development of neuropathic pain (Coyle 1998). To assess the extent of astrogliosis, expression of glial fibrillary acidic protein (GFAP, an astrocyte marker) was examined in the spinal dorsal horn prior to and after injury. Consistent with $Ca\alpha_2\delta_1$ levels, GFAP is upregulated following PSNL and expression is not altered by the loss of $LPA_{5,}$ quantified by pixel intensities and counts (data not shown) (Figure 3.4D-F).

pCREB expression through LPA_5 is associated with neuropathic pain

In the spinal cord dorsal horn, phosphorylated cAMP response element binding proteins (pCREB) are up-regulated in response to neuropathic pain induced

by two different models of neuropathic pain, chronic constriction injury (CCI), and PSNL (Ma and Quirion 2001; Song, Cao et al. 2005). Furthermore, down-regulation of pCREB is associated with anti-hyperalgesic effects (Liou, Liu et al. 2007; Wang, Cheng et al. 2011). Interestingly, in contrast to LPA_1, exposure of LPA_5 to LPA stimulation results in increased cellular cAMP levels (Kotarsky, Boketoft et al. 2006; Lee, Rivera et al. 2006). This result led us to determine whether or not the loss of LPA_5 can affect pCREB expression in the spinal cord dorsal horn after nerve injury. Six days after PSNL, the ipsilateral-to-contralateral ratio of pCREB expression in the spinal cord dorsal horn Laminae I-II was markedly reduced in *Lpar5* null mice as compared to wildtype controls (Figure 3.5). This suggests that in the absence of LPA_5, upregulation of pCREB in response to PSNL is abrogated, leading to protection from neuropathic pain.

3.5 Discussion

Prior studies showed the importance of LPA signaling through LPA_1 in the initiation of neuropathic pain. Here, through the use of the first reported *Lpar5* deficient mice, a distinct LPA receptor, LPA_5, was identified as a new influence on neuropathic pain as assessed by PSNL. The mechanism through which LPA_5 produces protection appears to be distinct from LPA_1 (Inoue, Rashid et al. 2004), demonstrating that different receptors for the same endogenous ligand can affect pain development through non-identical molecular and cellular pathways.

LPA_5 gene expression in DRG and spinal cord dorsal horn neurons, as revealed by *in situ* hybridization, suggests a possible role in pain. Interestingly, normal pain sensation is not altered in LPA_5 deficient mice, whereas the development of nerve-injury induced neuropathic pain is abolished. This protective effect is not seen in heterozygous mice that show wildtype PSNL susceptibility, indicating that a single allele of *Lpar5* is sufficient for the development of neuropathic pain. These results also suggest that LPA_5 is activated during injury but not under basal conditions, which is consistent with the increased levels of LPA detected in spinal cord dorsal horn and dorsal roots following nerve injury (Ma, Uchida et al. 2010).

In the initial report describing the involvement of LPA_1 in neuropathic pain development, a significant reduction of demyelination in the dorsal root area of *Lpar1* deficient mice was proposed to be the protective mechanism (Inoue, Rashid et al. 2004). Subsequent studies validated that LPA-LPA_1 signaling was responsible

for the dorsal root demyelination (Nagai, Uchida et al. 2010). In contrast, no difference in nerve-injury induced demyelination between wildtype and *Lpar5* deficient mice was detected, despite maintained LPA_1 expression, indicating that LPA_5 loss does not prevent demyelination, despite protecting against the development of neuropathic pain. This observation provides the first evidence that, despite sharing the same endogenous ligand and partially overlapping expression patterns, LPA_1 and LPA_5, participate in neuropathic pain development through distinct pathways.

In DRG, $Ca\alpha_2\delta_1$ expression has been shown to increase with nerve injury-induced neuropathic pain and is thought to increase the excitability of DRG neurons (Luo, Chaplan et al. 2001; Luo, Calcutt et al. 2002). The activation of this protein is greatly reduced in *Lpar1* null mice (Inoue, Rashid et al. 2004). Combined with the reduction of demyelination, these phenomena suggest that the loss of LPA_1 signaling prevents abnormal pain signaling transmission from the periphery into the central nervous system. By contrast, $Ca\alpha_2\delta_1$ still increases in *Lpar5* null mice when challenged with nerve injury, indicating relatively normal pain transmission through the DRG to the spinal cord dorsal horn. Surprisingly, no significant elevation of PKCγ was observed in any genotype and treatment, despite the clear production of the pain phenotype in wildtype PSNL animals, possibly reflecting background strain differences of prior studies.

Astrocytes are known to be activated during neuropathic pain as manifested by astrogliosis and are thought to contribute to the development of neuropathic pain

(Gao and Ji 2010). Upon activation, astrocytes can release cytokines/chemokines including interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), and monocyte chemotactic protein 1 (MCP-1), which can promote the neuropathic pain phenotype (Milligan and Watkins 2009). These molecules are also important in maintaining synaptic connectivities required for proper neuronal signaling, interacting with neurons through the release of neurotransmitters such as glutamate (Bezzi, Domercq et al. 2001). Astrogliosis in the L4-L6 spinal dorsal horn was identified by GFAP immunostaining, which demonstrated that astrocytes are indeed activated in all injured animals. Nevertheless, no significant differences between *Lpar5* knockout vs. wildtype animals were detected. These data indicate that the spinal cord dorsal horn continues to receive normal pain signals despite LPA_5 loss and the accompanying abrogation of the pain phenotype.

In wildtype animals exposed to PSNL, LPA_5 could contribute to increased pCREB expression through at least two different pathways. Increased levels of LPA following nerve injury provides a high concentration of ligand to activate LPA_5 in dorsal horn neurons that could produce an increase in cAMP and pCREB, which would be associated with increased sensitivity of dorsal horn neurons to pain stimuli. In addition, the increased LPA concentration may also activate a subgroup of DRG neurons expressing LPA_5 to increase their stimulation of neurons in the dorsal horn, thus leading to increased pCREB expression within the spinal cord. Additional experiments are required to elucidate the detailed mechanism, however,

the abrogated pCREB activation and failure to develop neuropathic pain observed in *Lpar5* null mice was unambiguous and distinct from endpoints observed with loss of LPA_1. We speculate that the loss of LPA_5 activity reduces neuronal signaling required to induce neuropathic pain.

Interestingly, cellular cAMP levels have been shown to increase in response to LPA through LPA_5 signaling and this is the opposite response observed with LPA_1-$G_{\alpha i}$ activation that reduces cAMP (Kotarsky, Boketoft et al. 2006; Lee, Rivera et al. 2006), representing a possible explanation for the differences observed between *Lpar5* and *Lpar1* null mutants. Increased cAMP can lead to CREB phosphorylation and hyperalgesia via central sensitization (Hoeger-Bement and Sluka 2003; Kawasaki, Kohno et al. 2004). Decreased pCREB expression is also associated with increased pain threshold (Liou, Liu et al. 2007; Wang, Cheng et al. 2011). Consistent with previous reports, a low level of pCREB expression was observed in the dorsal horn of naïve mice, however this was strongly increased in animals challenged with nerve injury (Ji and Rupp 1997; Ma and Quirion 2001). Importantly and consistent with a loss of cAMP production, PSNL-induced pCREB expression was markedly reduced in *Lpar5* null mice. This reduced expression may in part account for the protective effects of LPA_5 loss, as increased pCREB expression is strongly associated with neuropathic pain after nerve injury (Liou, Liu et al. 2007; Wang, Cheng et al. 2011). Furthermore, this specific reduction in pCREB expression was not observed in LPA_1 knockout mice (data not shown),

further demonstrating that each receptor subtype has distinct effects that separately contribute to neuropathic pain.

In conclusion, LPA_5 appears to contribute to neuropathic pain through central pCREB activation. The protective effects observed following PSNL in animals deficient for *Lpar5* is mechanistically distinct from that mediated through LPA_1, in part reflecting the opposing actions of these different LPA receptors on cAMP levels and the production of pCREB. These data further support LPA signaling in the development of neuropathic pain, and suggest that inhibition of LPA_1 and/or LPA_5 via pharmacological antagonism could be relevant for novel neuropathic pain therapeutics.

3.6 Acknowledgement

Chapter 3, in full, has been submitted for publication, Lin, Mu-En; Rivera, Richard R.; Chun, Jerold. The dissertation author was the primary investigator and author of this paper.

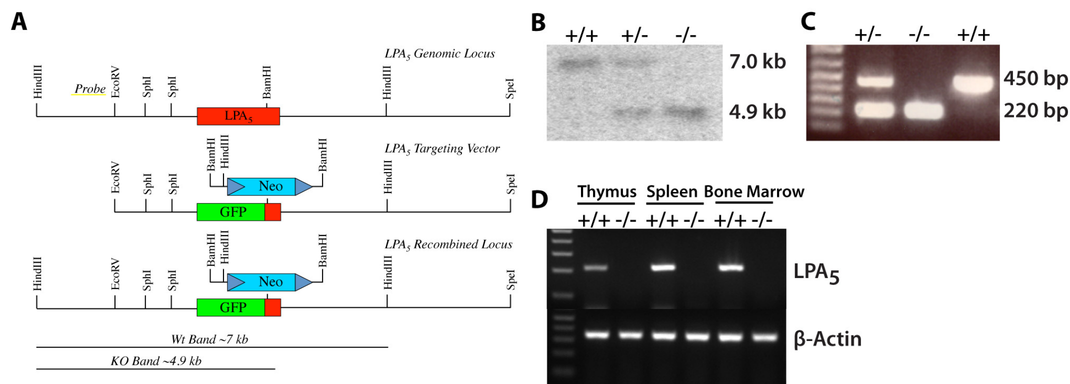

Figure 3.1 Targeted disruption of the *Lpar5* genomic locus and generation of knockout mice by homologous recombination. (**A**) Schematic diagram of the *Lpar5* gene targeting strategy. To generate the *Lpar5* targeting vector a portion of the *Lpar5* coding region was removed and replaced in frame by EGFP (middle panel). ES cell clones positive for homologous recombination were identified by digestion of genomic DNA with HindIII and Southern blotting with the external probe shown in the top panel. ES cell clones with non-homologous recombination events showed a 7 kb band while clones with homologously recombined DNA produced a 4.9 kb band. (**B**) Southern blot showing the properly recombined product for wildtype (7 kb) and knockout animal (4.9 kb). (**C**) PCR genotyping showing the *Lpar5* wildtype (450bp) and mutant (220bp) products, primers are indicated in (**A**). (**D**) RT-PCR of cDNA from thymus, spleen, and bone marrow from wildtype and *Lpar5* homozygous mutant mice shows an absence of *Lpar5* mRNA in tissues from knockout animals. b-actin control for all tissues is shown.

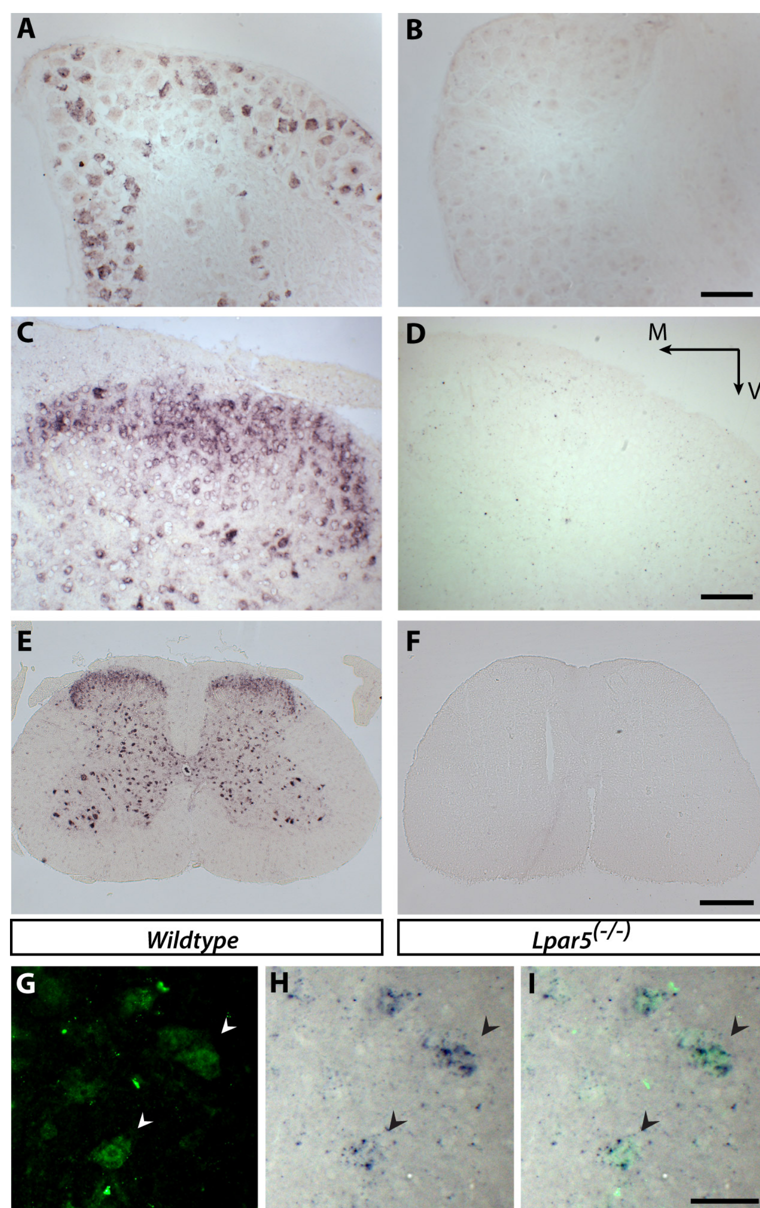

Figure 3.2 ***In situ*** **hybridization and immunolabeling of tissues from wildtype and null mutant mice with *Lpar5* DIG labeled anti-sense probes and NeuN antibody.** Sections of dorsal root ganglia (DRG) from wildtype (**A**) and *Lpar5* null mice (**B**); note that the expression of *Lpar5* is limited to small to medium size DRG neurons and is absent in DRG from *Lpar5* null mutant mice. Sections of spinal cord dorsal horn from wildtype (**C**) and *Lpar5* null mice (**D**) showing *Lpar5* expression in the dorsal horn area. Scale bar = 100 µm. M = Medial, V = Ventral. Low magnification of whole spinal cord sections from wildtype (**E**) and *Lpar5* null mice (**F**). Note that both dorsal horn and ventral horn neurons are labeled. Scale bar = 400 µm. Double labeling using anti-NeuN antibody (**G**) and *in situ* hybridization against *Lpar5* (**H**) confirmed *Lpar5* expression in neurons (**I**). Arrowheads indicate the same cells from G-I. Scale bar = 50 µm.

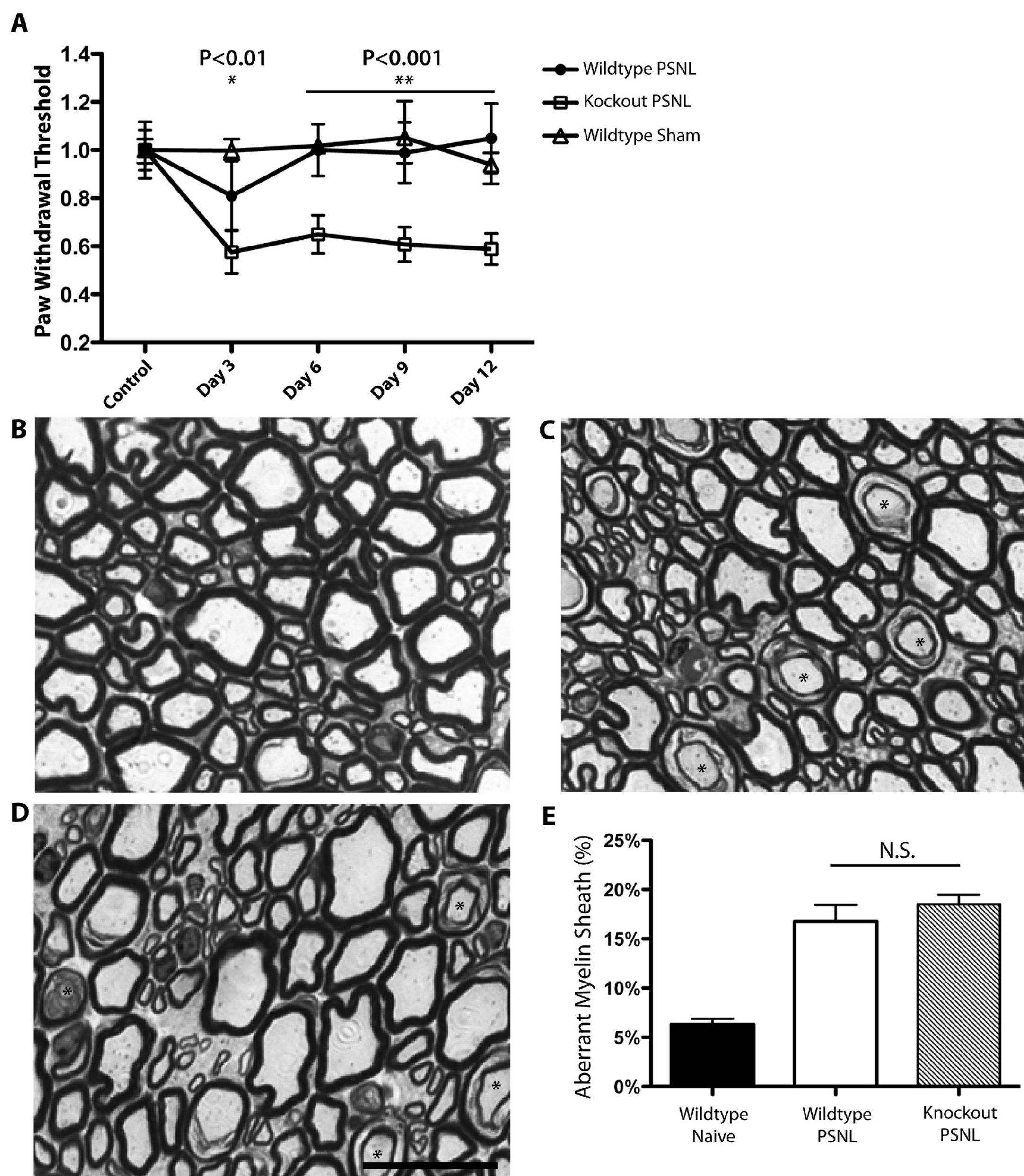

Figure 3.3 Loss of partial sciatic nerve ligation (PSNL)-induced neuropathic pain in *Lpar5* null mice. (**A**) Paw withdrawal threshold against mechanical stimuli after PSNL or sham operation. P-value compares homozygous null mutants with wildtype mice with PSNL. * P<0.01; ** P<0.001. Representative semi-thin sections of L5 dorsal roots, six days after PSNL, from naïve wildtype (**B**), PSNL wildtype (**C**) and PSNL LPA_5 null mice (**D**). Scale bar = 20 μm.

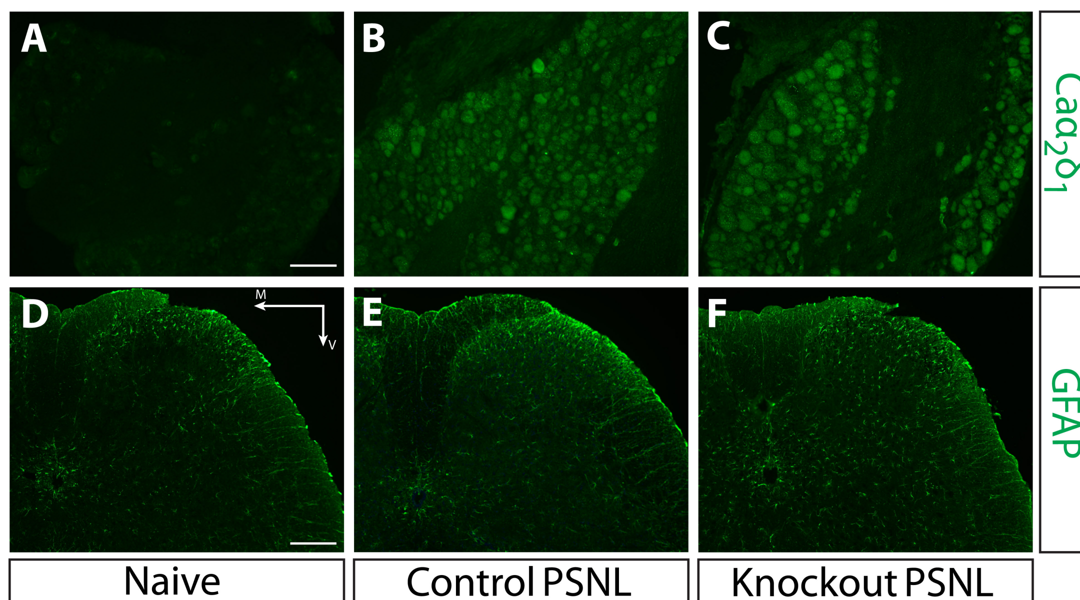

Figure 3.4 Immunohistochemistry showing $Ca\alpha_2\delta_1$ immunoreactivity in DRG as well as PKCγ, and GFAP immunoreactivity in the L5 spinal cord region six days after PSNL. (**A, B, C**) $Ca\alpha_2\delta_1$ is significantly up-regulated in DRG after PSNL, however, no significant difference was observed between heterozygous control and null mutant mice. Scale bar = 100 µm. (**D, E, F**) GFAP was significantly increased in PSNL mice, indicating astrocyte activation, however, *Lpar5* null mutant mice showed similar, if not increased, GFAP immunostaining compared to the heterozygous control. Scale bar = 200µm. M = Medial, V = Ventral.

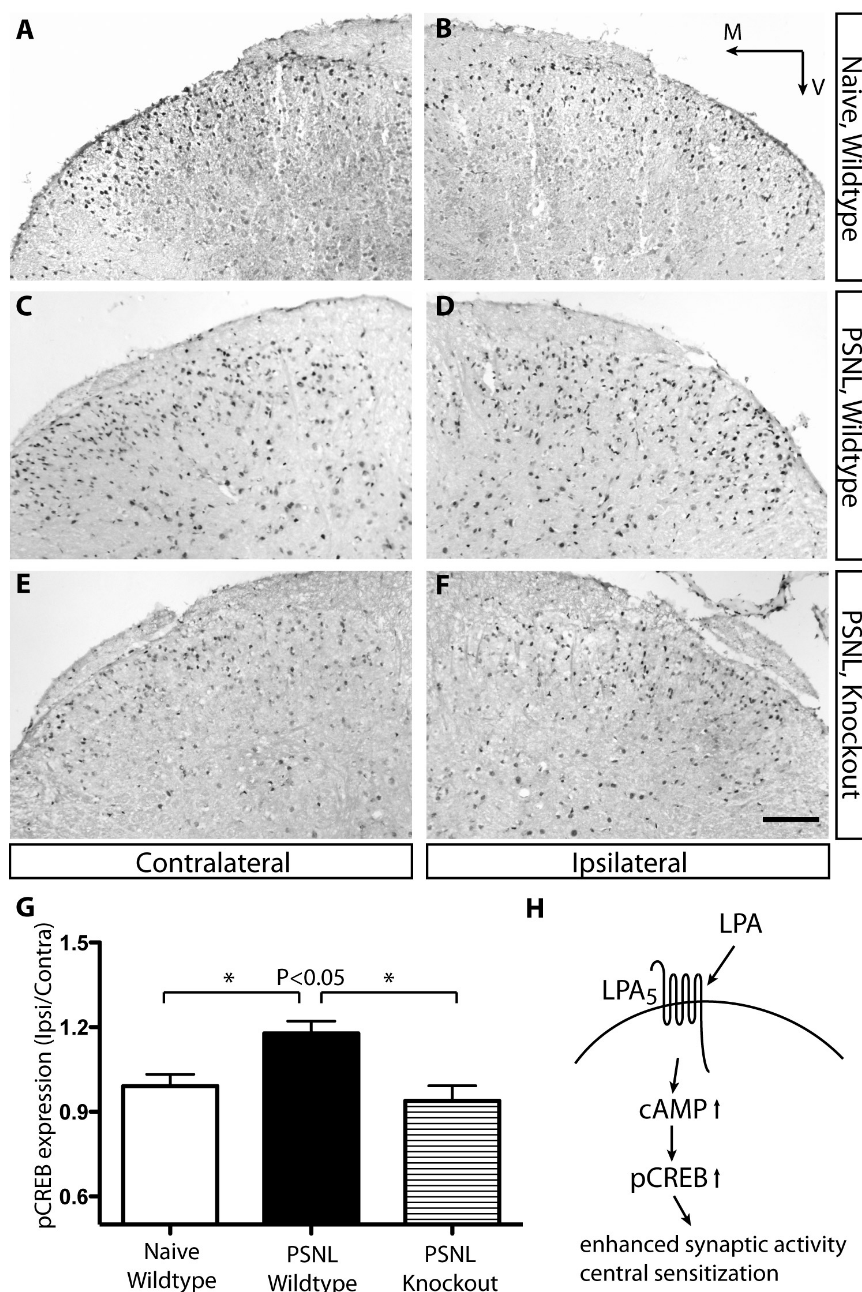

Figure 3.5 pCREB expression in the spinal dorsal horn L4-6, six days after PSNL. Immunolabelling of pCREB is markedly increased in control mice after PSNL (**C, D**) compared to naïve mice (**A, B**). Attenuated expression of pCREB was observed in *Lpar5* null mice that were subjected to the same nerve injury (**E, F**). Scale bar = 100μm. M = Medial, V = Ventral. (**G**) Quantification of pCREB immunostaining, represented by ratios of labeled cells between ipsilateral and contralateral side of the same section. * $P<0.05$ (**H**) Schematic diagram of proposed LPA_5 involvement in neuropathic pain development.

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IV.

Activation of LPA_1 through neuronally secreted LPA induces Schwann cell migration *in vitro* and impacts axonal segregation and myelination in peripheral nerves

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4.1 Abstract

Schwann cell (SC) migration is an important step preceding myelination and remyelination in the peripheral nervous system. Various factors including neuregulin are known to regulate these important SC differentiation processes. Here we present evidence that biologically active lysophosphatidic acid (LPA) is a novel soluble factor influencing both peripheral myelination and SC migration. The LPA_1 null mutant lacking one out of six known G protein-coupled receptors for LPA showed a significant reduction in the thickness of peripheral nerve myelin sheaths as compared to controls. In addition, at postnatal day 24 some axon bundles in the sciatic nerve of LPA_1 null mutants were still not ensheathed by SCs while others revealed polyaxonal myelination suggesting that axonal segregation is also affected in the absence of LPA_1. *In vitro*, LPA produced by neurons isolated from dorsal root ganglia proved to be a strong inducer of Schwann cell migration through activation of LPA_1. The heterotrimeric G-alpha protein, $G_{\alpha i}$, and the small GTPase, Rac1, were identified as important downstream signaling components. These results suggested that production of LPA by neuronal tissues and activation of LPA_1 act to influence SC migration and subsequent axonal segregation and myelination.

4.2 Introduction

Various developmental and differentiation steps are necessary for Schwann cells (SCs), the myelinating glial cells in the peripheral nervous system (PNS), in order to properly segregate axons, establish a 1:1 relationship with a large caliber axon and finally form a myelin sheath around the axon (Jessen and Mirsky 2005). In contrast to the myelinating SCs, non-myelinating SCs only ensheath multiple small diameter axons by large membrane extensions. Several neuronal signals have been reported to control SC proliferation, migration, and differentiation, as well as the myelination process itself. These signaling molecules include neuregulin-1 (NRG) and brain-derived neurotrophic factor (BDNF) that signal through ErbB2/ErbB3 receptor tyrosine kinases (Garratt, Britsch et al. 2000; Michailov, Sereda et al. 2004; Lemke 2006; Nave and Salzer 2006) and the $p75^{NTR}$ receptor (Chan, Cosgaya et al. 2001; Cosgaya, Chan et al. 2002; Yamauchi, Chan et al. 2004), respectively. In addition to these signals, a role for the omnipresent lysophosphatidic acid (LPA) in myelinating SCs and oligodendrocytes has been suggested due to the high expression level of LPA_1, a LPA-specific G protein-coupled receptor (GPCR) (Allard, Barron et al. 1998; Weiner, Hecht et al. 1998; Contos, Fukushima et al. 2000; Cervera, Tirard et al. 2002). Indeed, the influence of LPA/LPA_1 signaling in several important physiological and pathophysiological SC processes that are likely to impact myelin formation has started to unravel. The role of LPA in the prevention of SC apoptosis that manifests in an increased percentage of SCs undergoing apoptosis in the LPA_1 null mutant has been described

(Weiner and Chun 1999; Contos, Fukushima et al. 2000) in addition to a pronounced LPA/LPA$_1$-induced rearrangement of the SC actin cytoskeleton and regulation of cellular adhesion properties (Weiner, Fukushima et al. 2001).

Another clue that LPA signaling might influence the myelination process emerged from the observation of Ueda and colleagues who described a prominent role of LPA$_1$ and downstream activation of the Rho/ROCK pathway in the induction of neuropathic pain and demyelination in the PNS (Inoue, Rashid et al. 2004). Demyelination is associated with dedifferentiation of SCs, whereupon myelinating SCs downregulate expression of myelin-specific proteins, reexpress cell adhesion molecules and growth factors, and start to proliferate again (Jessen and Mirsky 2008). The absence of LPA$_1$ prevents not only the induction of neuropathic pain and demyelination, but also the downregulation of myelin protein expression (Nagai, Uchida et al. 2010). These observations suggest that LPA/LPA$_1$ signaling mechanisms might not only impact SC dedifferentiation and demyelination under pathophysiolocial conditions such as neuropatic pain or nerve injury, but also SC differentiation into either myelinating or non-myelinating cells during development. Therefore, the potential effects of LPA signaling on the myelination process required clarification which led us to investigate the relationship between LPA signaling and myelination. Observations in sciatic nerves of LPA$_1$ null mice indicated a role for LPA$_1$ in the segregation of axons that also prompted us to investigate LPA-mediated effects on SC migration.

4.3 Material and Methods

G-ratio determination

Sciatic nerves were isolated from 13-26 week old wildtype (WT) (*Lpar1*$^{+/+}$) and LPA_1 null mice (*Lpar1*$^{-/-}$) and fixed overnight *in situ* in freshly made 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4 containing 50 mM $CaCl_2$, and processed as described below. Semi-thin cross sections (1-2 μm) were stained with 0.1% toluidine blue for light microscopy. Image capture from the sections and measurement of the g-ratio was processed based on blinded determinations. Value of the g-ratio was obtained by dividing axonal diameter by fiber diameter as described (Little and Heath 1994). Two image fields were taken from each nerve using a bright-field microscope equipped with a 100X oil objective lens and an AxioCam digital camera (Zeiss). For measuring the g-ratio, myelin thickness was determined using MetaMorph image analysis software (v6.3, Molecular Devices, USA).

Electron microscopy

Sciatic nerves were removed after fixation from WT (*Lpar1*$^{+/+}$) and null mutant mice (*Lpar1*$^{-/-}$) for LPA_1, respectively, contrasted with 1% osmium tetroxide in 0.12 M sodium cacodylate with 3.5% sucrose for 4 hr at 4 °C, washed, dehydrated through graded ethanols and acetone, and embedded in Epon/Araldite epoxy resin. Sections were made on a Leica Ultracut ultramicrotome for light and

electron microscopy. The grids were contrasted with uranyl acetate and Reynold's lead citrate.

Primary Schwann cell (SC) culture

Murine SCs were isolated from dorsal root ganglia (DRG) at embryonic day 13.5 as previously described (Kim 1997). Isolated SCs were immunopositive for $p75^{NTR}$ (Chemicon), GFAP (Sigma), and S100 (Dako). The purity of the SCs was approximately > 96% as assessed by cell size and morphology. After the first passage, SCs were cryoprotected in fetal bovine serum (FBS) with 10% DMSO and stored in liquid N_2. After thawing, cells were passaged on poly-$_L$-lysine (PLL)-coated dishes for up to 4 passages. DMEM (Invitrogen) was supplemented with 10% FBS, 20 μg/mL pituitary extract (BD Biosciences), 2 μM forskolin (Sigma), and penicillin-streptomycin. Before migration and pull-down assays, SCs were serum-starved overnight in a modified "Sato" medium (Milner, Wilby et al. 1997) consisting of DMEM (Invitrogen) complemented with 1x N2 supplement (Invitrogen), 20 μg/mL pituitary extract, 0.1 mg/mL fatty-acid free bovine serum albumin (FAFBSA, Sigma), 400 ng/mL of each T3 and T4 (Sigma), 4 μM forskolin, and penicillin-streptomycin. Receptor-deficient SCs and WT control cells from littermates were isolated from embryos at E13.5.

Transwell migration assay

Cell migration was measured using transwells in a 24 well format (BD Bioscience) as previously described with minor modifications (Yamauchi, Chan et

al. 2003). Briefly, 1.5-2.5 x 10^5 SCs in 300 μL Sato medium were loaded onto collagen-coated polyethylene terephthalate transwell filters (8 μm pore size) and placed in wells containing 500 μL Sato medium. If cell-permeable inhibitors (50 μM LY294002, 100 nM Wortmannin, and 100 μM NSC23766) were used, cells were pretreated for 30-45 min before migration was induced. PTX was added to the cells overnight during serum-starvation at a concentration of 150 ng/mL. To induce directed SC migration across the membranes, LPA was added to the medium in the lower compartment at a final concentration of 500 nM unless otherwise indicated. After incubation at 37˚C in 7.5% CO_2, the cells in the upper compartment were removed and migrated cells at the bottom side of the filters were stained with crystal violet and counted at six fields per filter. All experiments were done in triplicate and repeated two to four times.

For conditioned medium, murine DRGs at E13.5 were dissociated and plated at high densities in DMEM with 10% FBS and 50 ng/mL NGF (Harlan Bioproducts) onto PLL/laminin-coated glass coverslips. The next day, the medium was changed to serum-free conditions using DMEM/F12 medium (Invitrogen) containing 1x N2-supplement, 50 ng/mL NGF, and 2.5 μg/mL gentamycin (Invitrogen). To purify DRG neurons, 3 cycles of antimitotic treatment using 10 μM 5-Fluoro-deoxy uridine (Sigma) and 10 μM uridine (Sigma) were performed. Conditioned medium was collected 5 days after the last medium change. Alternatively, antimitotic treatment was omitted to collect conditioned medium from SC/neuron co-cultures. Conditioned medium was subsequently used as a

chemoattractant in the lower compartments of transwells for SCs plated in DMEM/F12 medium supplemented with N2, NGF, and gentamycin onto the filters.

Migration assay along axon bundles

Induction of SC migration along axon bundles of cultured DRG neurons was performed as described (Yamauchi, Chan et al. 2004). Briefly, murine DRG neurons were grown in parallel lanes along collagen stripes on glass coverslips. During antimitotic treatments DRG neurites became fasciculated. Finally, GFP-expressing WT and LPA_1 null SCs were reaggregated overnight on a non-permissive substrate with constant agitation. The next day, SC aggregates were plated onto the bundled DRG neurites, and migration of SCs away from the aggregates and along the neurites was examined either in the presence or absence of 1 μM LPA. After 6-7 hr, the cultures were fixed and immunostained for neurofilaments (αNF-200, Serotec). Nuclei were stained with DAPI and images were acquired using a fluorescence microscope fitted with an AxioCam camera (Carl Zeiss, Thornwood, NY). Migration of all SCs detached from individual aggregates was determined by measuring the distance from the single SC nuclei to the periphery of the aggregates.

LPA measurement in the conditioned medium

All extraction processes were done using 1.5 mL low adhesion tubes (Fisher Scientific) and low adhesion pipette tips (Axygen) on ice to prevent LPA loss. In all samples, 1 μM of C^{17} LPA (Avanti Polar Lipids) was added immediately before

extraction as an internal standard. Conditioned medium (1 mL/sample) was collected. The same volume of MeOH-HCl 10:1 mix and half volume of chloroform were added sequentially and vortexed. The mixture was then incubated on ice for 40 min. Saturated NaCl in water (280 μL) was then added and mixed gently by inverting the tube. After 10 min, bottom phase (organic phase) was collected after centrifugation at 14,000 rpm at 4°C. Samples were dried down using a Vacufuge (Eppendorf) and reconstituted with 50 μL of methanol. Mass spectrometry was done in TSRI's Mass Spectrometry Core using an Agilent 6410 triple quad mass spectrometer coupled to an Agilent 1200lc stack. Samples (5 μL) were injected into the LC system. The LC was run at following conditions: A=95:5 H2O:MeOH 0.1% NH4OH, B=60:35:5 IPA:MeOH:H2O 0.1% NH4OH. Starting with 50:50=A:B for 3 min and ramped up to 100% B in 10 min. The LC column is extended C18, 21x150 mm, 3.5 μm and flow rate is 150 μL/min. Mass spectrometry was set up as follows: detecting for LPA (m/z: 435 -> 153 and 435 -> 79) and C^{17} form LPA (m/z: 423 ->153 and 423 ->79). Negative ion is monitored and instrument set up as follows: Fragmentor voltage=160V, collision energy=20V, drying gas flowrate=10 L/min, temp=350C, nebulizer pressure=30psi, and capillary voltage=4500V.

Rac pulldown

The PBD pulldown assay for measuring Rac GTPase activation including expression and purification of the GST-PAK1 PBD fusion protein has been described in detail elsewhere (Benard and Bokoch 2002). Briefly, SC cultures were

serum-starved overnight in Sato medium. Cells were stimulated with 1 μM LPA for indicated times. Cells were washed twice with ice-cold PBS and lysed in 800 μL ice-cold lysis buffer (25 mM Tris buffer, pH 7.5, 40 mM NaCl, 30 mM $MgCl_2$, 1 mM DTT, 0.5% NP-40, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin). Cell lysates were cleared by centrifugation at 14000 rpm for 10 min at 4˚C. Cleared lysates (10 μL) were used for total protein control. The remaining lysates were incubated with 20-30 μg GST-PAK1 PBD beads for 1 hr at 4˚C. Subsequently, the beads were washed 4x with 0.5 mL lysis buffer before bound Rac was eluted from the beads and analyzed by standard SDS-PAGE and Western blotting techniques using a monoclonal anti-Rac1 antibody (Upstate Biotechnology). Film images were scanned for densitometric analysis.

Reverse Transcriptase-PCR

RNA from DRGs isolated at E13.5 was isolated using the RNAqueous Micro Kit (Ambion) as described in the manual. RNA from cultured SCs or from lung and spleen was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RT-PCR was performed as previously described (Lee, Rivera et al. 2006). All primer pairs, with one exception, amplified a product across two exons. The primer pair for *Lpar4* did not cross an intron-exon boundary. Therefore, PCR amplification of RNA without reverse transcription was performed to ensure that *Lpar4* signals did not derive from contaminating genomic DNA (not shown).

SC migration assay

Murine SCs were isolated from the dorsal root ganglia (DRG) at embryonic day 13.5 as previously described (Kim 1997). Receptor-deficient SCs and WT control cells from littermates were isolated from embryos at E13.5 emerging from *Lpar2*$^{+/-}$ x *Lpar2*$^{+/-}$ and *Lpar3*$^{+/-}$ x *Lpar3*$^{+/-}$ crosses, respectively. Isolated SCs were cultured with growth medium: DMEM (Invitrogen) supplemented with 10% FBS, 20 μg/mL pituitary extract (BD Biosciences), 2 μM forskolin (Sigma), and penicillin-streptomycin. Before the migration assay, SCs were serum-starved overnight in a modified "Sato" medium (Milner, Wilby et al. 1997) consisting of DMEM (Invitrogen) complemented with 1x N2 supplement (Invitrogen), 20 μg/mL pituitary extract, 0.1 mg/mL fatty-acid free bovine serum albumin (FAFBSA, Sigma), 400 ng/mL of each T3 and T4 (Sigma), 4 μM forskolin, and penicillin-streptomycin. Cell migration was determined using transwells in a 24 well format (BD Bioscience) as previously described with minor modifications (Yamauchi, Chan et al. 2003). SCs (1.5-2.5 x 10^5) were loaded onto collagen-coated polyethylene terephthalate transwell filters (8 μm pore size) and placed in wells containing 500 μL Sato medium. If cell-permeable inhibitors (PD98059, SB203580, SP600129, and Y27632) were used, cells were pretreated for 30-45 min before migration was induced. To induce directed SC migration across the membranes, LPA was added to the medium in the lower compartment at a final concentration of 500 nM. After incubation at 37˚C in 7.5% CO_2, the cells in the upper compartment were removed and migrated cells at the bottom side of the filters were stained with

crystal violet and counted at six fields per filter. All experiments were done in triplicate and repeated two to four times.

4.4 Results

Deficiency of LPA_1 increases the g-ratio in the sciatic nerve

To identify whether LPA_1-mediated signaling is indeed involved in myelination, sciatic nerves were isolated from LPA_1 null mice (*Lpar1*$^{-/-}$) and wildtype littermates (WT, *Lpar1*$^{+/+}$). The g-ratio (axon diameters/fiber diameters), a parameter for the thickness of the myelin sheath in relation to the axon diameter, was determined and compared between adult LPA_1 null (n=7) mice and WT (n=5). A significant decrease in myelin thickness was observed in sciatic nerves from LPA_1 null mice when compared to sciatic nerves from WT mice (Figure 4.1A-D). The statistically significant increase in the mean g-ratio from 0.677 ± 0.010 in WT controls to 0.713 ± 0.009 in LPA_1 null mice (Figure 4.1C) was not accompanied by changes in axonal size, as the percentages of axons with a given axonal diameter were similar between LPA_1 null mice and WT controls (Figure 4.1F).

Reduced axonal segregation and polyaxonal myelination in LPA_1 null mice

Electron microscopy analysis of sciatic nerves from mice of different ages (from postnatal day 5 to 15 weeks old) revealed signs of defective segregation of axons by SCs (Figure 4.1G-K). In nerves from 5-day old (P5) LPA_1 null mice, axonal segregation of small caliber axons seems to be less advanced than in the WT controls. Although SCs in the absence of LPA_1 were able to establish a 1:1 relationship with large caliber axons and to initiate myelination, many of the small caliber axon bundles were without interacting SCs (Figure 4.1H, arrowhead). In

contrast, in the nerves from WT mice, SCs started to ensheath the small caliber axons and to form Remak bundles (Figure 4.1G). At postnatal day 24 (P24), formation of Remak bundles in WT mice is completed (Figure 4.1I), while in LPA_1-deficient nerves naked small caliber axons were still frequently observed (Figure 4.1J, double asterisk). However, at adult stages, naked axons were no longer observed indicating that the ensheathment of small caliber axons and the formation of Remak bundles is delayed rather than interrupted in the LPA_1 null mice (not shown). In addition to this phenotype, an increased incidence of polyaxonal myelination became evident in LPA_1 null mice at P24, as bundles of small caliber axons became enclosed by a single thin myelin sheath (Figure 4.1J, asterisk). It has been reported that such polyaxonal myelination can occasionally occur in young WT mice during active myelination but becomes corrected during further development (Rasi, Hurskainen et al. 2010). However, in LPA_1 null mice, bundles of small axons were still frequently wrapped by a single myelin sheath in adult mice of 15 weeks of age (Figure 4.1K, asteriks) and 57 weeks of age (not shown). In adult WT littermates, no polyaxonal myelination was observed. Overall, the analysis of sciatic nerves of LPA_1 null mice indicates that LPA_1-mediated phospholipid signaling plays a role in the segregation of axons and in the myelination process.

LPA enhances SC migration in a dose-dependent manner

The delay in axonal segregation observed in LPA_1 null mice could be explained by altered SC-axon interactions, impaired formation of SC processes, or

by delayed or insufficient SC migration. As the first two possibilities seemed unlikely due to fact that LPA_1-deficient SCs were readily able to myelinate large caliber axons, we focussed on the latter possibility and investigated effects of LPA signaling on SC migration.

First, we investigated the effects of LPA on migration of primary mouse SCs using a transwell chamber approach. LPA added at different concentrations to the lower compartment of the transwell, turned out to be a strong inducer of SC migration (Figure 4.2A and B). Concentrations as low as 10 nM LPA were sufficient to promote a nearly 2-fold enhancement of SC migration, whereas 500 nM LPA increased SC migration 4-5 fold over control levels.

LPA-mediated SC migration through LPA_1

The low LPA concentration required for induction of SC migration suggested that this response is receptor-mediated. Indeed, primary SCs isolated from newborn mice and rats are known to express a variety of LPA receptors (Weiner and Chun 1999; Weiner, Fukushima et al. 2001; Li, Gonzalez et al. 2003). Data from RT-PCR analysis showed that cultured embryonic mouse SCs express $LPA_{1\text{-}4}$ receptors (Figure 4.S1). Similarly, $LPA_{1\text{-}4}$ expression was detectable in the dorsal root ganglia (DRG) at E13.5, the source of mouse primary SCs (Figure 4.S1). In contrast, LPA_5 was not expressed in embryonic SCs or DRGs. To identify the receptors that mediate SC migration in response to LPA, we isolated SCs from LPA_1 null mice and also included SCs from LPA_2 (*Lpar2*$^{-/-}$) and LPA_3 (*Lpar3*$^{-/-}$) null mice in the analysis. Whereas SCs deficient for LPA_2 (Figure 4.S2A) or LPA_3

(Figure 4.S2B) showed similar migration rates in response to LPA as WT littermate cells, we observed a complete loss of LPA-induced SC migration in LPA_1 null cells (Figure 4.2C). Instead of a 4-fold increase in migration as observed with WT SCs, the migratory response of LPA_1 deficient SCs in response to LPA was completely abolished. However, LPA_1 null SCs were still able to migrate in response to sphingosine 1-phosphate (S1P), another phospholipid that signals through cognate G protein-coupled receptors (data not shown). S1P-induced migration of LPA_1 deficient SCs indicated that migration *per se* was not impaired in these cells. In summary, these results clearly demonstrate that LPA_1 is the receptor mediating LPA-induced SC migration.

LPA released by DRG neurons mediates SCs migration

LPA/LPA_1-induced SC migration could only be considered as physiologically relevant, if DRG neurons produce and release LPA that then leads to an enhancement of SC migration. Using a combination of HPLC and MS techniques, we found that LPA was present in the conditioned medium of purified and unpurified mouse DRG neurons at a concentration of 3.322 ± 0.158 and 2.345 ± 0.237 nM, respectively (Figure 4.2D). To examine whether LPA produced endogenously from DRG neurons mediates SC migration, we used conditioned media from DRG neurons as a migration stimulus in a transwell assay. Conditioned media from either murine DRG neuron/SC co-cultures (unpurified) or purified DRG neurons markedly enhanced WT SC migration (Figure 4.2E and F). Subsequently, we compared the ability of WT and LPA_1 null SCs to migrate in response to the

endogenous LPA present in conditioned media. On average, media from neuron/SC co-cultures or from purified neurons enhances migration of WT SCs 5-6.5 fold over control levels within 5-6 hr (Figure 4.2E and F). In the absence of LPA_1, however, SC migratory responses to conditioned media from DRG neuron cultures were greatly reduced: LPA_1-deficient SCs showed a 65-70% reduction in migration compared to WT SCs (Figure 4.2E and F). These observations strongly suggest that LPA secreted by DRG neurons is a potent inducer of SC migration, thereby supporting a role of LPA/LPA_1 signaling in SC migration *in vivo*.

SC migration along DRG neurites

We next examined whether LPA can also induce SC migration along neurite track. Instead of studying migration across transwell membranes, we measured SC migration along purified mouse DRG axons in culture. GFP-labeled WT or LPA_1 null SCs were aggregated, seeded onto DRG axons, and allowed to migrate out from the aggregates for 6-7 hr either in the presence or absence of exogenous LPA. The addition of 1 μM LPA significantly increased the average migration distance of WT SCs by approximately 20% (Figure 4.3D-F, M). Since exogenous LPA did not enhance migration of LPA_1 null SCs along DRG axons, this increase is clearly LPA_1 dependent (Figure 4.3J-L, M). Furthermore, in two out of three independent experiments, LPA_1-deficient cells showed a reduction in migratory response under control conditions. In the absence of exogenous LPA, the average migration distance of LPA_1 null SCs (Figure 4.3G-I, M) was reduced to about 27% of that seen in littermate WT cells (Figure 4.3A-C, M). These results do not only reveal

that LPA_1 mediates SC migration along DRG axons, but they also suggest that endogenous LPA is present at levels sufficient to induce LPA_1-induced migration along DRG neurites.

LPA_1 enhances SC migration through G_i proteins and the small GTPase Rac

LPA receptors are known to couple to various heterotrimeric G proteins, thereby linking the receptors to several signaling pathways (Choi, Herr et al. 2010). We examined the signaling mechanisms involved in LPA-induced migration to determine which signaling pathways are involved in mediating the LPA-induced migration response by using different inhibitors in a transwell migration assay. Treatment of WT SCs with either pertussis toxin (PTX) (Figure 4.4A), a specific inhibitor for G_i proteins, or NSC23766 (Figure 4.4B), an inhibitor of the small GTPase Rac1, completely abolished LPA-induced SC migration. We next analyzed the role of PI3K, a signaling mediator between G_i protein and Rac1, in the LPA-mediated SC migration assay. Unexpectedly, both PI3K inhibitors, LY294002 and wortmannin, did not specifically block LPA-induced SC migration (Figure 4.4C). In fact, inhibition of PI3K reduced overall migration, including basal migration under control conditions. LPA, however, still markedly increased SC migration to levels comparable to those observed in control cells. Similarly, inhibitors of other key migratory molecules, including the mitogen-activated protein kinases (MAPKs), Erk1/2, p38 and JNK, (Figure 4.S3A and B) or the small GTPase Rho and its associated kinase ROCK (Figure 4.S3C), only partially reduced overall SC migration without specifically blocking LPA-induced migration. These

observations suggest that cell type-specific signaling mechanisms underlie lysophospholipid-induced cell migration and show that signaling pathways downstream of Rac1 diverge for LPA-induced migration in SCs.

So far, our inhibitor studies identified G_i proteins and Rac1 as mediators of LPA-induced SC migration. To directly verify activation of Rac1 through LPA_1, we measured the activation state of endogenous Rac1 using a GST-PBD pull-down assay. In WT SCs, LPA enhanced Rac1 activation in a time-dependent manner (Figure 4.4D). Maximum levels of activated Rac1 were obtained at 30 min following stimulation with LPA and the elevated levels of activated Rac1 persisted for at least 90 min after stimulation (not shown). We typically observed a 2-3 fold increase in GTP-bound Rac1 compared to non-stimulated cells. In contrast to WT SCs, no Rac1 activation was observed in LPA_1-deficient SCs after stimulation with LPA (Figure 4.4D). We therefore concluded that LPA-induced Rac1 activation through LPA_1 is the main component in SC migration, in agreement with Rac1 playing a role in the myelination process (Yamauchi, Miyamoto et al. 2005; Benninger, Thurnherr et al. 2007).

4.5 Discussion

This study demonstrates a novel role for LPA_1 signaling in peripheral nerve development and identifies LPA_1/G_i/Rac as the signaling pathway inducing SC migration towards LPA that can be secreted by DRG neurons (schematic overview shown in Figure 4.S4).

Migration of SC precursors along outgrowing axons precedes other important cellular processes, such as axonal segregation of small axons and radial sorting of large diameter axons, that are required for SC differentiation to either nonmyelinating or myelinating cells (Voyvodic 1989; Jessen and Mirsky 2005). Thus, impaired or delayed SC migration could contribute at least to some of the defects observed in the sciatic nerves of LPA_1 null mice. In particular, the large amount of naked or insufficiently enveloped small axon bundles detected at P5, might be a direct consequence of decreased SC migration. During further development, the naked bundles of small axons become more and more ensheathed by SCs indicating that the reduced axonal segregation observed at P5 is due to delayed, rather than defective, axonal segregation. Finally, in adult LPA_1 null mice, all non-myelinated small caliber axons were ensheathed by SCs and organized into Remak bundles. However, in order to prove that lack of LPA_1-mediated SC migration is indeed the reason for the delay in axonal segregation, it will be necessary to quantify SC precursors at different stages of nerve development, an analysis that might be hampered by the previously described role of LPA_1 in SC survival *in vivo* (Contos, Fukushima et al. 2000).

Additional alterations observed in peripheral nerves of LPA_1 null mice included a reduced thickness of myelin sheaths and an increased incidence of polyaxonal myelination of small axon bundles. The key myelination trigger, determining whether axons are ensheathed or myelinated and also governing the thickness of the myelin sheaths, is type III β1a neuregulin (NRGβ1a type III) (Michailov, Sereda et al. 2004; Taveggia, Zanazzi et al. 2005). Low axonal NRG type III expression results in ensheathing SCs, while high axonal expression induces the formation of a myelin sheath. Thereby, the thickness of the sheaths correlates closely to the axonal expression level of NRGβ1a type III; reduced expression results in thinner myelin sheaths, whereas increased expression of NRGβ1a type III results in hypermyelination (Michailov, Sereda et al. 2004; Taveggia, Zanazzi et al. 2005). In addition, NRGβ1a type III is also required for proper segregation and ensheathment of small caliber axons by SCs (Taveggia, Zanazzi et al. 2005). Mice heterozygous for the NRGβ1a type III isoform revealed an increased number of small caliber axons ensheathed by a single nonmyelinating SC, while the axons were frequently incompletely segregated into the SC pockets (Taveggia, Zanazzi et al. 2005). Interestingly, overexpression of the second known NRG type III isoform, NRGβ3 type III, revealed profound effects on Remak bundles, as the small caliber axons of the bundles were closely packed and no longer segregated from one another by SC cytoplasma (Gomez-Sanchez, Lopez de Armentia et al. 2009). In addition, a significant number of these bundles were myelinated as a single unit, similar to what was observed in the LPA_1 null mice. The similarities between the consequences of deregulated neuregulin type III signaling and LPA_1 deficiency are

striking. It is noteworthy in this context that a similar function for LPA/LPA_1 and NRG signaling has been previously reported for SC survival (Weiner and Chun 1999). Although LPA and NRG bind to different receptor classes, NRG type III isoforms to ErbB receptor tyrosine kinases and LPA to G protein-coupled receptors, they are able to activate similar downstream signaling pathways, as shown for SC survival. Both NRG and LPA prevented SC apoptosis through activation of PI3K and Akt (Weiner and Chun 1999; Li, Tennekoon et al. 2001). Besides activation of PI3K, axonal neuregulin also activates RAS-MAPK pathways in SCs (Taveggia, Zanazzi et al. 2005). Since both pathways can also be activated through LPA_1 (Anliker and Chun 2004), it is tempting to speculate that LPA/LPA_1 signaling might modulate, to some extent, the activation state of the downstream effectors of NRG/ErbB signaling pathways in SCs.

Several ligands including NRG, NT3, and nerve growth factor (NGF) that were shown to induce SC chemotaxis *in vitro*, could potentially be involved in the regulation of developmental processes associated with SC movements (Anton, Weskamp et al. 1994; Mahanthappa, Anton et al. 1996; Meintanis, Thomaidou et al. 2001; Yamauchi, Chan et al. 2003; Jessen and Mirsky 2005). The present study reveals LPA as another strong candidate for regulating SC movements *in vivo*. LPA not only revealed a potent chemoattractive effect on SCs and the ability to promote migration directly along neurites, it also seems to be the most important pro-migratory factor released from DRG neurons *in vitro*. Using conditioned medium from DRG neurons as a chemoattractant, we observed a reduction of 65-75% in the

migratory response of LPA_1-deficient SCs compared to WT cells. The remaining induction of SC migration in LPA_1-deficient cells was marginally due to S1P, another lysophospholipid, as observed by comparison with SCs deficient for both LPA_1 and $S1P_3$, the receptor mediating S1P-induced SC migration (unpublished data). Thus, only 20-25% of the increase in SC migration by conditioned media was independent of lysophospholipid signaling mechanisms. This observation is particularly striking in view of the lack of SCs along peripheral nerves in NRG type III-, ErbB2- and ErbB3-deficient mice or zebrafish (Garratt, Britsch et al. 2000; Lyons, Pogoda et al. 2005). The lack of SCs along axons in these mutants either reflects defective growth/survival and/or migratory mechanisms. In fact, at least in zebrafish, NRG signaling through ErbB2/ErbB3 receptor tyrosine kinases exhibited an essential role for directed SC migration along axon bundles (Lyons, Pogoda et al. 2005). A direct proof of defective SC migration is more difficult to be recapitulated in mice. Morris et al. investigated the migratory behavior of WT and ErbB2-deficient SCs in response to NRG using *in vitro* assays (Morris, Lin et al. 1999). Unexpectedly, NRG did not reveal a promigratory effect when mouse WT DRGs from embryonic day E12.5 were used for studying SC migration out of the ganglia. Moderate NRG-induced migration was only observed when DRGs from newborn mice or rats were used (Mahanthappa, Anton et al. 1996; Morris, Lin et al. 1999; Woldeyesus, Britsch et al. 1999). This observation suggested that NRG is capable of inducing SC migration of neonatal mouse SCs, but not of SC precursors isolated at E12.5. Differences in the differentiation state of SCs might also explain the apparent discrepancy between our findings and the observations of Yamauchi and

colleagues. These authors observed a similar induction of rat SC migration in response to conditioned medium from DRG neurons which could be significantly diminished by adding ErbB3-Fc, a scavenger for NRG (Yamauchi, Miyamoto et al. 2008). Thus, their results suggested that NRG is the key inducer of SC migration present in conditioned medium from DRG neurons (Yamauchi, Miyamoto et al. 2008). However, they used SCs isolated from newborn animals, while we isolated the SCs from mouse DRGs at E13.5. This discrepancy implies that differentiation of SC precursors to immature SCs might correlate with a change in their responsiveness to migratory stimuli. In fact, the possibility that the lack of LPA/LPA_1-induced migration of SC precursors might be overcome at later stages by NRG-induced migration, which might provide an explanation about why defects in peripheral nerves of LPA_1 null mice were not more pronounced.

While the underlying signaling mechanisms for SC survival are comparable for LPA and NRG, the migratory response seems to be differentially regulated (Weiner and Chun 1999; Li, Tennekoon et al. 2001; Meintanis, Thomaidou et al. 2001). Migration in response to neuregulin was partially mediated through MAPKs and PI3Ks (Meintanis, Thomaidou et al. 2001). In contrast, LPA-induced migration was not blocked by MAPK or PI3K inhibitors. The latter result was unexpected since it is well known that the $\beta\gamma$-subunits of G_i proteins activate PI3Ks, whose phosphoinositide products subsequently activate Rac-GEFs such as Tiam1 or P-Rex-2b (Van Leeuwen, Olivo et al. 2003; Li, Paik et al. 2005). Furthermore, in glioma cells, LPA was found to induce migration partially through the

LPA_1/G_i/PI3K/Rac/JNK signaling pathway (Malchinkhuu, Sato et al. 2005). It is possible that the pathway mediating cell migration in SCs is different than the one observed in glioma cells. On the other hand, we cannot exclude the possibility that a PI3K subtype with a reduced sensitivity to LY294002 and wortmannin is involved in LPA-induced SC migration. The cloning of PI3K-C2α, a class II PI3K, revealed a PI3K that was at least one order less sensitive to both PI3K inhibitors compared to class I PI3Ks (Domin, Pages et al. 1997). In addition, another class II PI3K, PI3K-C2β, has been reported to be crucial in LPA-dependent migration of human cell lines (Maffucci, Cooke et al. 2005). However, the inhibitor concentrations used in the present study (50μM for LY294002 and 100nM for wortmannin) were sufficient to block PI3K-C2β involved in LPA-induced migration of human cells (Maffucci, Cooke et al. 2005). Similarly, we did not observe an inhibition of LPA-induced SC migration using SP600125, a specific JNK inhibitor. JNK has been implicated in cell migration of several cell types (Huang, Jacobson et al. 2004). In SCs, activation of JNK either through the TrkC/PI3K/Rac pathway or through ErbB2, Rac1, and Cdc42 induced migration towards NT-3 and NRG, respectively (Yamauchi, Chan et al. 2003; Yamauchi, Miyamoto et al. 2008). In summary, these observations suggested cell type-specific signaling mechanisms underlying LPA-induced cell migration and reveal a divergence in the signaling pathway downstream of Rac for LPA-induced SC migration as compared to NT-3- or NRG-induced migration.

Given its previously described functions in SC survival, adhesion, and actin rearrangement (Weiner, Fukushima et al. 2001), the new roles for LPA_1 in SC migration, axonal segregation, and myelination emphasizes the importance of LPA_1 signaling during SC development.

4.6 Acknowledgement

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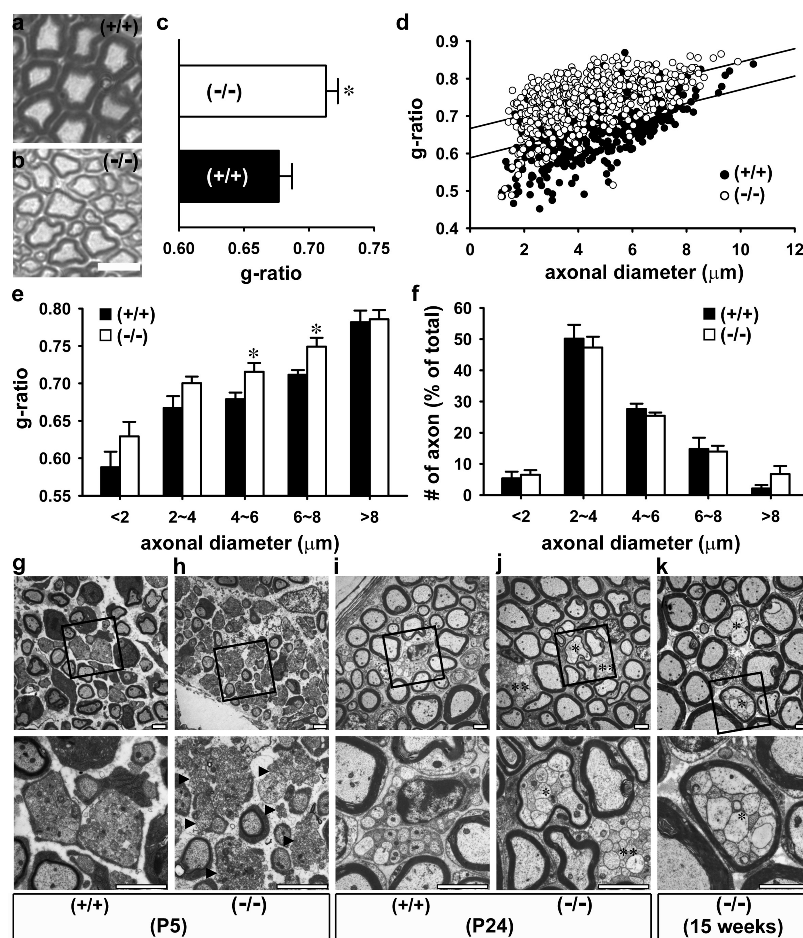

Figure 4.1 LPA$_1$ null mice reveal defects in myelination and axonal segregation. **a-f**, Semithin cross sections (2 μm) were prepared from sciatic nerves of adult WT (*Lpar1*$^{+/+}$, n=5) and LPA$_1$ null mice (*Lpar1*$^{-/-}$, n=7) and stained for myelin. Representative pictures of adult sciatic nerve of WT (**a**) and LPA$_1$ null (**b**) mice are shown. **c**, The mean value of g-ratio was calculated from all nerves processed (mean ± s.e.m. $^*P < 0.05$ vs. WT, *t*-test). **d**, G-ratio of individual fibers from 2 mice per group are depicted as a scatter plot **e**, Mean values of g-ratio from all WT and LPA$_1$ null mice were calculated and grouped according the axonal diameter (means ± s.e.m. $^*P < 0.05$ vs. WT, *t*-test). **f**, The percentage of axons within individual groups as determined by axonal diameter is unchanged in sciatic nerves of LPA$_1$ null mice. **g-k,** Sciatic nerves from littermate WT (**g, i**) and LPA$_1$ null (**h, j, k**) mice were isolated at different age and processed for electron microscopy. Representative pictures from sciatic nerves at postnatal day 5 (P5) (**g, h**), postnatal day 24 (P24) (**i, j**), and at 15 weeks of age (15 weeks) are shown (**k**). Arrowheads indicate naked axon bundles (h); ∗ indicates polyaxonal myelination of small axons (j, k); ∗∗ indicates axon bundles that are not ensheathed by SCs (j). (+/+) and (-/-) represent WT and LPA$_1$ null mice, respectively. Scale bars, 10 μm (**a, b**), 2 μm (**g-k**).

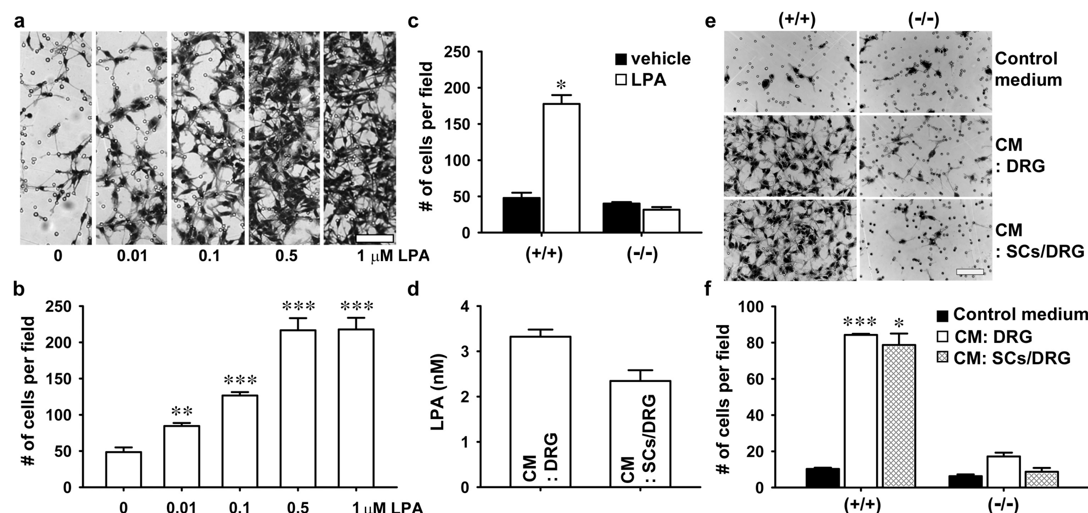

Figure 4.2 LPA_1 mediates SC migration induced by neuronally secreted LPA. **a, b**, Increasing concentrations of LPA were added to the lower compartments of transwell chambers to induce migration of SCs across a membrane with 8μm pore size. Migrated SCs at the bottom side of the membranes were stained with crystal violet (**a**) and quantified after 5-6 h (**b**). Shown are means ± s.e.m. (n=5, *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$ vs. control (0.1% BSA), *t*-test) (**b**). **c**, Migration of LPA_1 null SCs towards LPA as compared to WT SCs. Mean ± s.e.m. of one representative example of four independent experiments (n=3, *$P < 0.05$ vs. basal migration under control conditions, *t*-test). **d**, HPLC/MS was used for measuring LPA concentration in the conditioned medium from purified DRG neurons (CM: DRG) or DRG neurons/SC co-cultures (CM: SCs/DRG) that were cultured on 12-well plates. mean ± s.e.m. (n=6). **e**, **f**, Migration of LPA_1 null and WT SCs was examined using either control or conditioned media from purified DRG neurons (CM: DRG) or DRG neuron/SC co-cultures (CM: SCs/DRG) as a stimulus in the lower compartments of transwells. Representative photographs are shown after 5-6h of migration (**e**), when migrated cells at the bottom side of the transwells were quantified (**f**). Mean ± s.e.m. of a representative example of four independent experiments (n=3, *$P < 0.05$, ***$P < 0.0005$ vs. control, *t*-test). (+/+) and (-/-) represent WT and LPA_1 null mice, respectively. Scale bar, 100 μm (**a, e**).

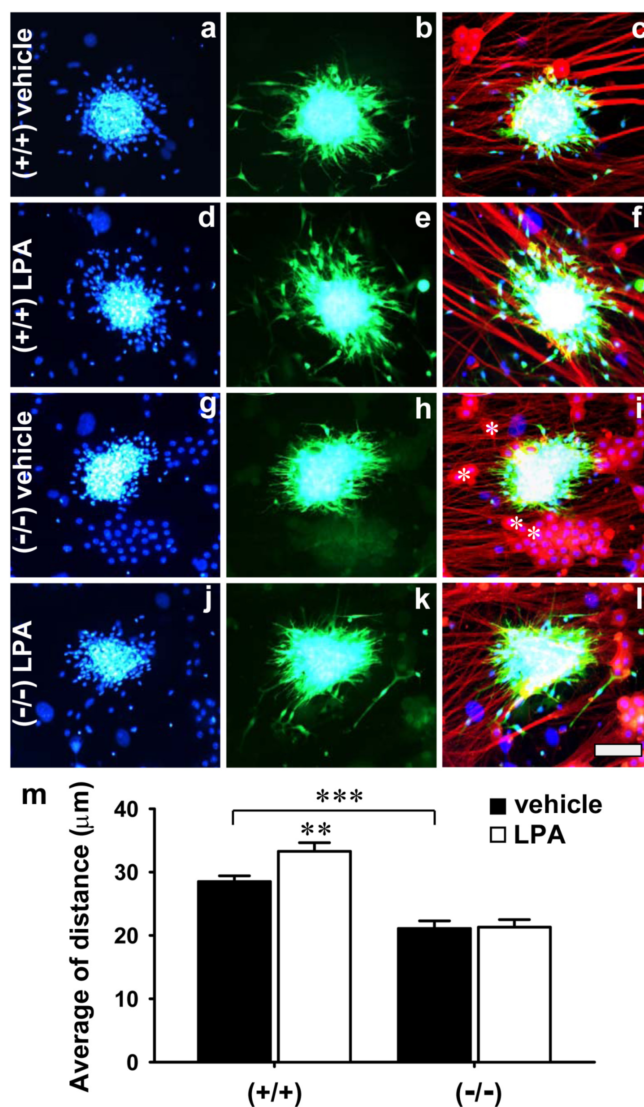

Figure 4.3 LPA induced SC migration along purified DRG neurons through LPA$_1$. a-l, Aggregated WT (**a-f**) or LPA$_1$ null SCs (**g-l**) on a GFP-transgenic background were added to purified DRG neuronal cultures and incubated in the presence of vehicle (0.1% BSA, **a, b, c, g, h, i**) or 1 μM LPA (**d, e, f, j, k, l**) for 6-7 hr. DAPI staining displays the nuclei of SCs and neurons (**a**, **d**, **g**, **j**). In addition, SCs were detected via GFP fluorescence (**b, e**, **h**, **k**), and DRG neurons were stained for neurofilaments to visualize axons (red in **c**, **f**, **i**, **l**). Merged images are also shown (**c**, **f**, **i**, **l**). Some of the neuronal cell soma are indicated with asterisks (**i**). Scale bar, 100 μm. **m**, LPA-induced migration from the aggregates along the fasciculated DRG axons was quantified by measuring the average distance of migrated SCs from the periphery of the aggregates. Mean ± s.e.m. of a representative example of three independent experiments (n=8, $^{**}P < 0.005$, $^{***}P < 0.0005$, vs. migration of WT cells under control conditions, *t*-test). (+/+) and (-/-) represent WT and LPA$_1$ null mice, respectively.

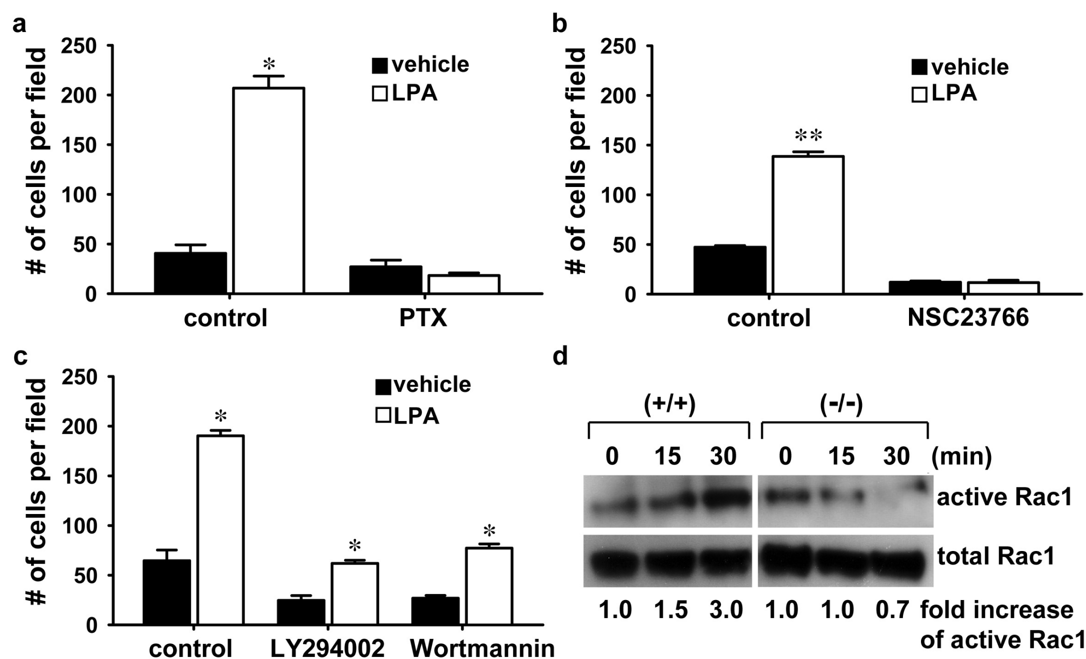

Figure 4.4 G_i proteins and the small GTPase Rac1 are involved in LPA/LPA_1 signaling-mediated SC migration. **a-c**, WT SCs were pretreated overnight with 150ng/ml pertussis toxin to inhibit G_i proteins (**a**), or treated for 30-45 min with either 100 μM NSC23766 to block Rac1 (**b**) or 50 μM LY294002 and 100 nM wortmannin, respectively, to inhibit PI3K (**c**) before SC migration was induced by adding 500 nM LPA to the lower transwell compartment. After 5-6h LPA-induced migration was quantified and compared to the vehicle (0.1% BSA)-induced migration of SCs treated with the respective inhibitors and to the responses of untreated SCs (**a-c**). Means ± s.e.m. of representative examples of 2-4 independent experiments (n=3, *$P < 0.05$, **$P < 0.005$, vs. vehicle-induced migration of cells pretreated with the respective inhibitor, *t*-test). **d**, Activation of endogenous Rac1 upon treatment with 1 μM LPA in WT or LPA_1 null SCs. GTP-bound Rac1 was pulled down from cell lysates at the indicated time points after addition of 1 μM LPA using a GST-tagged PAK-binding domain. Active GTP-bound and total Rac1 levels were subsequently analyzed by Western blotting. The fold increase of activated Rac1 at the different time points was measured and normalized against the total Rac1 levels. Shown are representative examples of 2-3 independent experiments. (+/+) and (-/-) represent WT and LPA_1 null mice, respectively.

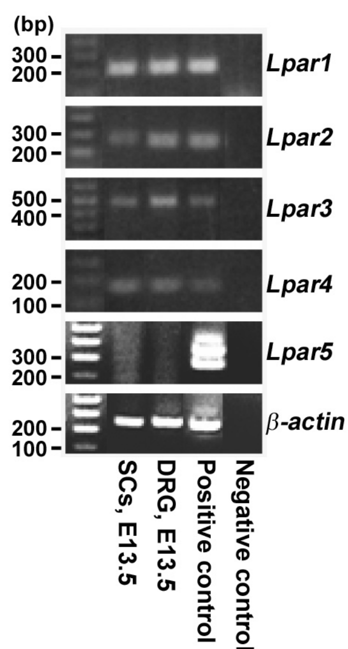

Figure 4.S1 Expression of LPA receptors in cultured SCs and DRG neurons. Detection of LPA receptor (*Lpar1-5*) expression by RT-PCR on RNA isolated from cultured embryonic mouse SCs or from DRG at E13.5. cDNA from lung and spleen was used as a positive control for *Lpar1-4*-specific and *Lpar5*-specific primer pairs, respectively. Amplification of β-actin served as a control of cDNA input.

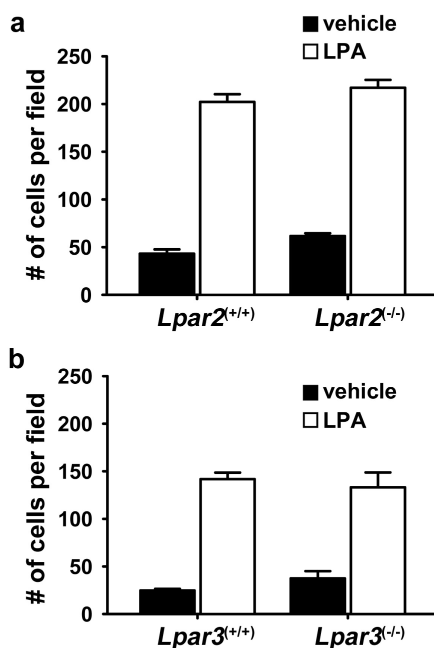

Figure 4.S2 Neither LPA_2 nor LPA_3 is involved in LPA-induced SC migration. **a**, Migration of WT (*Lpar2*$^{+/+}$) and LPA_2 null (*Lpar2*$^{-/-}$) SCs towards LPA (500 nM) was analyzed in transwell chambers. After 5-6h, migrated SCs werequantified and compared to the migration rate of vehicle (0.1% BSA)-treated SCs. Mean ± s.e.m. of one representative example of three independent experiments (n=3). **b**, Migrated WT (*Lpar3*$^{+/+}$) and LPA_3 null (*Lpar3*$^{-/-}$) SCs towards LPA (500 nM) were quantified and compared to the migration rate of vehicle (0.1% BSA)-treated SCs. Mean ± s.e.m. of one representative example of three independent experiments (n=3).

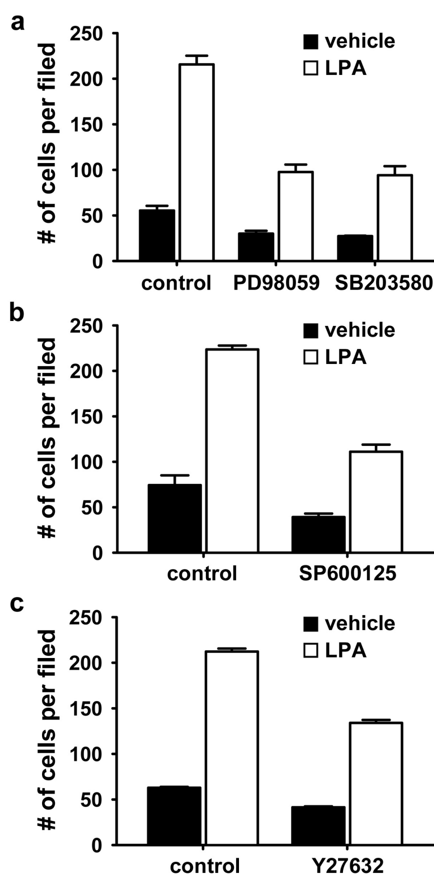

Figure 4.S3 LPA-induced SC migration is independent of MAPKs or Rock activation. a and b, The involvement of MAPKs including ERK1/2, p38, and JNKs was determined using specific inhibitor for each protein. WT SCs were pretreated for 30 min with 50 μM PD98059 (**a**), 20 μM SB203580 (**a**), or 10 μM SP600129 (**b**) to inhibit the activation of ERK1/2, p38, or JNKs before migration was induced by adding 500 nM LPA to the lower compartments of transwells. **c,** Role of the small GTPase Rho and its associated kinase Rock was also determined. SCs were pretreated for 30 min with 10 μM Y27632, an inhibitor for Rock, and then exposed to 500 nM LPA for 5 hr. Values represent mean ± s.e.m. of representative examples of 2 independent experiments (n=3).

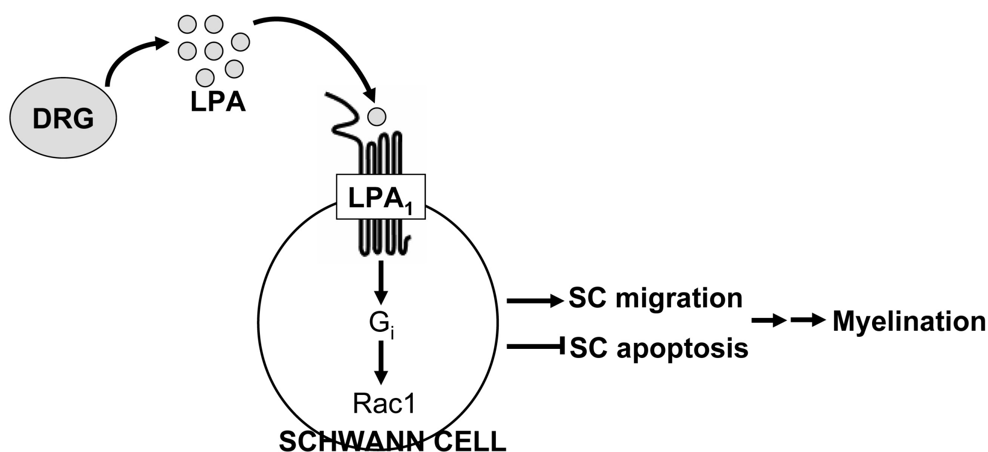

Figure 4.S4 Schematic model of LPA/LPA$_1$ signaling in SCs and its effects on SC developmental processes. Endogenous LPA secreted by DRG neurons increases SC migration through LPA$_1$ signaling coupled to G_i proteins and activation of the small GTPase Rac1. This effect on SC migration might subsequently lead to a delayed axonal segregation and impact myelination of the peripheral nerves during developmental stages. Combined with the previous results that LPA/LPA$_1$ signaling regulates SC apoptosis during developmental stages(Contos, Fukushima et al. 2000; Weiner, Fukushima et al. 2001), LPA/LPA$_1$ signaling is emerging as a multifunctional regulator of SC biology that either directly or indirectly also affects the myelination process.

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V.

LPA and S1P signaling interaction in Schwann cell migration and gene regulation

5.1 Introduction

We have demonstrated the involvement of LPA in regulating Schwann cell physiology including myelination and migration through LPA_1 in the previous chapter (Chapter Three). LPA also contributes to demyelination through LPA_1 signaling in both an *in vivo* and *in vitro* setting (Inoue, Rashid et al. 2004; Fujita, Kiguchi et al. 2007). As discussed above, LPA is also capable of promoting Schwann cell survival, preventing apoptosis, regulating the cell skeleton and adhesion (Weiner and Chun 1999; Weiner, Fukushima et al. 2001). It is also important in Schwann cell differentiation, which is an important step in both myelination and demyelination (Li, Gonzalez et al. 2003; Fukushima, Shano et al. 2007).

Although structurally similar, it is still unclear how S1P functioning through S1P receptors affects Schwann cells. By single cell RT-PCR, it has been demonstrated that $S1P_2$, $S1P_3$ and $S1P_4$ are expressed in myelinating Schwann cells (Kobashi, Yaoi et al. 2006). Similar to LPA, S1P can activate RhoA and Rac1; induce Schwann cell cytoskeleton rearrangement, lamellipodia formation and migration (Barber, Mellor et al. 2004). However, further investigation of receptor signaling is needed to understand the role of S1P in regulating Schwann cell biology.

We investigated the involvement of LPA_1 in neuropathic pain and demonstrated that LPA_1 acts in a spatially and temporally distinct fashion: an initial CNS effect and a later PNS component. We also demonstrate an interaction between LPA_1 and $S1P_3$ signaling in the development of neuropathic pain, where

the loss of $S1P_3$ signaling antagonized the protective effect of LPA_1 loss. This in turn led us to further investigate the role of S1P in Schwann cell biology. Using primary culture, we showed that S1P signaling can regulate cell migration and myelinating gene expression in Schwann cells. These effects are thought to be mediated through the activation of receptor subtypes $S1P_2$ and $S1P_3$. Activation of $S1P_2$ inhibits migration and also causes the down-regulation of myelin basic protein (MBP), whereas activation of $S1P_3$ promotes migration and results in the down-regulation of Krox20, a transcription factor controlling myelination. Together with current understanding of LPA signaling, these data suggest that LPA and S1P signaling interact to provide a fine-tuning mechanism that ensures proper demyelination and remyelination.

5.2 Further dissection of LPA_1 effect on neuropathic pain and $S1P_3$ interaction

As previously shown by Inoue *et al., Lpar1* null mice do not develop neuropathic pain while challenged with PSNL (Inoue, Rashid et al. 2004). In addition, the myelin sheath in the dorsal root area is protected from injury-induced demyelination (Inoue, Rashid et al. 2004). Subsequent experiments showed that LPA induces demyelination in *ex vivo* dorsal root cultures (Fujita, Kiguchi et al. 2007). However, exactly where LPA_1 exerts its effect remains unclear. We used an inhibitor of ATX, the major LPA production enzyme, together with conditional LPA_1 knockout animals to further investigate the involvement of LPA_1.

Two ATX inhibitors, AP104859 and AP104704, were utilized to block ATX activity. Both reagents were delivered by gavage twice daily starting one hour before surgery and continuing throughout the behavior testing period. The compound AP104859 has been shown to penetrate the blood-brain barrier and can suppress ATX activity in CNS when given at a dose of 100 mg/kg but not at 30mg/kg, while AP104704 has poor CNS penetration (unpublished data, Amira Pharmaceuticals). Interestingly, when challenged with nerve-injury, mice that recieved a high dose of AP104859 showed a complete protection from neuropathic pain development. A lower dose of AP104859 and AP104704 only provide some protective effect at later stage (Figure 5.1A). These results suggest that LPA_1 in the central nervous system is important for the initiation of neuropathic pain and development during the first few days of injury.

LPA_1 is widely expressed in the nervous system, and thus makes it difficult to determine which cell types are important for neuropathic pain. With the generation of LPA_1 conditional knockout mice, we were able to determine specific cell types responsible for the LPA_1 contribution. We generated P0-Cre LPA_1 and Nestin-Cre LPA_1 to specifically delete LPA_1 from Schwann cells and all neural cells respectively. The generation of other LPA_1 conditional knockout mice, including mice with LPA_1 deleted in neuronal cells and glial cells are still in progress. The P0-Cre LPA_1 mice showed a partial protective effect when challenged with PSNL. These mice still showed a reduced threshold to mechanical stimulation the first week after injury and the pain was ablated by the second week. However, since Nestin-Cre LPA_1 essentially removes LPA_1 from all PNS and CNS cell types, the protection from neuropathic pain development resembles a constitutive LPA_1 null mutant mouse (Figure 5.1B). These data are consistent with the use of ATX inhibitors; a central LPA_1 involvement is required for neuropathic pain development in the early phase where a peripheral mechanism, possibly acting through Schwann cells, occurs later. We hope that with the generation of other cell type specific LPA_1 knockout mice we can specifically determine which LPA_1 expressing cell types are involved in neuropathic pain development, for now at least two different cell types are clearly involved.

We are also interested in the involvement of another closely related lysophospholipid, S1P, in this system. We challenged $Lpar1^{(-/-)}/S1pr3^{(+/-)}$ and $Lpar1^{(-/-)}/S1pr3^{(-/-)}$ mice with PSNL and monitored their responses to mechanical

stimulus over 12-day period. Consistent with the previous report, *Lpar1*$^{(-/-)}$/*S1pr3*$^{(+/-)}$ mice are resistant to the development of neuropathic pain similar to LPA_1 null mice. However, double knockout animals no longer show the protective effect and have a lowered mechanical stimulus threshold over the 12-day period (Figure 5.1C). This suggests an interaction between LPA_1 and $S1P_3$ signaling during neuropathic pain development.

In conclusion, we have demonstrated that LPA_1 signals initially through a CNS component that is then followed by a PNS effect, possibly mediated through Schwann cells. Further studies targeting specific cell types in both systems are needed to further understand the LPA_1 contribution. In addition, we have shown that the loss of $S1P_3$ signaling reverses the protective effect that occurs through loss of LPA_1 on neuropathic pain development. Further studies on how $S1P_3$ signaling effects Schwann cell physiology and myelination regulation will be described next (See below).

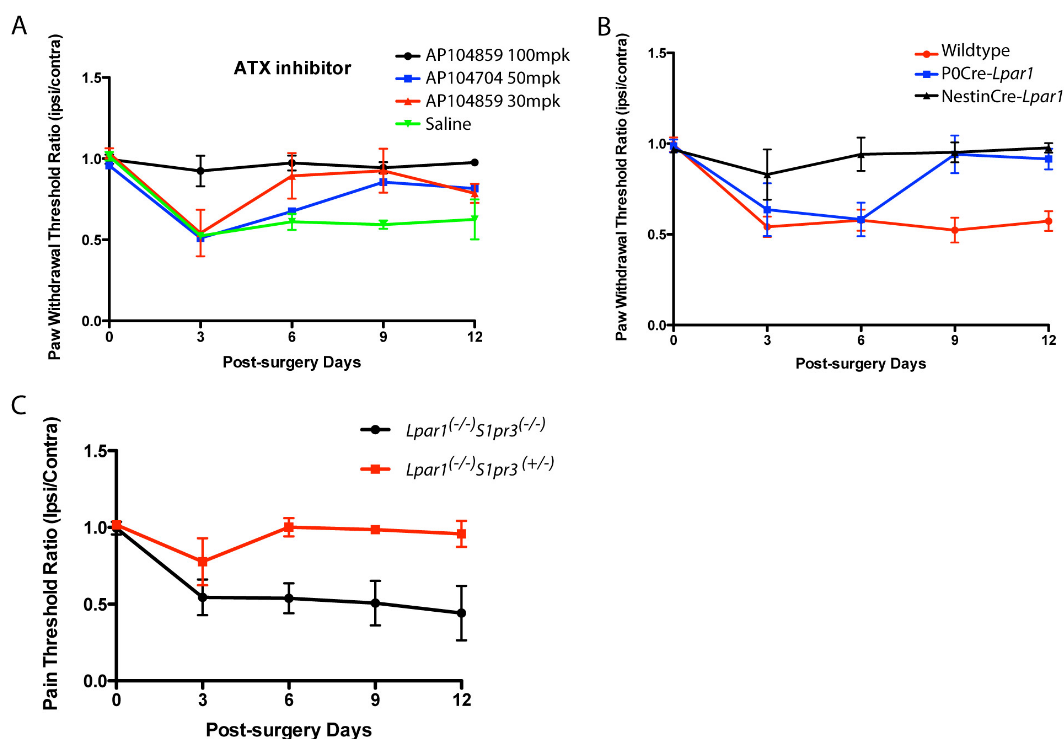

Figure 5.1 Behavior test using ATX inhibitor, LPA_1 conditional and $LPA_1/S1P_3$ double knockout mice. (A) Behavior test of paw withdrawal threshold against mechanical stimulus on wildtype mice receiving the ATX inhibitor AP104859 and AP104704 by gavage twice a day and challenged by PSNL. (B) Behavior test of paw withdrawal threshold against mechanical stimulus on P0-Cre LPA_1 and Nestin-Cre LPA_1 mice challenged by PSNL. (C) Behavior test of paw withdrawal threshold against mechanical stimulus after PSNL on LPA_1 and $S1P_3$ knockout animals. Value represents mean ± s.e.m. of withdrawal threshold ratio between ipsilateral and contralateral paws, N = 5.

5.3 LPA induced Schwann cell migration is affected by S1P signaling

We have demonstrated that LPA signaling through LPA_1 enhances Schwann cell migration and this signaling is necessary for proper myelin formation (Chapter Four). Previous experiments with mice deficient for both LPA_1 and $S1P_3$ have demonstrated that the loss of $S1P_3$ signaling can antagonize the protective effect resulting from the loss of LPA_1. Since the protective effect of LPA_1 is thought to be due to the prevention of demyelination, we are interested in how signaling through $S1P_3$ affects Schwann cells.

We have shown that LPA enhances SC migration through LPA_1 signaling. This led us to test if the related lysophospholipid, S1P, had a similar effect. Surprisingly, utilizing Boyden chambers to assess the migratory effects of LPA and/or S1P, we found that S1P antagonizes the effect of LPA (Figure 5.2A). This inhibitory effect contradicts a previous report that claims that S1P enhances Schwann cell migration (Barber, Mellor et al. 2004). A possible reason for this discrepancy is that the expression levels of $S1P_2$ and $S1P_3$ may be different in the different cell lines employed. $S1P_2$ and $S1P_3$ signaling have been shown to antagonize each other, especially the Rac and Rho interaction (Taha, Argraves et al. 2004). While both receptors can activate Rac and Rho, $S1P_2$ activation leads to higher Rho activation and subsequent inhibition of Rac through Rac-GAP while $S1P_3$ mainly activates the Rac pathway (Taha, Argraves et al. 2004). Rac activation is known to be essential for cell migration and inhibition of Rac that occurs through $S1P_2$ activation can be responsible for the inhibition of migration. Furthermore,

LPA and S1P induced Schwann cell migration is also reduced in Schwann cells deficienct for $S1P_3$ (Figure 5.2A). Interestingly, when LPA and S1P are administrated together, Schwann cell migration is even further reduced, suggesting an even greater enhancement of Rho signaling.

We have shown that LPA and S1P signaling interact to influence the Rac and Rho pathways, in addition they may interact to effect receptor expression levels. Using quantitative PCR to determine the expression levels of *Lpar1*, *S1pr2* and *S1pr3* in receptor deficient Schwann cells we found that *Lpar1* mRNA expression is highly upregulated in the absence of *S1pr3* but not *S1pr2*, while the expression level of both S1P receptors is upregulatd in *Lpar1* deficient cells (Figure 5.2B and C). This suggests possible antagonizing effects on LPA_1 and $S1P_3$ signaling and is in line with our previous finding that the loss of $S1P_3$ reversed the protective effect of LPA_1 loss.

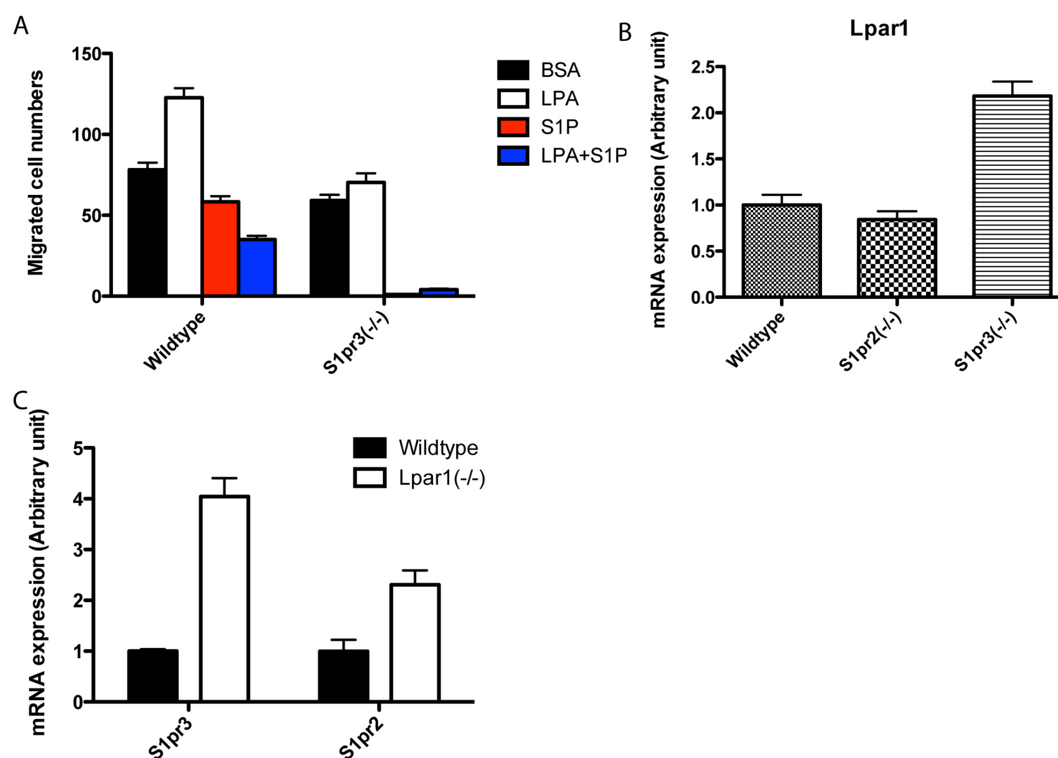

Figure 5.2 LPA and S1P regulate Schwann cell migration and receptors expression. (**A**) Numbers of cells migrating towards LPA and S1P using wildtype and *S1pr3* deficient Schwann cells. Bars represent mean ± s.e.m. of number of cells migrated per field. N = 3. (**B**) Expression levels of *Lpar1* mRNA in wildtype, *S1pr2,* and *S1pr3* deficient Schwann cells. (**C**) Expression levels of *S1pr2* and *S1pr3* mRNA in *Lpar1* deficient Schwann cells. Bars represent mean ± s.e.m. of fold increase over control. N = 3.

5.4 S1P signaling modifies myelination genes regulated by LPA

Consistent with the observation that LPA induces demyelination through LPA_1, we were able to demonstrate that LPA is involved in the regulation of several genes important for myelination in Schwann cells, including myelin basic protein (MBP) and the transcriptional factor Krox20. In order to understand how LPA and S1P are involved in this process, Schwann cells were activated with dbcAMP for three days and then treated with BSA or 1 μM LPA for four hours. cDNA was then prepared from RNA extracted from the cells and subject to qPCR analysis.

As shown in Figure 5.3, when applied to wild type Schwann cells, LPA represses the expression of both MBP and Krox20. However, this does not happen in Schwann cells deficient for LPA_1, indicating that LPA downregulation of MBP and Krox20 occurs in a LPA_1 dependent manner. Interestingly, the removal of either $S1P_2$ or $S1P_3$ dramatically influences how LPA regulates the expression level of these genes. *S1pr2* deficient Schwann cells do not show an LPA dependent down regulation of MBP demonstrating that the LPAs ability to reduce MBP expression requires $S1P_2$ signaling (Figure 5.3A). Likewise, $S1P_3$ signaling is essential for LPA induced down regulation of Krox20 (Figure 5.3B). This differential control mechanism is also in agreement with the signaling properties of S1P receptors discussed above. We used two different compounds, ROCK inhibitor Y27632 and Rac inhibitor NSC23766, to further validate the signaling pathways used. Y27632 completely blocks LPAs ability to decrease MBP expression, while NSC23766 blocks the LPA dependent down regulation of Krox20 (Figure 5.3C). This

observation is in agreement with the validated $S1P_2$ and $S1P_3$ signaling properties, which show that $S1P_2$ mainly activates the Rho pathway and $S1P_3$ activates Rac.

Here we demonstrated for the first time that an LPA and S1P signaling interaction controls the expression level of genes essential for myelination. More interestingly, this result demonstrates that the expression of different genes can be fine-tuned by modulating the activation of different S1P receptors during the demyelination and/or remyelination processes. A proposed mechanism is summarized in Figure 5.4. While LPA stimulation down regulates both MBP and Krox20 during demyelination, S1P signaling can adjust the degree or timing of the down-regulation. However, further experiments are needed to determine if the demonstrated LPA and S1P interaction control demyelintion and possibly remyelination *in vivo*. These novel data regarding how LPA and S1P regulate expression of genes important for myelination could lead to exciting new treatments for patients suffering from myelination related diseases.

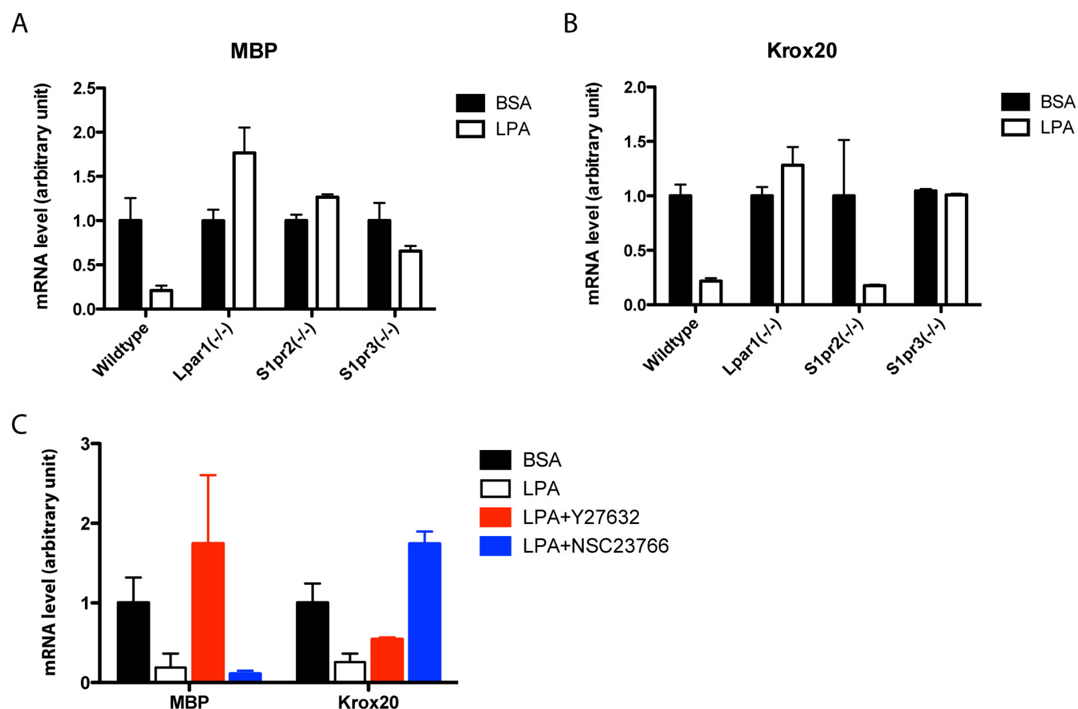

Figure 5.3 MBP and Krox20 mRNA expression levels. (**A**) LPA regulates MBP mRNA expression levels in LPA_1, $S1P_2$, and $S1P_3$ deficient Schwann cells. (**B**) LPA regulates Krox20 mRNA expression levels in LPA_1, $S1P_2$, and $S1P_3$ deficient Schwann cells. (**C**) LPA regulates MBP and Krox20 mRNA expression levels in wildtype Schwann cells under ROCK and Rac inhibitors, Y27632 and NSC23766. Bars represent mean ± s.e.m. of LPA induced mRNA fold increase against BSA control. N = 3.

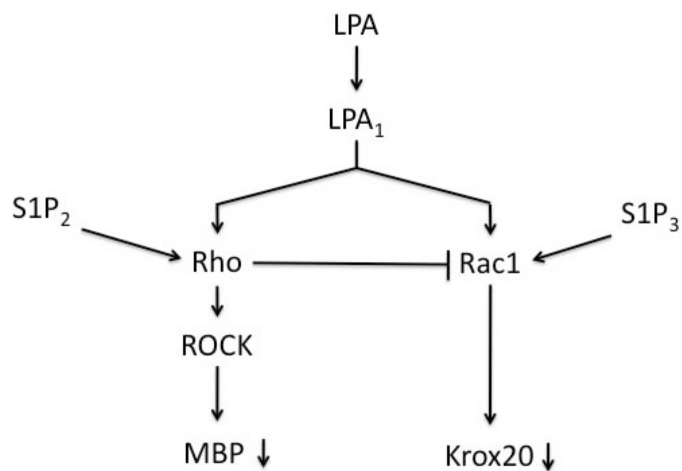

Figure 5.4 Proposed mechanism of LPA and S1P signaling interaction in regulating myelination gene expression in Schwann cells.

5.5 Conclusion

LPA is well known to regulate various aspects of Schwann cell biology including proliferation, survival, migration and differentiation. The data showing involvement of LPA_1 in injury-induced neuropathic pain further proves its essential role in Schwann cell physiology. Here we further dissected the role of LPA_1 in neuropathic pain using both conditional knock out animals and chemical inhibitors against autotaxin. We demonstrated there are two phases of LPA action in nerve injury models, an early CNS component and a later PNS component involving Schwann cells.

Another closely related lysophospholipid, S1P, has not previously been shown to affect Schwann cells nor to be involved in neuropathic pain. We demonstrated for the first time using LPA_1 and $S1P_3$ double knockout animals that an interaction between LPA and S1P signaling affects neuropathic pain development. Mice lacking both receptors are no longer protected from PSNL induced neuropathic pain as seen in LPA_1 deficient mice. Although detailed mechanism remains unclear, an interaction between these two signaling molecules in Schwann cells might provide an explanation. Using receptor deficient primary Schwann cells, we were able to show that S1P interfered with LPA induced Schwann cell migration depending on the receptor subtypes expressed. Activation of Rho and subsequent Rac inhibition through $S1P_2$ is most likely responsible for the inhibitory effect while activated Rac signaling through $S1P_3$ likely enhances migration.

This interaction between LPA_1 and $S1P_{2/3}$ does not only occur at the G protein level but also occurs at the receptor expression as well. In LPA_1 deficient cells, expression of both S1P receptors are elevated, while an increase in *Lpar1* mRNA is only observed in $S1P_3$ deficient cells. Thus, the expression of these receptors can antagonize each other and so can their downstream Rac/Rho signaling. This also proves to be true when it comes to regulation of genes involved in myelination. We showed that the regulation of genes involved in myelination that are upregulated by LPA can be further fine-tuned by S1P. Two genes essential for proper myelination, MBP and Krox20, were monitored following the administration of LPA. Both genes were down regulated after addition of exogenous LPA, consistent with the result that LPA can induce demyelination. Moreover, the expression of these genes can be further modified by $S1P_2$ and $S1P_3$ signaling. The down regulation of MBP requires $S1P_2$, most likely by signaling through Rho as shown by the use of Y27632, a ROCK inhibitor. Although also down regulated by LPA_1, Krox20 is differentially regulated by $S1P_3$ and is downstream of Rac signaling as shown by the use of NSC23766, a Rac inhibitor.

In conclusion, we have demonstrated that interactions between LPA and S1P occur at various levels ranging from cell signaling to responses to neuropathic pain. These interactions suggest a complicated system of regulation in neuropathic pain development and Schwann cell biology. Although LPA receptors have been shown to be important in these systems, the involvement of S1P has not been explored. How these interactions regulate Schwann cell genes may provide an explanation to

the behavioral outcomes and insights into the fine-tuning of the demyelination and remyelination processes. Further experiments are needed to resolve these issues and we hope that this initial study will draw more attention to this newly discovered area of lysophospholipid signaling.

5.6 References

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VI.

CONCLUDING REMARKS

This dissertation focused on lysophosphatidic acid and its involvement in neuropathic pain and Schwann cell biology. As previously demonstrated, LPA acting through LPA_1 causes demyelination and subsequent neuropathic pain development. However, the exact mechanism and the contribution of other receptors, still remain unclear. Here for the first time, we demonstrated: 1) involvement of another LPA receptors, LPA_5, in neuropathic pain development, 2) LPA regulates Schwann cell migration and is critical for proper myelination, and 3) interaction between LPA and S1P signals is critical in both neuropathic pain and Schwann cell biology.

Starting with the modification of LPA measurement protocol, we tested various extraction conditions and combined this with HPLC-Tandem Mass Spectrometry to detect LPA distribution. The refined extraction protocol is critical to the measurement as *in vitro* degradation and production of LPA, as well as low recovery could lead to false results. We modified an extraction protocol using 1-butanol and successfully reduced *in vitro* conversion of LPC to LPA and still maintained a good recovery rate. With this technique, we determined LPA concentration in various tissues in the nervous system. The highest concentration were observed in the spinal cord, among all the tissue examined, and thus provided an important clue to the action of LPA in neuropathic pain.

Secondly, we demonstrated that LPA_5, previously known as GPR92, is also required in neuropathic pain development. Interestingly, the mechanism is distinct from what is proposed for LPA_1, which consists of lowered PKCγ and Caα2δ1

expression, as well as protection against demyelination. Instead, LPA_5 activation appears to induce pCREB signaling in the dorsal horn area and the loss of LPA_5 could provide protection via lowering dorsal horn neuron sensitivity. This is a novel contribution of LPA to neuropathic pain as LPA_5 could directly contribute to the modulation of post-synaptic neuron activity. Although experiments to dissect the action site of LPA_5 are still needed, the expression data suggest dorsal horn neurons and DRG cells are the best candidates. Upon activation, LPA_5 can increase cAMP levels and could lead to pCREB activation. However, direct studies on these neurons during the nerve injury event are still needed to prove this hypothesis. A direct measurement of neuron excitability and cAMP levels will be ideal using PSNL model. Further studies which include the use of LPA_1 and LPA_5 double knockout mice, as well as specific chemical antagonists, will be of interest and could lead to potential therapeutic treatments.

We are also interested in determining the action of LPA_1 signaling on demyelination and Schwann cell biology. With a primary Schwann cell culture system, we demonstrated a regulatory role of LPA in Schwann cell migration in both a Boyden chamber and DRG neuron co-culture. LPA_1 is also required to form a proper myelin sheath during development since LPA_1 null mice showed a thinner myelin sheath and abnormal axonal segregation. These regulations could be attributed to the ability of LPA to activate small G-proteins such as Rac and Rho, which are important in cell migration as well as in cell shape change to form the myelin sheath. While activated during the nerve injury events, LPA_1 signaling could

lead to demyelination through the mechanism described above. Given the high concentration of LPA present in the nervous system, additional studies on how LPA regulates Schwann cells and myelin sheath formation *in vivo* could give us an understanding into the dynamic of LPA signaling.

Not only can LPA regulate Schwann cell physiology, but another closely related lysophospholipid, S1P, is also involved and can modify LPA's action. In a Boyden chamber study, S1P is administrated alone caused a slight decrease in Schwann cells migration. However, when added with LPA, S1P can block the LPA induced cell migration, indicating an interaction between their signaling pathways. More interestingly, this antagonizing effect depends on the signal strength balance between $S1P_2$ and $S1P_3$, which activates the downstream small G-proteins Rho and Rac, respectively. Another interesting interaction between LPA and S1P is observed at the behavioral level. When challenged with PSNL, LPA_1 null mice do not develop neuropathic pain as previously described, however, the loss of $S1P_3$ reverses this protection and the double null mice develop a similar hyperalgesia phenotype as wildtype control mice. Combining these results, we hypothesize that the interaction between LPA and S1P in Schwann cells could lead to differences in neuropathic pain development.

Given that the LPA_1 is responsible for the demyelination event and neuropathic pain development during nerve injury, we are interested in knowing if S1P signaling, especially $S1P_2$ and $S1P_3$, can affect myelination. The expression of MBP and Krox20 are important events in myelination, as the interruption of either

gene can cause severe demyelination. Therefore, we examined how LPA regulates the expression level of these two genes in Schwann cells lacking LPA_1, $S1P_2$, or $S1P_3$. While LPA acts through LPA_1 and decreases both MBP and Krox20 expression as expected, $S1P_2$ acts through the Rho-ROCK pathway and is required for LPA to suppress MBP expression. On the other hand, $S1P_3$ acts through Rac signaling and is required for Krox20 suppression caused by LPA. This is the first time a modification of LPA signaling by S1P is demonstrated. It also suggests that both LPA and S1P should be taken into consideration while studying lysophospholipid signaling in Schwann cell biology and neuropathic pain development. Many more studies need to be done to elucidate the fine-tuning mechanism provided by the interaction between LPA and S1P in the course of demyelination, and possibly also remyelination processes.

This dissertation aims to provide a greater understanding of how lysophosphatidic acid signaling affects neuropathic pain and Schwann cell biology. Although many more studies need to be done in order to get a clearer picture, I hope that our findings provide an important step into this area that can serve as a basis for future research that ultimately leads to better therapeutic options for people with neuropathic pain.