Spinal cord injury

Role of endothelial differentiation gene family lysophosphatidic acid receptors

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Summary

Although the spinal cord is well protected by the bones of the spinal column, it can be damaged in many ways: by cut, bruise or compression, and less frequently injured by infection, damaged when its blood supply is cut off, or affected by diseases that alter its nerve function. Any of these spinal cord injuries result in a disruption of the pathways that carry information between brain and body, as well as of neuronal networks related to many physiological functions. As central nervous system (CNS) axons of adult mammals do not regenerate following lesion, and dead cells are not successfully replaced, this results in irreversible functional loss in patients suffering from spinal cord injury (SCI).

The pathophysiology of SCI involves two stages of tissue degeneration known as primary and secondary injury. The first results from the direct mechanical trauma to the spinal cord which is followed by a secondary wave of tissue degeneration that occurs over a period of several weeks that include inflammation and other mechanisms triggered by injury. Secondary tissue damage that occurs after SCI contributes significantly to permanent functional disabilities. Although regeneration of damaged axons and replacement of lost neurons after SCI are important goals, the secondary damage to axons, neuronal cell bodies, myelin and glial cells that follows the initial trauma is likely to be more easily amenable to treatment. Preventing or minimizing such secondary damage after SCI can be expected to substantially reduce the functional disability. This is of crucial interest since there is currently no effective treatment for reducing the functional disabilities in SCI patients.Lysophosphatidic acid (LPA) is an extracellular lipid mediator with many physiological functions, which signals through 6 known G protein-coupled receptors (LPA_{1-6}). A wide range of LPA effects has been identified in the CNS, including neural progenitor cell physiology,

astrocyte and microglia activation, neuronal cell death, and axonal retraction. *In vivo* studies also show that LPA is involved in the etiology of fetal hydrocephalus, as well as the development of neuropathic pain after sciatic nerve injury and cerebral ischemia. A recent work reveals that the administration of the B3 antibody, which binds to LPA and other lysophospholipids preventing them from interacting with their receptors, promotes functional recovery after spinal cord hemisection in mice. Although the hemisection model is not a clinical relevant model of SCI, this study provided the first insights on the potential deleterious actions of LPA in SCI. More recently, the B3 antibody was shown to promote neuroprotection after brain trauma. Due to the wide variety of LPA receptors it is likely that LPA may exert helpful or harmful effects in the CNS depending on the receptors it signals through, as has been already observed in other lipid mediators such as prostaglandins. There is therefore a need to know which LPA receptors contribute to neurodegeneration, and those, if any, that could mediate neuroprotection. In the present thesis we aimed at dissecting out the contribution of the three LPA receptors belonging to the endothelial differentiation gene (Edg) family (LPA_{1-3}) to SCI.

Our results reveal that LPA levels increase in the spinal cord parenchyma after contusion injury. We show that administration of LPA into the intact spinal cord leads to inflammation and demyelination. Moreover, we found LPA-mediated demyelination is triggered by activation of microglia LPA_1 and LPA_2 rather to a direct toxic effect of LPA via oligodendrocyte LPA_1 and LPA_2 signaling. We also reveal that activation of LPA_2 leads to neuronal cell death. Interestingly, we demonstrate that the lack of LPA_1 and LPA_2 signaling after SCI enhances functional recovery and myelin sparing as well as neuronal protection in case LPA_2 activity is lacking. In addition, we show that LPA_3 does not contribute to SCI. Overall, these results reveal for the first time a detrimental contribution of LPA_1 and LPA_2 in SCI, and suggest LPA_1 and LPA_2 as novel therapeutic targets for the treatment of acute SCI, but also for other CNS conditions in which demyelination and neuronal cell death contribute to the pathology.

INTRODUCTION

SPINAL CORD: description and basic anatomy

Functionally, the spinal cord is the part of the central nervous system (CNS) that controls the voluntary muscles of the limbs and trunk, and which receives sensory information from these regions. It also controls most of the viscera and blood vessels of the thorax, abdomen and pelvis.

Structurally, the spinal cord is the part of the CNS that is connected to the brain and lies within the vertebral canal. Although the adult spinal cord is visually a continuous cylinder of central nervous tissue, it is described as being made up of a series of segmental components, which are determined by the pattern of emergence of the spinal nerves. Along all the spinal cord length, pairs of spinal nerves arise from each side of every medullary segment, constituting the dorsal and ventral roots. Spinal roots leave the spinal cord through the intervertebral foramina and convey sensory (for the dorsal) and motor (for the ventral) information. The bodies of the sensory neurons are allocated into the dorsal ganglia, while the somas of the motor neurons are into the spinal cord. The spinal cord starts at the foramen magnum, at the base of the skull, and ends at the level of the first or second lumbar vertebra. However, the spinal nerves continue down until they reach its correspondent vertebral segment, and form a bundle of nerves called the cauda equina.

Besides the vertebral column, the spinal cord is protected by the spinal meninges, three membranes that surround all the CNS. In mammals, these membranes are the dura mater, which is the outermost layer, the arachnoid mater, and the pia mater, that is directly adhered to the surface of the brain and the spinal cord. The meninges are separated from each other by the subdural and the subarachnoid spaces,

respectively. This second encloses the cerebrospinal fluid, which also provides a mechanical and immunological protection.

The spinal cord is composed of gray and white matter. In a transverse section the gray matter can be distinguished in the middle, in the shape of an H, while the white matter surrounds it.

Organization of the gray matter

The gray matter of the spinal cord is occupied by neuronal cell bodies, dendrites, axons and glial cells. It is macroscopically divided in two dorsal and two ventral horns, which correspond with the dorsally and ventrally projecting arms of the H, respectively. The central connecting region is called intermediate gray matter. Only in the thoracic and upper lumbar spinal cord there is two small lateral projections of the intermediate gray matter, the intermediolateral horns, which contain the cells of origin of the autonomic nervous system.

The neuronal bodies in the gray matter are organized in ten successive layers, from dorsal to ventral, called the Rexed laminae. Laminae I-VI are located in the dorsal horns, laminae VII-IX are in the lateral and ventral gray matter and lamina X is in the center, surrounding the central canal. Whereas the dorsal interneurons are involved in sensory input, medial are related to autonomic functions and ventral are involved in modulating descending motor control. Below there is a brief description of the main projections and functions of each laminae (Anderson *et al.*, 2009) (Figure 1).

Lamina I is a very thin layer previously known as the marginal layer of the dorsal horn. Its neurons receive input principally from Aδ and C fibers innervating the skin, viscera, muscles and joints. These fibers travel through the dorsal roots and carry information about pain and temperature. Lamina I also respond to innocuous mechanical stimuli conveyed by Aβ fibers. The primary target of lamina I in the thalamus and axons from lamina I make up about half of the spinothalamic tract.

Lamina II, also known as substantia gelatinosa, has greater cell density

than lamina I. It is also a principal region of termination of Aδ and C fibers and its neurons mainly functions as interneurons that modulate nociceptive transmission integrating primary afferent input and modulating the output of ascending projections in the surrounding laminae.

Lamina III contains many myelinated fibers and neurons are less densely packed and larger than in lamina II. Its neurons respond to tactile stimulation from Aβ fibers and contact with dendrites from laminae IV-VI.

Lamina IV, formerly known as the head of the dorsal horn, is thicker than lamina III. It also receives axons from Aβ fibers and responds to light and noxious mechanical stimuli. Some neurons arise ascending projections to the thalamus via the spinothalamic tract.

Lamina V, formerly called the neck of the dorsal horn, is the thickest layer in this area. Contains neurons whose dendrites are in lamina II receiving monosynaptic nociceptive information from Aβ and C fibers from viscera. From this lamina, projections arise to the thalamus and the brainstem through the spinothalamic tract. Descending rubrospinal and corticospinal connections are also received in this layer.

Lamina VI is the most ventral layer of the dorsal horn and is especially large in the cervical and lumbar enlargements. Propioceptive information from muscle spindles arrive to this layer, from where it is originated the spinocerebelar tract. This lamina contains numerous propiospinal interneurons, mainly involved in reflex pathways.

Lamina VII contains interneurons that communicate the dorsal and the ventral horns and mainly act as relay points in the transmission of visceral information. It is also involved in the regulation of posture and movement. Descending motor pathways control motoneurons by means of connection with interneurons in lamina VII.

Lamina VIII is found in the ventromedial or ventral region of

the spinal cord and its size is reduced in the cervical and lumbar enlargements, since the motoneuron pools from lamina XI are larger there. The cells of this layer are propiospinal interneurons and the large ones project to motoneurons on the same and opposite side. Motor interneurons in this layer modulate the motor activity through gamma-motoneurons, which innervate the intrafusal muscle fibers.

Lamina IX is mainly occupied by columns of alpha-motoneurons and smaller beta- and gamma- motoneurons, which are somatotopically organized. It is especially large in the cervical and lumbar enlargements, since all motoneurons responsible for limb muscle control are allocated there. The big alpha-motoneurons launch their axons through the ventral roots to innervate extrafusal fibers or skeletal muscles, while gamma-motoneurons innervate the intrafusal fibers.

Lamina X is found surrounding the central canal and contains neurons that project to the contralateral side of the spinal cord. It is also known as central gray matter.

Organization of the white matter

The white matter consists mostly of longitudinally running axons and also glial cells. It can be divided according to different classification criteria. For a location criterion, it is divided by the horns of the gray matter. A group of axons in a given area is a funiculus and they are named dorsal, lateral and ventral funiculus depending on the position into the spinal cord. However, the terms "tract" and "pathway" are used when there is a common function for a nerve fiber bundler. A tract is a group of nerve fibers with the same origin, course, termination and function (e.g. corticospinal tract), and a pathway is a group of tracts with a related function (e.g. neospinothalamic pathway). Thus, ascending tracts and pathways convey sensorial information, while descending ones consist in motor fibers. There are also many fibers, called propiospinal fibers, which connect one spinal cord segment with another. A few differences are found between the tract organization of the spinal cord in different species ranging from rodents, cats,

primates and humans. One of the major differences is the location of the corticospinal tract: while in humans is placed in the lateral and ventral funiculi, rodents present an important dorsal component of the corticospinal tract (Armand, 1982). Below, main ascending and descending spinal tracts in humans are briefly described (Figure 1).

Ascending tracts arise from primary neurons whose somas are in the dorsal root ganglia or from interneurons in the dorsal horn, and convey sensory informat ion to higher areas of the CNS. Occupying the dorsal column ascend the gracile and cuneatus tracts, which carry information related to tactile, two point discrimination, vibration, position and movement sense and conscious proprioception. In the lateral column, the lateral spinothalamic tract carries pain, temperature and crude touch information from somatic and visceral structures. Nearby, the dorsal and ventral spinocerebellar tracts carry unconscious proprioception information from muscles and joints of the lower extremity to the cerebellum. In the ventral column, the anterior spinothalamic tract carries pain, temperature and touch associated information to the brain stem and diencephalon; the spinoolivary tract carries information from Golgi tendon organs to the cerebellum; the spinoreticular tract carries pain information to the thalamus; and the spinotectal tract carries pain, thermal and tactile information to the superior colliculus for spinovisual reflexes.

Descending tracts originate from different cortical areas and from brain stem nuclei and carry information associated with maintenance of motor activities such as posture, balance, muscle tone, and visceral and somatic reflex activity. These include the lateral corticospinal (pyramidal) and the rubrospinal tracts, located in the lateral column, which carry information associated with voluntary movements. The reticulospinal, vestibulospinal and anterior corticospinal tracts, in the ventral column, mediate balance and postural movements. Lissauer's tract, which is wedged between the dorsal horn and the surface of the spinal cord, carries the descending fibers of the dorsolateral funiculus, which regulate incoming pain sensation at the spinal level and intersegmental fibers.

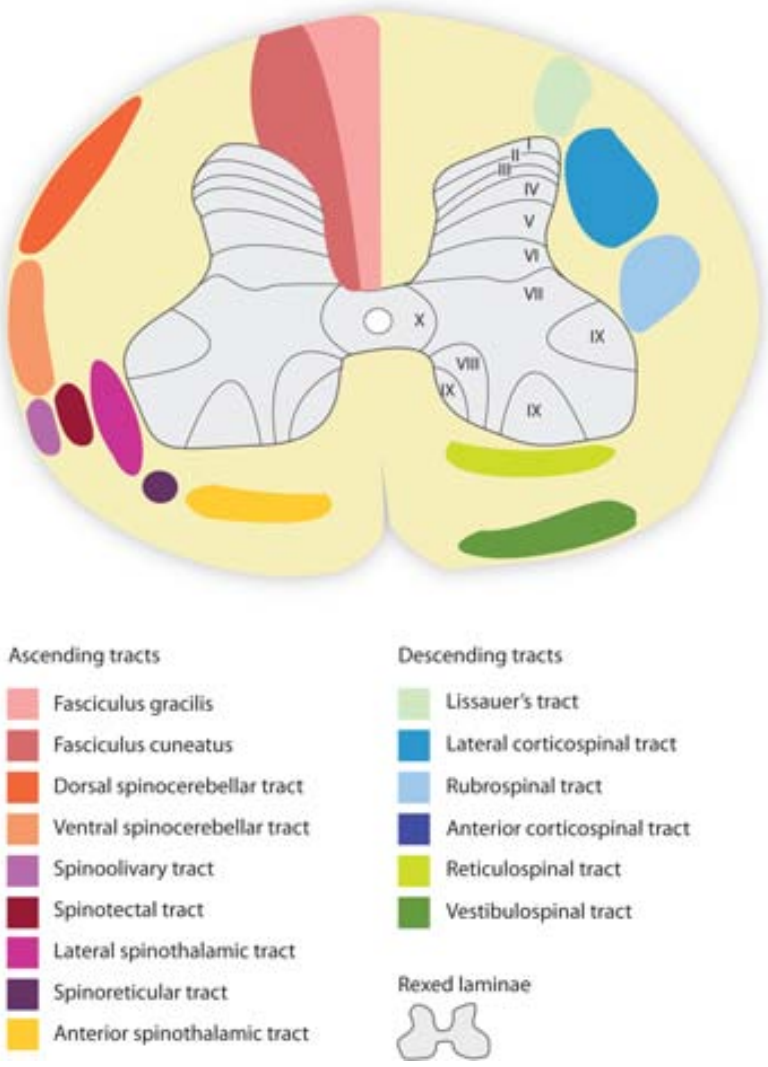

Figure 1. Schematic diagram of the organization of the gray and white matter of the spinal cord.

SPINAL CORD INJURY: description, causes and epidemiology

Although the spinal cord is well protected by the bones of the spinal column, it can be damaged in many ways: by cut, bruise or compression in an accident, and less frequently injured by infection, damaged when its blood supply is cut off, or affected by diseases that alter its nerve function. Any of these spinal cord injuries result in a disruption of the pathways that carry information between brain and body. As CNS axons of adult mammals do not regenerate following lesion, this results in a dramatic functional loss below the site of injury. A traumatic spinal cord injury (SCI) is then an event that conditions the rest of the life of those who suffer it.

The first spinal cord injuries were documented more than two thousand years ago and it still does not exist any treatment for them. In fact, the prognosis and the life span of SCI patients only improved 50 years ago. Nowadays treatments and medical care make possible to them to present a life span very close to those without an SCI, despite the loss of functionality below the lesion site, the appearance of secondary complications, and the consequent reduction in quality of life.

Etiology and epidemiology of the SCI

SCI is a major cause of death and disabilities. The incidence is higher in young people (18-35 years) and more frequent in men than in women, with a 4:1 ratio. Incidence of traumatic SCI varies between 13.1 and 52.2 cases per million inhabitants in developed countries, and between 12.7 and 29.7 in developing countries (Chiu *et al.*, 2010). In Spain, 10,274 new patients were admitted for traumatic SCI between 2000 and 2009, with an annual incidence of 23.5 per million (Pérez *et al.*, 2012).

Among all types of SCI, traumatism is the most frequent (70-80%), and therefore the most studied. Motor vehicle accidents are the main cause (46%), followed by falls (18%), that is the most common cause in people over 60 years old, violence (17%) and sports (13%) (Mazaira *et al.*, 1998). The non-traumatic SCI have also an important impact in Spain (20-30%) and the main causes are tumors (40%), infections (30%) and vascular accidents (20%), among others (Mazaira *et al.*, 1998).

Level and severity of the SCI

When the spinal cord is damaged all its functions can be compromised below the lesion site, resulting in total or partial loss of movement (tetraplegia or paraplegia), sensation (anesthesia or hypoesthesia), autonomic deficits (sexual dysfunction, loss of control of the sphincters, etc.) and pain. The level of the spinal cord, the severity (partial or complete), and the type of injury (contusion, compression, section, etc.) are important factors that will determinate the magnitude of final deficits.

The level of injury determines the type of dysfunction, so that the higher the injury is, the more dysfunctions can occur (Figure 2).

High-cervical injuries (C1-C4) are the most severe. They lead to tetraplegia, and patients affected with these lesions may not be able to breathe on their own, cough, nor control bowel or bladder movements. Ability to speak is sometimes impaired. These patients require complete assistance with all daily living activities. Patients with **low-cervical injuries (C5-C8)** may be able to breathe on their own and speak normally. They may also have some function on upper-limbs, as bend wrists, straighten the arms or move the shoulders. Although assistance of a second person is needed with most activities of daily living, these persons are able to move from one place to another independently with a power wheelchair.

In **high-thoracic lesions (T1-T5)** arm and hand function is usually normal, making possible the use of manual wheelchairs. In these injuries, trunk and legs are affected, what is known as paraplegia.

Patients with **low-thoracic lesions (T6-T12)** are usually able to control and balance trunk while seated and can have little control of bowel or bladder function.

Lumbar injuries (L1-L5) generally result in some loss of function in the hips and legs. Patients way need a wheelchair depending on leg strength.

Sacral injuries (S1-S5) also use to affect hips and legs, but patients are mostly able to walk.

Functional deficits also depend on the severity of the lesion. Complete

Figure 2. Functional deficits associated with each spinal cord injury level.

injuries refer to those that result in the complete loss of sensory and motor functions, as well as in the alteration of reflexes, below the point of injury. However, this not necessarily implies total disconnection of all descending and ascending spinal pathways. On the contrary, incomplete injuries refer to those in which some feeling or movement is still evident below the point of injury. In these cases, the remaining of motor and sensory function may differ between patients depending on the percentage of motor and sensory pathways preserved. To evaluate the degree of impairment, the American Spinal Injury Association (ASIA) elaborated an international classification; the Standards for Neurological Classification of Spinal Cord Injury. For this classification it is used the ASIA Impairment Scale (AIS), which indicates the neurological level (the last unaffected segment) and if the lesion is complete or not. This scale does not assess pain and the bowel, bladder and sexual functions (Kirshblum *et al.*, 2011).

AIS A (Complete). No sensory or motor function is preserved in the sacral segments S4-S5.

AIS B (Sensory incomplete). Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-S5. Besides, no motor function is preserved more than three levels below the motor level on either side of the body.

AIS C (Motor incomplete). Motor function is preserved below the neurological level, where more than half of key muscle functions have a muscle grade less than 3 (muscle contraction or even active movement with gravity eliminated).

AIS D (Motor incomplete). Motor function is preserved below the neurological level, where at least half of key muscle functions have a muscle grade > 3 (active movement with full range of motion against gravity, or more).

AIS E (Normal). Although patient had prior deficits, sensation and motor function are normal in all segments.

The spinal cord normally goes into what is called spinal shock after it has been damaged, a transitory state in which all spinal functions and reflexes are abolished. As a consequence, the true extent of many incomplete injuries isn't fully known until 6-8 weeks post injury. Someone who is completely paralyzed at the time of injury may get a partial recovery after spinal shock has subsided.

Pathophysiology of the SCI

At the pathophysiological level, the events following a traumatic SCI involve two phases. First, there is the **primary injury**, characterized by the mechanical trauma and the subsequent disruption of axons, blood vessels, membranes and myelin. This leads to hemorrhage, axonal and neuronal death, mainly by necrosis, and to an important edema (Figure 3). This uncontrollable and unpredictable injury is typically focal and usually implies the fracture of bones and disk displacement within the spinal canal, that compresses and contusions the spinal cord. Little can be done to reduce these events.

From hours to days after the initial trauma a range of secondary cellular and molecular events occur, constituting the **secondary injury**. This phase is characterized by multiple mechanisms acting simultaneously, such as disruption of vasculature and ischemia, edema, excitotoxicity, ionic imbalance, free radical production, inflammation, necrosis and apoptosis, up-regulation of an array of inhibitory molecules and formation of a dense glial scar around the injury site (Rowland *et al.*, 2008; Silver & Miller, 2004; Yiu & He, 2006) (Figure 3). In this phase, a centripetal and rostro-caudal necrotic wave spread for up to two vertebral levels both above and below the initial lesion, in an irreversible process by 8 hours after the injury (Profyris *et al.*, 2004). This makes the secondary injury the major cause of tissue damage and functional deficits. Moreover, alterations produced in the primary afferent pathways frequently result in the induction of neuropathic pain, which greatly augments patient suffering (Ondarza *et al.*, 2003). Unfortunately, primary injury cannot be avoided, but secondary injury presents possibilities of modulation, being more amenable to therapeutic interventions.

Vascular dysfunction and ischemia

Immediately after injury to the spinal cord, vasospasm of the superficial vessels and intraparenchymal hemorrhage is produced. Hemorrhage is initially localized in the highly vascularized and most vulnerable central gray matter, and this damage impairs tissue perfusion. Disruption of blood-spinal cord barrier results in vasogenic edema, and vasoactive factors are released to counteract it, including leukotrienes, thromboxanes, platelet aggregation factors, serotonin and endogenous opioids. This situation leads to ischemia by hypoperfusion, hypoxia and hypoglycemia (Tator & Fehlings, 1991; Mautes *et al.*, 2000). Moreover, systemic responses resulting from the loss of autoregulation, as posttraumatic hypotension, bradycardia, and decreased cardiac output, exacerbate the ischemic damage (Guha & Tator, 1988). Additionally, intravascular thrombosis may also contribute to this post-traumatic ischemia (Dumont *et al.*, 2001). Ischemia propagates necrosis in the tissue, which initiates signaling cascades that will eventually expand the area of tissue damage (Armand, 1982; Nelson *et al.*, 1977). Although this situation is transitory, the reperfusion implies the production of reactive oxygen species (ROS), contributing also to secondary lesion (Chiu *et al.*, 2010; Basu *et al.*, 2001). On the other hand, hypoxia increases the anabolic metabolism, leading to the production of acidic products such as lactic acid. The acidification of the environment would alter ATP production, affecting ion pumps and ion homeostasis, stimulate pathologic ROS formation, and inhibit astrocytic glutamate uptake, contributing to excitatory neuronal damage (Pérez *et al.*, 2012; Chu & Xiong, 2013). Moreover, recent findings state that acidosis can induce neuronal injury by activating a family of acid-sensing ion channels (Mazaira *et al.*, 1998; Chu & Xiong, 2013).

Excitotoxicity

Disruption of cell membranes results in massive release of glutamate into the extracellular space, together with a global alteration of ion equilibrium. Increased concentrations of glutamate provoke

persistent neuronal depolarization, which will lead to excitotoxic cell death (Mazaira *et al.*, 1998; Park *et al.*, 2004). Depolarization is initiated primarily by activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, followed by the activation of sodium voltage-dependent channels. This results in sodium influx and further depolarization (Kirshblum *et al.*, 2011; Doble, 1999). Then, passive influx of chloride occurs in order to maintain ionic equilibrium (Rowland *et al.*, 2008; Rothman, 1985; Silver & Miller, 2004; Yiu & He, 2006), and all this ion imbalance induces changes in the osmotic gradient, causing the entry of water. Finally, cellular lysis release cell contents to the extracellular medium (Profyris *et al.*, 2004;

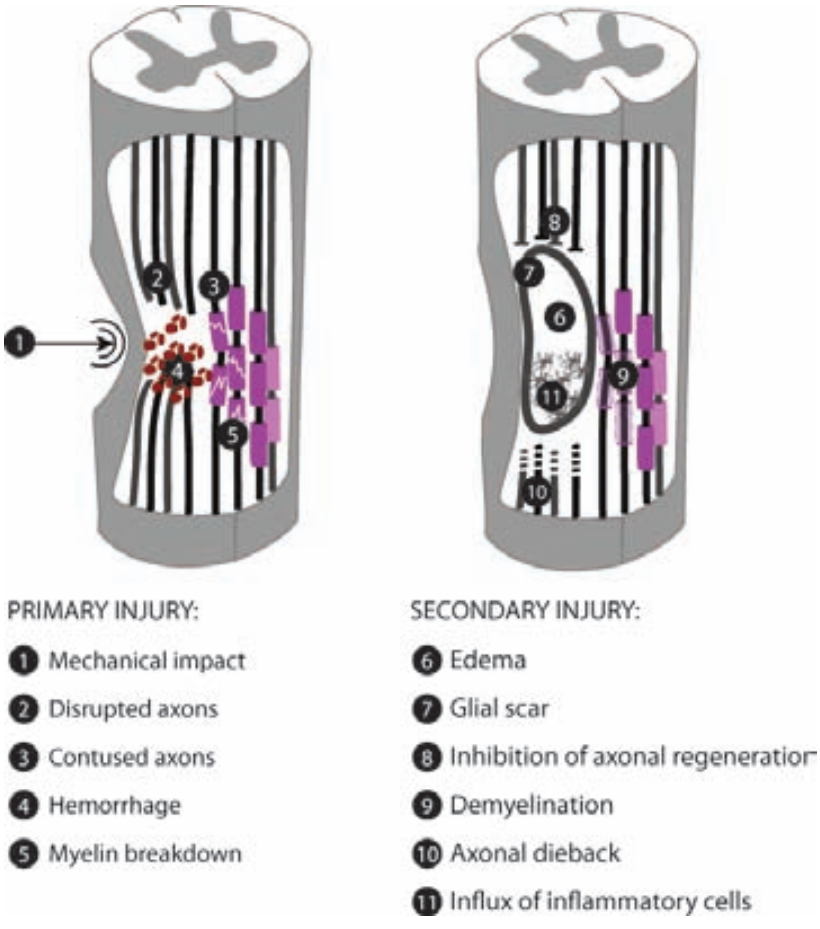

Figure 3. Pathophysiology of the spinal cord injury

Doble, 1999). Moreover, N-methyl-D-aspartate (NMDA) receptors are also activated by the persistent presence of glutamate, leading to an excess of the calcium influx, which can stimulate several intracellular mechanisms that become detrimental for cell survival (Ondarza *et al.*, 2003; Doble, 1999). Some of these mechanisms include activation of nucleases that fragment DNA, activation of cytosolic proteases, such as calpain, which will attack the cytoskeleton and other organelles, or activation of cytosolic kinases and lipases, as protein kinase C and phospholipase A2 (PLA_2), which will disrupt cell function and attack cell and organelles membranes (Tator & Fehlings, 1991; Doble, 1999; Mautes *et al.*, 2000).

Oxidative stress

There are a number of potential sources for the formation of oxygen radicals after SCI. As mentioned above, re-exposure of endothelial cells to oxygen during reperfusion leads to an enzymatic reaction that gives rise to ROS formation (Guha & Tator, 1988; Basu *et al.*, 2001). On the other hand, activation of glutamate leads to an increase in intracellular calcium, which activates calcium-dependent phospholipases. Phospholipid hydrolysis leads to the formation of arachidonic acid and its subsequent metabolism in the cyclooxygenase pathway, producing prostaglandins and ROS. Another source of ROS are neutrophils and macrophages that infiltrate into the lesion, which induces oxidative bursts resulting in the production of ROS. In normal conditions, this mechanism has a powerful antibacterial effect (Dumont *et al.*, 2001; Carlson *et al.*, 1998). Recent studies also show that reactive iron is also a potent catalyst of ROS formation (Rathore *et al.*, 2008). All these highly oxidizing compounds can induce damage to cells by modifying their lipids, proteins and DNA. Their action on lipid peroxidation contributes to cell membrane disruption, promoting cell death and generation of more ROS in a positive feedback that can expand the secondary damage (Profyris *et al.*, 2004). They can modulate the action of several proteins and enzymes and oxidize amino acid side chains, causing fragmentation of proteins (Profyris *et al.*, 2004). ROS

can also react with the nucleobase thymine producing single strand breaks in the DNA (Profyris *et al.*, 2004). Moreover, ROS can enhance excitotoxicity by impairing glutamate uptake by astrocytes (Rao *et al.*, 2003).

Inflammation

Inflammation is a basic pathological process produced in response to the breaching of a tissue's integrity, and is strictly necessary for wound healing. However, peculiarities of CNS tissue contribute to its characteristics of inflammation, which follows a distinct course compared with other organs. The presence of a very selective barrier, the blood-brain barrier, makes changes in vascular permeability more dramatic because of the normally low permeability. Moreover, the CNS has no lymphatic vessels, as extra fluid drains into the cerebrospinal fluid. The CNS also contains a high density of endogenous immunological cells known as microglia, which participate importantly in the inflammatory response, but lacks other inflammatory cells as mast cells and dendritic cells (Hausmann, 2003). Small extracellular space also contributes to the particular inflammatory response. In contrast to other tissues, CNS inflammation becomes chronic after injury, contributing importantly to secondary damage, impaired regeneration and functional deficits. Nevertheless, this inflammatory response is not only detrimental, as it also contribute to repair.

SCI inflammation involves both cellular and humoral components. Immediately after the initial necrosis and blood-spinal cord barrier disruption, microglial cells activate and release pro-inflammatory cytokines, such as IL-1β and TNFα, that can promote the influx of polymorphonuclear cells and haematogenous macrophages from the circulation (David & Kroner, 2011). These cytokines, together with a number of other vasoactive substances released by glia and leukocytes (ROS, kinins, histamines, nitric oxide, elastase, etc.), may enhance vascular permeability (Donnelly & Popovich, 2008). Moreover, the damaged endothelial cells also secrete proinflammatory cytokines, such as IL-1α and β, TNFα and IFNγ, and express adhesion molecules, which

will further mediate the recruitment of immune cells. Neutrophils rapidly infiltrate into the tissue, reaching a peak at about 24 hours (Donnelly & Popovich, 2008). There, neutrophils produce ROS, eicosanoids and proteases that can cause neuronal and glial toxicity. A number of studies have reported improvement in functional outcome and tissue protection after depleting or neutralizing neutrophils (Taoka *et al.*, 1997; Gris, 2004; Lee *et al.*, 2011). However, they also support recovery through their ability to phagocytose cellular debris. In fact, a recent study show that depleting neutrophils more selectively, by using a monoclonal antibody against neutrophil marker Gr-1, worsens tissue damage and functional recovery (Stirling *et al.*, 2009). During this first hour after SCI and for the following 7 days, resident microglia proliferate and shift to a more phagocytic phenotype. Meanwhile, macrophages from peripheral circulation enter the injured cord within 24 hours and reach a peak by day 3-7 (Donnelly & Popovich, 2008). Both microglia and macrophages populations become fully activated and result indistinguishable in their morphology and antigenic phenotype. Microglia and macrophages also produce inflammatory mediators such as cytokines, interleukins and prostaglandins that cause cell death, demyelination and the formation of ROS. Moreover, activated microglia and macrophages are also thought to increase levels of extracellular glutamate, contributing to excitotoxicity (Piani *et al.*, 1991). Macrophages can be detected within the injured spinal segments until some months after the injury.

Together with microglia and peripheral inflammatory cells, resident glial cells also play an important role after the SCI. Astrocytes are the most abundant cells in the CNS and play an essential role in maintaining the homeostasis in the nervous tissue. A few hours after the SCI they become activated, and last in this state for months or even years. Astrocytes also secrete pro-inflammatory cytokines, contributing to the persistence of this inflammatory response. But they are also very important for wound healing, as they are the main responsible of filling the empty spaces caused by cell death. Once activated, they form the glial scar, which on one hand may inhibit axonal growth due to the expression of inhibitory molecules such

as chondroitin sulfate but, on the other hand, it will limit the extent of tissue damage (Rolls *et al.*, 2009). Astrocytes also act as buffers of glutamate, in order to reduce their increased concentrations, to which oligodendrocytes are especially sensitive (Matute *et al.*, 2006). Similarly, macrophages also exert beneficial effects during the inflammatory response that occurs after SCI. Macrophages are very effective phagocytes that clean rests of dead cells and myelin debris. They are also known to produce some neuroprotective molecules, as TGF-β1, that acts as immunosuppressant, promotes axonal growth and limits oligodendrocyte cytotoxicity (McTigue *et al.*, 2000; Merrill & Zimmerman, 1991). Activated macrophages also synthesize neurotrophic factors such as CNTF, NGF, BDNF, GDNF and NT-3. A very new line of study suggests that distinct macrophage activation is responsible for the differential beneficial or detrimental dual properties of these cells. This is referred as macrophage polarization, in which M1 macrophages, activated by IFNγ, induces them to produce pro-inflammatory cytokines, while IL-4 influences macrophages by inhibiting production of pro-inflammatory cytokines (David & Kroner, 2011). Although the inflammatory response is necessary to heal injured tissue, it is also one of the most detrimental features of the SCI, and a lot of efforts are being done to modulate its evolution in order to limit secondary damage and functional deficits.

Pharmacological approaches for the treatment of the SCI

Unlike the peripheral nervous system (PNS), the CNS of adult mammals has limited abilities for spontaneous self-repair, making damage resulting from primary injury irreparable without regenerative or replacement therapies. This is due to the poor intrinsic capacity of central axons to regenerate (Goldberg, 2002), the insufficient neurogenesis to replace dead neurons (Yang, 2006; Vessal *et al.*, 2007), and the negative environment induced by the insufficient expression of growth factors and the presence of inhibitory molecules at the injury site (Fawcett, 1997). However, damage from secondary injury is likely more ease to be prevented or reduced. Several pharmacological compounds targeting detrimental processes of

secondary injury have been clinically proven during the last two decades (Baptiste & Fehlings, 2006). Some of them are methylprednidolone, with an anti-inflammatory effect, or riluzole, a Na^+ channel blocker, which would minimize loss of ionic homeostasis due to membrane disruption (Xu *et al.*, 1992; Schwartz & Fehlings, 2001). NMDA and non-NMDA receptor antagonists improve tissue sparing and prevent loss of neurons and glia, targeting excitotoxicity (Baptiste & Fehlings, 2006). Minocycline, a synthetic tetracycline derivate antibiotic, also attenuates secondary injury by counteracting excitotoxicity and reducing apoptosis and inflammation (Tikka & Koistinaho, 2001; Wells *et al.*, 2003; Maier *et al.*, 2007). Some nonsteroidal anti-inflammatory drugs, as ibuprofen and indomethacin, have been shown to stimulate axonal growth *in vitro* and promote axonal sprouting after SCI by Rho inhibition, yet they have still not been clinically proven (Fu *et al.*, 2007). Rho is a small GTPase protein that mediate many of the growth-inhibitory effects in the mature CNS (Yiu & He, 2006). Cethrin (BA-210), another Rho inhibitor, has been tested in clinical trials in patients with SCI (Baptiste & Fehlings, 2006). Also targeting axonal growth inhibition, another clinically proven pharmacological treatment is anti-Nogo monoclonal antibodies, which have been shown to allow axonal regeneration and functional recovery in experimental models of SCI (GrandPré *et al.*, 2000; Bregman *et al.*, 1995).

Although all these drugs have been demonstrated to promote beneficial effects in experimental models of SCI, at present there are no pharmacological strategies of proven benefit in humans. Methylprednisolone has been the first widely used drug therapy for the treatment of SCI. This practice is based on the National Acute Spinal Cord Injury Studies (NASCIS) I and II, in which high dose methylprednisolone has been shown to induce functional outcomes if given within 6 hours of injury. However, the use of methylprednisolone in SCI has been hardly criticized, due to doubtful correctly analyzed data and important side effects (Hurlbert, 2000; Hurlbert, 2006). Patients in NASCIS III had higher rates of severe sepsis and pneumonia, as well as other complications found in other studies, such as higher rates of respiratory and urinary tract infections, gastrointestinal

complications, wound site infection and needs of longer intensive care and hospitalization (Pandya *et al.*, 2010). Because of this, it has been suggested that the application of high dose methylprednisolone after acute SCI should be abandoned in clinical practice (Hurlbert, 2006).

But despite these discouraging results, some promising preclinical results on protective effects of minocycline have led to the initiation of a clinical trial at University of Calgary (Canada). This trial was conducted between June 2004 and August 2008, and consisted in a randomized, placebo controlled and double-blinded pilot trial of 52 patients, in which intravenous minocycline was given within 12 hours of injury, and twice a day for the next 7 days (clinicaltrials.gov number: NCT00559494). The results favor minocycline, compared to placebo, in patients with cervical SCI in 14 points for ASIA motor recovery over 12 months (Casha *et al.*, 2012). Although these functional outcomes lacked statistical significance, this study is suggestive of improvement in patients receiving minocycline, and warrant further investigation in a multi-centre phase III future clinical trial.

Lysophosphatidic Acid

Lysophosphatidic acid (LPA) is a member of the glycerol phospholipid subfamily. It was originally described as a key intermediate in *de novo* lipid synthesis, until 1990, when it was identified also as an important intercellular phospholipid messenger. LPA is a pleiotropic molecule, present in low concentrations in all mammalian cells and tissues, that regulates a wide range of biological activities including cellular proliferation, cell growth, prevention of apoptosis, cell migration and morphology, cytokine and chemokine secretion, platelet aggregation, smooth muscle contraction, and neurite retraction. It has also been shown to play a major role in development, inflammation and neuropathic pain.

LPA synthesis

LPA, rather than a single molecular entity, is an array of structural analogs with a single functional glycerol alcohol phosphate, esterified to a variety of fatty acyl hydrocarbons of varied lengths and degrees of saturation. LPA is found in various body fluids, but it is highly present in plasma, where the most abundant forms are the 16:0, 18:2 and 18:1. The 18:1 form is the most commonly used laboratory reagent for activation of LPA receptors, and is the one used in the present thesis.

LPA is produced both intracellularly and extracellularly from membrane phospholipids. Intracellular LPA is thought to be fundamentally structural, aside from an intermediate for the lipid synthesis, but other synthesis pathways are responsible for the synthesis of the bioactive extracellular LPA pool. Thus, there are two major pathways for LPA production. In the first, mainly involved in cellular LPA production, membrane-derived phospholipids (PLs) are hydrolyzed by phospholipase D (PLD) to generate phosphatidic acid (PA), which is deacetilated by PLA_1 or PLA_2 to create LPA. In the

second, mainly involved in extracellular LPA production in bodily fluids, such as plasma, lysophospholipids (LPLs) generated by PLA_1 or PLA_2 are subsequently converted to LPA by autotaxin (ATX), an enzyme with lysoPLD activity (Aoki, 2008). Additional LPA-producing pathways also exist, like generation of PA by diacylglycerol kinase (DGK), and the subsequent transformation to LPA by PLA_1 or PLA_2, or acylation of glycerol-3-phosphate (G3P) by glycerophosphate acyltransferase (GPAT) (Figure 4).

Platelets constitute the main source of LPA production in plasma, which after being activated, release the LPLs used by ATX as substrate for LPA synthesis. However, several cellular types have been documented to produce LPA, including fibroblasts, neurons, astrocytes, erythrocytes, adipocytes and cancerous cells.

LPA receptors

LPA signals through defined G protein-coupled receptors (GPCRs) in many biological processes involving almost all vertebrate systems. There is seven current recognized LPA receptors, which can be classified into two subgroups: the **endothelial differentiation gene (Edg) family** and the **non-Edg family** (Figure 5). The first comprises LPA_1/EDG2, LPA_2/EDG4, LPA_3/EDG7 and the GPCR for sphingosine 1-phosphate (S1P). LPA_{1-3}, also known as the classic LPA receptors, share about 50-57% identities at the amino acid level. By using knockout mice, several important roles have been revealed for Edg family LPA receptors in the nervous system, cancer, cardiovascular function, reproduction and other processes noted below. The non-Edg family includes LPA_4/p2y9/GPR23, LPA_5/GPR92/93 and LPA_6/p2y5, phylogenetically related to P2Y receptors family. These LPA receptors were later identified and are associated to distinct functions of LPA, including vascular development, platelet activation and hair growth. Most LPA receptors are expressed in the nervous system, especially during development (Ohuchi *et al.*, 2008).

In an extracellular signaling context, the term LPA generally refers to 1-acyl-2-hydroxy-*sn*-glycero-3-phosphate, but distinct chemical forms

exist, such as 1-alkyl- or 2-acyl-LPA. These different forms of LPA, together with the distinct degree of saturation of the fatty acid, may have a differential affinity for each LPA receptor.

Below, a more detailed description of each LPA receptor is presented:

Lysophosphatidic acid receptor 1 (LPA_1) was the first receptor identified for any lysophospholipid. It is highly homologous to LPA_{2-3}, sharing a 50-60% of amino acid sequence identity. LPA_1 couples with three types of G_α proteins: $G_{12/13}$, $G_{q/11}$ and $G_{i/o}$, initiating signaling cascades through downstream molecules such as mitogen-activated protein kinase (MAPK), phospholipase C (PLC), Akt and Rho (Figure 5). Cellular responses induced by LPA_1 activation include cell proliferation and survival, cell migration and cytoskeletal changes, Ca^{2+} mobilization and adenylyl cyclase inhibition (Yung *et al*., 2014).

LPA_1 expression is widely distributed in both adult mice and humans, including in the brain, where it is abundantly expressed, uterus, testis, lung, small intestine, heart, stomach, kidney, spleen, thymus, placenta and skeletal muscle (Chun *et al*., 2013). LPA_1 has been reported to be involved in several biological functions in the nervous system, such as neurite retraction, growth cone collapse and migration of neuroprogenitor cells *in vitro*. In addition, LPA_1 signaling has been suggested to regulate astrocyte proliferation, Schwann cell survival, and oligodendrocyte maturation and myelination during development (Chun *et al*., 2013). A direct relationship between LPA_1 signaling and neuropathic pain and demyelination has also been found after peripheral nerve injury (Ueda *et al*., 2013). Besides to the nervous system, LPA_1 has been involved in other processes and diseases like differentiation and proliferation of adipose tissue and pulmonary and renal fibrosis (Chun *et al*., 2013). LPA receptors are expressed on most immune cells and organs, modulating inflammation, where LPA_1 plays a role (Choi *et al*., 2010).

Through the use of *Lpar1*$^{-/-}$ mice, it is known that this receptor is also very important during neurodevelopment. These mice present 50%

perinatal lethality, reduced body size and craniofacial dysmorphism. During colony expansion of the *Lpar1*$^{-/-}$ line, a variant arose spontaneously in a laboratory in Málaga, Spain (Estivill-Torrús *et al.*, 2008). This new mouse line, named maLPA$_1$, exhibits more severe developmental brain defects than the original line, yet has very few perinatal lethality (Estivill-Torrús *et al.*, 2008). This mouse line has been used in this thesis to study the role of LPA$_1$ on the pathophysiology of SCI.

LPA$_2$ shares 60% similarities to LPA$_1$ and couples to the same G$_\alpha$ proteins: G$_{12/13}$, G$_{q/11}$ and G$_{i/o}$ (Figure 5). Its expression is more restricted in adult mice and humans, compared to LPA$_1$, being expressed in testis and leukocytes. Lower levels of LPA$_2$ are also found in prostate, spleen, thymus and pancreas. *Lpar2* expression has been found in kidney, uterus and testis, and less abundantly in lung, stomach, spleen, thymus, heart and brain (Chun *et al.*, 2013). Similar to LPA$_1$, LPA$_2$ has been involved in nervous system development and neuropathic pain. Along with LPA$_3$, LPA$_2$ is also involved in cancer and, together with

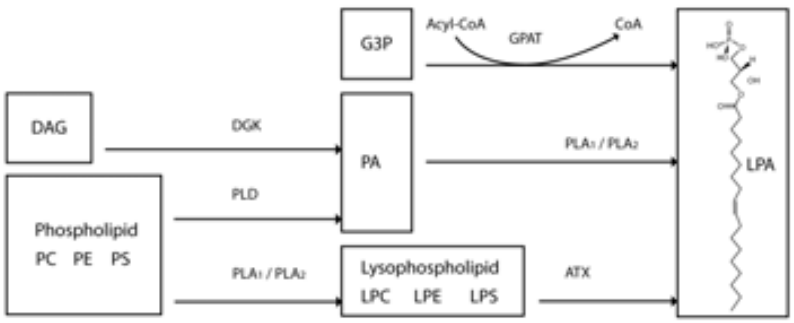

Figure 4. LPA Synthesis pathways. G3P, glycerol-3-phosphate; DAG, diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; GPAT, glycerophosphate acyltransferase; DGK, diacylglycerol kinase; PLA, phospholipase A; PLD, phopholipase D; ATX, autotaxin.

LPA_1, plays a role in the inflammatory response (Chun *et al.*, 2013).

Contrary to *Lpar1*$^{-/-}$, *Lpar2*$^{-/-}$ mice are phenotypically normal, with normal prenatal and postnatal viability (Chun *et al.*, 2013). This mouse line has been used in this thesis for the study the contribution of LPA_2 in the pathophysiology of SCI.

LPA$_3$ is 54% identical to LPA_1 and 49% identical to LPA_2 in amino acid sequence. It can couple to $G_{i/0}$, $G_{q/11}$ (Figure 5) and it has reported to prefer 2-acyl-LPA containing unsaturated fatty acids (Yung *et al.*, 2014). LPA_3 is expressed in human heart, testis, prostate, pancreas, lung, ovary and brain, and in mouse testis, kidney, lung, small intestine, heart, stomach, spleen, brain and thymus (Chun *et al.*, 2013). LPA_3 has been involved in cancer and inflammation, but despite its expression in the CNS, there are no reported neural deficits in *Lpa3*$^{-/-}$ mice (Yung *et al.*, 2014). *Lpar3*$^{-/-}$ mice are viable and normal, but as LPA_3 has an important function during embryo implantation this leads to a very reduced litter size (Chun *et al.*, 2013). This mouse line has been used in this thesis for the study of LPA_3 role on the pathophysiology of SCI.

LPA$_4$ was the first identified non-Edg LPA receptor. Previously known as an orphan GPCR named p2y9 for its similarity to P2Y purinergic receptors, it shares only 20-24% sequence homology to LPA_{1-3}. It couples to several G_α-proteins, including G_s, G_i, G_q and $G_{12/13}$ (Figure 5), through which modulates several cellular activities such as neurite retraction and inhibition of cell migration. LPA_4 shows an important expression during development, and it has been suggested an important role in brain and circulatory system development (Yanagida & Ishii, 2011). It has also been shown to be involved in osteogenesis regulation.

LPA$_5$ shares 35% homology with LPA_4, but is still more different to classic LPA receptors. It couples to $G_{12/13}$ and G_q (Figure 5), and has been involved in brain development, neurite retraction, intestinal water absorption and platelet activation (Ishii *et al.*, 2009). Other ligands different than LPA has been identified *in vitro* for LPA_5, such as peptone and farnesyl pyrophosphate, indicating a lack of specificity

to LPA for this receptor(Yanagida & Ishii, 2011).

LPA$_6$ is the most recently identified LPA receptor (Yanagida *et al.*, 2009). It shares the highest sequence homology with LPA_4 among all GPCRs, fact that helped in its identification. It couples to G_{13} (Figure 5) and so far it has been related to human hair loss (Pasternack *et al.*, 2008).

In the present thesis we focus our work to assess the contribution of the Edg-family LPA receptors (LPA_{1-3}) in the pathophysiology of SCI.

LPA in the nervous system

As previously mentioned, LPA is an intercellular signaling lipid that produces or regulates many physiological functions. Despite LPA evokes a variety of cellular responses in most tissue, little is known about the role of LPA in the nervous system, and most of its known functions arise from *in vitro* studies. LPA receptors are differentially expressed in the cells of the nervous system.

Here are summarized some of the cellular effects that LPA exert on neural and glial cells.

Neurons

LPA has been reported to induce cytoskeletal changes in neuronal cell lines, mainly dependent on the Rho/ROCK pathway. These changes lead to neurite retraction, cell rounding and growth cone collapse (Tigyi *et al.*, 1996). Other authors also found LPA-induced growth cone collapse and neurite retraction in primary cultures of different neuron populations (sympathetic ganglion cells, dorsal root ganglion (DRG) neurons, retinal neurons and cortical neurons) from embryonic animals (Saito, 1997; Fukushima *et al.*, 2002). However, these effects are not only found in young neurons, as LPA has also been found to induce neurite retraction in primary cultured DRG neurons from adult mice (Bouquet *et al.*, 2007). LPA also has effects on inhibiting neuronal migration in cortical explants of embryonic mice (Fukushima *et al.*, 2002). Moreover, LPA has the ability to modulate neuronal activity *in*

vitro, by inducing release of neurotransmitters as noradrenalin and dopamine (Nishikawa *et al.*, 1989; Shiono *et al.*, 1993). Further, LPA elicits neuronal activity also *in vivo*, in adult rat spinal cord neurons and in DRG neurons (Elmes *et al.*, 2004). Finally, LPA promotes neuronal death both by apoptosis and necrosis, associated with mitochondrial alterations and the generation of ROS (Holtsberg *et al.*, 1998; Steiner *et al.*, 2000).

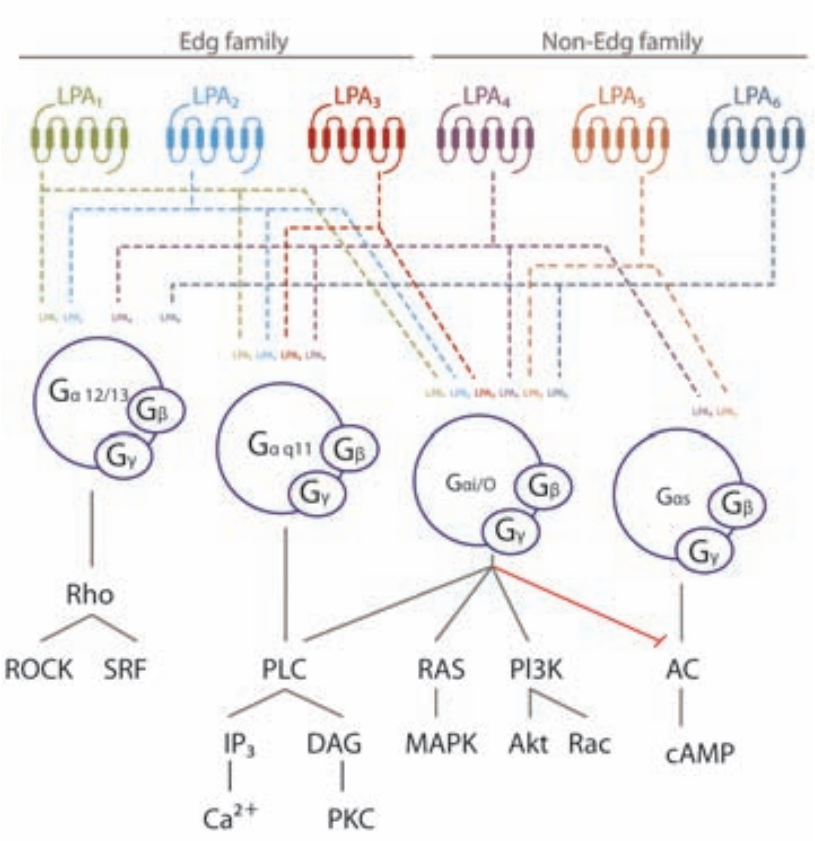

Figure 5. LPA receptors and signaling pathways.

Oligodendrocytes

LPA effect on oligodendrocytes appears to depend on the degree of maturation of these cells. In oligodendrocyte progenitor cells (OPCs) and immature differentiating oligodendrocytes LPA induces process

retraction and inhibits oligodendrocyte maturation (Dawson *et al.*, 2004). However, a more recent study found that LPA may play a role in regulating the later stages of oligodendrocyte maturation, as in these cells LPA induces an increase in the extension of oligodendrocyte's processes and an up-regulation of the myelin basic protein (MBP) (Nogaroli *et al.*, 2008). In primary cultured mature oligodendrocytes, LPA was observed to not influence survival, maturation, cytoskeleton organization or myelination (Stankoff *et al.*, 2002). Although in a rat immortalized oligodendrocyte cell line, LPA has been found to promote survival upon serum withdrawal and myelinogenesis (Matsushita *et al.*, 2005).

Astrocytes

LPA induces morphological changes in astrocytes through Rho/ROCK pathway, consisting in the reversion of astrocyte stellation induced by cAMP, a phenomenon that is associated with astrocyte activation because of its morphological similarities *in vivo* (Ramakers & Moolenaar, 1998). LPA can also stimulate astrogliosis *in vivo* and proliferation *in vitro* (Sorensen *et al.*, 2003). Astrocytes are known to regulate extracellular concentrations of glutamate and to supply neural energy demand. LPA has been found to decrease both glutamate and glucose uptake and increase lipid peroxidation in astrocytes (Keller *et al.*, 1996; Shano *et al.*, 2008). Such effect may have consequences on neuron health, as it exacerbates neurotoxicity and reduces energy supply. LPA has also been shown to induce astrocyte proliferation through LPA_1 signaling (Shano *et al.*, 2008). Moreover, LPA can exert indirect effects on neurons through stimulation on astrocytes. *In vitro* pretreated astrocytes stimulate neuronal differentiation and neurite outgrowth of cerebral cortical progenitors. This neuritogenesis may be mediated by an increase in the production of laminin, an extracellular matrix glycoprotein that would enhance astrocyte permissiveness to neurite outgrowth (E Spohr *et al.*, 2011). LPA stimulate the expression of various cytokine genes, including IL-1β, IL-3 and IL-6, suggesting a pro-inflammatory role that would influence wound healing after CNS trauma

(Tabuchi *et al.*, 2000). However, LPA stimulates the synthesis and secretion of NGF, suggesting also a neuroprotective role (Furukawa *et al.*, 2007; Tabuchi *et al.*, 2000).

Microglia

LPA receptors expression on microglial cells seems to be very controversial, as several microglial cells types have been studied *in vitro*. In contrast to our results, LPA_1 has not found to be expressed in microglial cells *in vivo* after SCI (Goldshmit *et al.*, 2010). Regarding cellular effects of LPA, it has been found to induce proliferation of mouse but not rat primarily cultured microglial cells (Möller *et al.*, 2001). However, in rat microglial cells LPA have been found to promote ATP release via LPA_3 signaling, and BDNF expression (Fujita *et al.*, 2008). In the adult mouse spinal cord, LPA is synthetized by microglia in their early phase of activation and would be responsible for neuropathic pain (Ma *et al.*, 2010).

Together, these *in vitro* and *in vivo* observations suggest that LPA contribute to inflammation, and other undesirable effects, such as axonal retraction, demyelination and neuronal cell death, in CNS pathologies, which would lead to functional and cognitive loss as well as the development of neuropathic pain. Despite the potential role for LPA in triggering these detrimental responses, no studies have so far addressed the role of LPA in CNS pathologies. Recently, a work revealed that the administration of B3, an antibody that binds to LPA and other Lysophospholipids preventing them from interacting with their receptors, promotes functional recovery after spinal cord hemisection in mice (Goldshmit *et al.*, 2012). Although the B3 antibody does show a selective binding for LPA, and despite the hemisection model is not a clinical relevant model of SCI, this study may suggest and detrimental role for LPA in SCI.

AIMS

AIMS

The general objective of the present thesis is to study the potential role of the endothelial differentiation gene family LPA receptors (LPA_{1-3}) in the physiopathology of spinal cord contusion injury. The thesis has been divided in three chapters according to the following specific aims:

CHAPTER I: Lysophosphatidic receptor 1 (LPA_1) contributes to the pathophysiology of the spinal cord injury

- To assess the LPA levels in the intact and injured spinal cord parenchyma and the changes in the expression of the enzymes involved in its synthesis.
- To study the effects of intraspinal injection of LPA into the intact spinal cord.
- To assess the constitutive expression of LPA receptors in the spinal cord tissue.
- To evaluate the contribution of LPA_1 to demyelination triggered by intraspinal injection of LPA.
- To assess whether LPA_1 is involved in the physiopathology of spinal cord injury.
- To study the mechanisms underlying oligodendrocyte cell death by LPA_1.

CHAPTER II: Lysophosphatidic acid receptor type 2 (LPA_2) mediates secondary damage following spinal cord injury in mice

- To quantify the changes in the expression of LPA_2 mRNA levels in the spinal cord after contusion injury
- To evaluate the contribution of LPA_2 to LPA-triggered demyelination in the intact spinal cord.
- To assess whether the lack of LPA_2 enhances functional and histological outcomes after spinal cord contusion injury.
- To study the mechanisms involved in LPA_2-triggered oligodendrocyte and neuronal cell death.

CHAPTER III: Lysophosphatidic acid receptor type 3 (LPA_3) does not contribute to secondary damage after spinal cord injury

- To study the changes in LPA_3 expression in the spinal cord tissue after contusion injury.
- To assess whether the lack of LPA_3 exerts enhanced functional and histological outcomes after spinal cord injury.
- To evaluate whether the activation of LPA_3 worsens locomotor skills and secondary tissue damage after spinal cord contusion injury.

METHODOLOGY

Methodology

1.- Spinal cord contusion injury model

Contusion injury to the mouse spinal cord was performed using the Infinite Horizons Impactor device (Precision Scientific Instrumentation, Fairfax Station, VA) (Figure 1). This instrumentation is capable of inflicting consistent contusion injuries to the spinal cord of small rodents, by the application of a controlled impact defined in terms of force (kilo dynes; kdynes). Force is user-selectable between 30 and 300 kdynes, allowing to perform graded magnitudes of injury. Displacement produced to the spinal cord as a consequence of the impact is also registered. To induce the injury, animals were anesthetized with ketamine:xylacine (90:10 mg/kg). The spinal cord was exposed by means of a laminectomy at the desired vertebral level, and the column was rigidly stabilized with the aid of two Adson forceps attached to two articulated support arms. In this study, moderate contusion injuries at the thoracic T11 level have been performed to mice, by applying a force of 50 ± 5 kdynes, and accepting a range of 400-600 μm in spinal cord displacement.

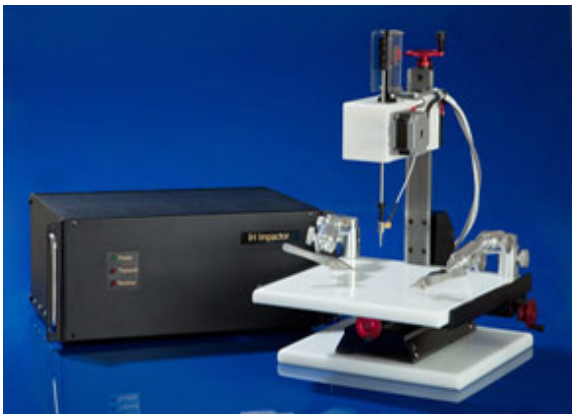

Figure 1. The Infinite Horizons Impactor device.

Injury inflicted by this device is very similar to what is observed in humans, in terms of histological changes (i.e. loss of gray matter, few spared white matter and intact dura mater). The injury leads to functional deficits below the level of the lesion, affecting the hind limbs and the trunk. Spontaneous recovery of some motor function is produced, allowing to detect changes due to potential treatments.

2.- Intraspinal injection of LPA

Mice were anesthetized with ketamine:xylacine (90:10 mg/kg). The spinal cord was exposed by means of a laminectomy at the T11 vertebral level, and the column was rigidly stabilized with the aid of two Adson forceps attached to two articulated support arms. Intraspinal injections were performed using a glass needle (30 µm internal diameter, Eppendorf, Hamburg, Germany) coupled to a 10 ml Hamilton syringe (Hamilton #701, Hamilton Co, Reno, NV, USA). 1 µl of saline, saline containing 5 nmoles of LPA (18:1 LPA; Sigma-Aldrich) or LPA plus a selective LPA_1 antagonist (AM095; 0.22 nmoles) was injected in the dorsal funiculi of the uninjured spinal cord. Injections were made at a perfusion speed of 2 µl/min controlled by an automatic injector (KDS 310 Plus, Kd Scientific, Holliston, MA, USA), and the tip of the needle was maintained inside the cord tissue 3 min after each injection to avoid liquid reflux.

3.- Behavioral scoring

After injury, spontaneous locomotor recovery of mice was assessed by using the Basso Mouse Scale (BMS) (Table 1). BMS is a widely used 9-point scale in which two blinded observers score the animal's motor performance. Animals are allowed to move freely in a circular open field (90 cm diameter x 24 cm wall height) for 5 min, and then the consensus score is taken. When the animal is able to step frequently (5 points), a sub-score of 11-point scale is

0	No ankle movement
1	Slight ankle movement (less than 90°)
2	Extensive ankle movement (more than 90°)
3	Plantar placing of the paw with or without weight support of the body
4	Occasional plantar stepping
5	Frequent (> 50%) or consistent (less than 5 missed steps) plantar stepping, *no* coordination (between fore and hind limbs) **or** Frequent or consistent plantar stepping, *some* coordination and paws *rotated* at initial contact and lift off phases of the step
6	Frequent or consistent plantar stepping, *some* coordination and paws *parallel* (to the body) at initial contact of the step **or** Frequent or consistent plantar stepping, *mostly* coordinated and paws *rotated* at initial contact and lift off phases of the step
7	Frequent or consistent plantar stepping, *mostly* coordinated and paws *parallel* at initial contact and *rotated* at lift off phase of the step **or** Frequent or consistent plantar stepping, *mostly* coordinated, paws *parallel* at initial contact and lift off phases of the step, and *severe* trunk instability (lean or sway of the trunk)
8	Frequent or consistent plantar stepping, *mostly* coordinated, paws *parallel* at initial contact and lift off phases of the step, and *mild* trunk instability **or** Frequent or consistent plantar stepping, *mostly* coordinated, paws *parallel* at initial contact and lift off phases of the step, *normal* trunk stability, and tail *down* or *up & down*
9	Frequent or consistent plantar stepping, *mostly* coordinated, paws *parallel* at initial contact and lift off phases of the step, *normal* trunk stability, and tail *always* up (normal mouse locomotion).

Table 1. Scores for the Basso Mouse Scale (BMS), used in the evaluation of spontaneous locomotor recovery after spinal cord injury in mice.

Plantar stepping (score both paws)	Frequent	0
	Consistent	1
Coordination	None	0
	Some	1
	Most	2
Paw position (score both paws)	Rotated thru out	0
	Parallel and rotated	1
	Parallel thru out	2
Trunk instability	Severe	0
	Mild	1
	Normal	2
Tail	Down	0
	Up & Down	0
	Up	1

Table 2. Basso Mouse Scale (BMS) subscores, used to complement BMS scores. This scale evaluates more fine aspects of locomotion. Maximal score of 11 points.

performed in parallel, in order to assess fine aspects of the locomotion (Table 2). BMS was performed at 1, 3, 5, 7, 10, 14, 21 and 28 days after injury.

DigiGaitTM Imaging System (Mouse Specifics, Inc., Boston, MA) device has also been used to evaluate the maximum speed at which animals were able to run in a forced locomotion situation. The device is provided with a motorized treadmill belt that allows the user to set a constant speed ranging from 0 to 99 cm/s. Animals were placed on the treadmill belt and allowed to explore the compartment for 5 min. Then, speed was gradually increased and stopped at the maximum speed at which animals were able to perform a minimum of 5 seconds.

4.- Electrophysiological tests

Electrophysiological tests were used to quantitatively assess central motor conduction after SCI. After lesion, few white matter is preserved at the epicenter of the lesion, as we can observe by histological analyses. However, motor evoked potentials (MEPs) are effective to directly quantify the functionality of those spinal tracts that are still preserved.

MEP monitoring requires transcranial electric stimulation of the motor cortex to produce a descending response that traverses the spinal cord tracts and peripheral nerves to eventually generate a measurable response in the form of muscle activity. The wave of depolarization activates corticospinal tract, which will descend from the motor cortex to the brainstem, where most fibers (90%) will cross the midline, descending in the contralateral spinal cord (Figure 2).

Conduction test were carried out at 28 days after injury, in order to detect differences between central conduction preservation on control and treated animals. Animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p., Sigma) and placed prone over a warmed flat coil controlled by a hot water circulating pump to maintain body temperature. Compound muscle action potentials (CMAPs) and MEPs from the tibialis anterior and gastrocnemius muscles were registered.

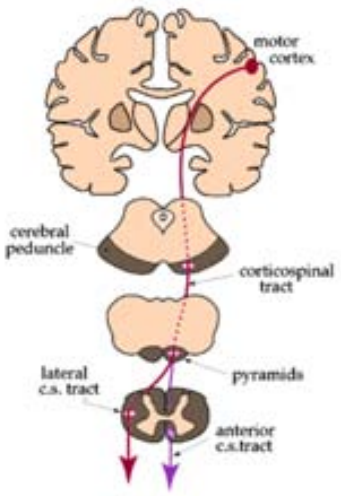

Figure 2. The pyramidal system, constituted by the corticospinal tract, is the basic motor pathway responsible for the voluntary movements of the body. It begins with the large pyramidal neurons of the motor cortex, and travels through the pyramids of the brainstem, where most fibers (90%) will cross over to the opposite side in the pyramidal decussation, and descend through the lateral funiculus of the contralateral spinal cord (lateral corticospinal tract). The remaining fibers (10%) will remain uncrossed and descend into the ipsilateral spinal cord (anterior corticospinal tract). An 8% of these fibers will cross the midline at the level they leave the spinal cord.

In this case, the lesion does not affect peripheral nerve conduction. However, CMAPs were recorded for several reasons: for internal control of peripheral normal conduction, for normalization of MEPs values by individual's own CMAP for the same muscle, and for ensure right position of electrodes before MEPs register.

CMAPs

For motor nerve conduction tests, the sciatic nerve was stimulated percutaneously by means of single electrical pulses of 0.02 ms duration (Grass S88) at supramaximal intensity. Pulses were delivered through a pair of needle electrodes; the cathode inserted deep into the sciatic notch, and the anode near the first, subcutaneously and more rostrally.

CMAPs were recorded from the tibialis anterior and gastrocnemius muscles of the right paw, by means of microneedle electrodes inserted superficially in the muscle. Reference and ground electrodes were inserted at the third toe and the base of the tail, respectively. Recording microneedles were placed under a magnifier lens to ensure reproducibility. Muscle responses were amplified and displayed in a digital oscilloscope (Tektronix 450S). The amplitude and latency of the maximal M and the reflex H waves were recorded (Figure 3).

MEPs

For evaluation of the motor central pathways, MEPs were elicited by transcranial electrical stimulation of the motor cortex by single rectangular pulses of 0.1 ms duration. Needle electrodes were inserted subcutaneously over the skull; the cathode overlaying the sensorimotor cortex and the anode at the nose. Recording electrodes were not moved after positioning for CMAP register. Acceptable MEP responses were polyphase with a consistent latency. Latency and maximum amplitude were taken (Figure 3).

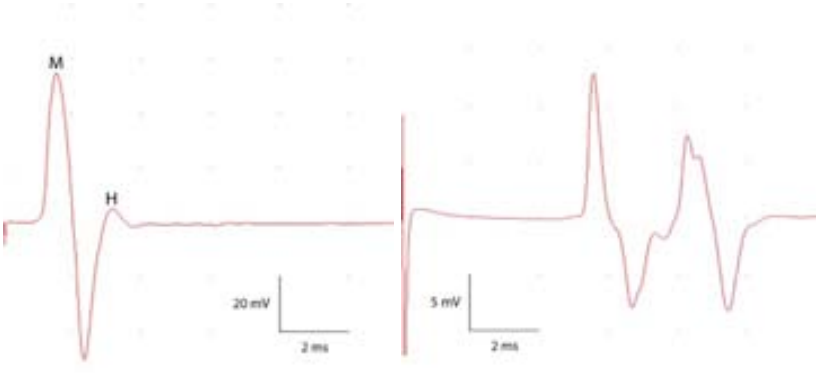

Figure 3. Representative recordings of a compound muscle action potential (CMAP; left) and a motor evoked potential (MEP; right) from tibialis anterior muscle of an intact mouse. CMAP recording present two wave forms: the M wave, pointed by an "M", and the late H reflex response, pointed by an "H".

5.- Histological assessment

Mice were deeply anaesthetized using Dolethal (pentobarbital sodium; Vétoquinol E. V. S. A.) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Seven mm length of spinal cord containing the injection or the lesion site centered was harvested, post-fixed for 1 hour in 4% paraformaldehyde in 0.1 M PB and cryoprotected with 30% sucrose in 0.1 M PB at 4ºC, for a minimum of 48 hours. Spinal cords were fast-frozen at -60ºC in cryoembedding compound (Tissue-Tek® OCT, Sakura) and cut on a cryostat (Leica). Ten series of 10-µm-thick transversal sections were picked up on glass slides, so adjacent sections on the same slide were 100 µm apart.

Luxol Fast Blue (LFB)

Tissue sections were rehydrated in 0.1 M phosphate buffered saline (PBS) and gradually dehydrated in 50%, 70%, 80% and 95% ethanol. Then, sections were placed in a 1 mg/ml LFB solution in 95% ethanol and 0.05% acetic acid overnight at 37ºC. Sections were washed in 95% ethanol and distilled water before placing them into a solution of 0.5 mg/ml Li2CO3 in distilled water for 1 min at room temperature. After washes in distilled water, sections were dehydrated and mounted in DPX mounting media (Sigma).

Regular fluorescent immunohistochemistry

Tissue sections were rehydrated in PBS and blocked with blocking buffer (BB; 5% FBS in 0.3% Triton-PBS(PBST)) for 1 hour at room temperature. Sections were then incubated overnight at 4ºC with primary antibodies diluted in BB. After several washes in PBST, sections were incubated for 1 hour at room temperature with the appropriate Alexa 594- or Alexa 488-conjugated secondary antibodies (Invitrogen) diluted in BB. Finally, sections were washed with PBST, PBS and PB and coverslipped in Mowiol mounting media containing DAPI (1 µg/ml; Sigma).

Toluidine blue

Spinal cord samples were fixed with 2.5% glutaraldehyde in 0.1 M PB overnight at 4ºC. Then, samples were post-fixed with 2% osmium tetroxide for 1 hour at room temperature, gradually dehydrated in 50%, 70% and 97% ethanol, and embedded in Epon812. Thin sections (1 µm) were cut from each block and stained with alkaline toluidine blue. The stained sections were observed under an electron microscope (technique elaborated in collaboration with Samuel David's laboratory).

For histopathological analyses, images of stained sections were acquired with a digital camera attached to the microscope (Olympus) and then analyzed using NIH ImageJ software (NIH, Bethesda, MD).

Determination of intraspinal injection/injury epicenter

Epicenter of intraspinal injection or injury impact was determined for each mouse spinal cord by localizing the tissue section with less tissue sparing along the spinal cord, regarding GFAP-positive staining.

Quantification of demyelination after intraspinal injection

Demyelination was measured by delineating LFB non-stained white matter in the dorsal funiculi after intraspinal injection. Quantifications were done at the epicenter and in sections every 200 µm until 1400 µm at both, rostral and caudal sides of the epicenter (15 sections per mouse).

Quantification of tissue and myelin sparing after SCI

Tissue and myelin sparing were calculated by delineating total GPAP- and LFB-stained areas, respectively, on each spinal cord section. Quantifications were done at the epicenter and in sections every 200 µm until 1400 µm at both, rostral and caudal sides of the epicenter.

Quantification of neuronal survival

Neuronal survival was assessed by manually counting $NeuN^+$ cells in

the ventral horn of spinal cord slices corresponding to the epicenter, and every 200 µm until 1400 µm at both, rostral and caudal sides of the epicenter.

Quantification of tissue affected length

Length of microglia/macrophage activation, astrogliosis and demyelination after intraspinal injection was calculated by localizing the first and the last affected tissue section. The length corresponded to the distance in µm between these two sections.

6.- Animal genotyping

Mice lacking LPA_{1-3} were used in this thesis. maLPA_1 mice, as well as wild type (WT) littermates were gently donated by Guillermo Estivill-Torrús laboratory from Instituto de Investigación Biomédica de Málaga. LPA_2 null mice and LPA_3 were gently provided by Jerold Chun from The Scripps Research Institute (La Jolla, EEUU) and colonies were established at our animal facility. PCR analysis was used to detect the presence of WT and mutated alleles. For tissue sampling, the tip of the tail was cut at the time of weaning the mice. Then, genomic DNA extraction was carried out by using the ArchivePure DNA Purification System (5PRIME). Briefly, tail tissue was digested in 0.1 mg/ml proteinase K

	LPA_2	LPA_3
Forward primer	5´- AGTGTGCTGGTATTGCTGACCA -3´	5´-GAAGAAATCCGCAGCAGCTAA-3´
Reverse primer	5´- CTCTCGGTAGCGGGGATGG -3´	5´-TGACAAGCGCATGGACTTTTTC-3´
Mutation primer	5´- CAGCTGGGGCTCGACTAGAGGAT -3´	5´-AGCGCCTCCCCTACCCGGTAGAAT-3´
PCR conditions	95ºC 95°C 30 sec 56°C 30 sec 36 Cycles 72°C 1 min 72°C 5 min	95°C 30 sec 56°C 30 sec 36 Cycles 72°C 1 min 72°C 5 min
Product sizes	Wild type: 576 bp Mutation: 328 bp	Wild type: 216 bp Mutation: 170 bp

Table 3. Primers sequences, PCR conditions and product sizes used for genotyping wild type and mutated alleles from *lpa2* and *lpa3* genes in mice colonies.

solution (5PRIME) diluted in lysis buffer at 56 ºC overnight. After digestion, proteins were eliminated by precipitation using a protein precipitation buffer and centrifugation 3 min at 15000 g. Supernatants were then mixed with 2-propanol (Panreac) to DNA precipitation. Samples were then centrifuged and pellets washed with ethanol 97%. Finally, DNA pellets were completely dried at room temperature before DNA solubilization by adding 50 µl DNA hydration solution at 65ºC for 1 hour. Allele amplification was performed by PCR reaction, using the Taq DNA Polymerase kit (Invitrogen), and PCR products were analyzed by standard electrophoresis in 1% (LPA_2) and 2% (LPA_3) agarose gels. Primers sequences, PCR conditions and product sizes are detailed in Table 3.

7.- Real-Time PCR

To evaluate the expression level of mRNAs for LPA receptors subtypes (LPA_1, LPA_2, $LPAt_3$, LPA_4 and LPA_5) in the intact mouse spinal cord, quantitative real-time (RT)-PCR was performed. Mice were perfused with sterile saline and 5 mm length of intact spinal cord was harvested. Tissue was homogenized with QIAzol lysis reagent (Qiagen) and RNA extracted using RNeasy Lipid Tissue kit (Qiagen), according to the

	Forward	Reverse
LPA_1	5'-TGTCCTGGCCTATGAGAAGTTCT-3'	5'-TTGTCGCGGTAGGAGTAGATGA-3'
LPA_2	5'-CTCACTGGTCAATGCAGTGGTATAT-3'	5'-GAAGGCGGCGGAAGGT-3'
LPA_3	5'-GGGACGTTCTTCTGCCTCTTTA-3'	5'-GAAAGTGGAACTTCCGGTTTGT-3'
LPA_4	5'-GATGGAGTCGCTGTTTAAGACTGA-3'	5'-TGTTTGATCACTAACTTCCTCTTGGATA-3'
LPA_5	5'-CCGTACATGTTCATCTGGAAGAT-3'	5'-CAGACTAATTTCTCTTCCCACCT-3'
LPA_6	5'-ACTGCTGCTTTGACCCTATTG-3'	5'-AAGGTCTGTAGGTTGTGTTGG-3'
GAPDH	5'-TCAACAGCAACTCCCACTCTTCCA-3'	5'-ACCCTGTTGCTGTAGCCGTATTCA-3'

Table 4. Primer sequences used in Real Time-PCR reaction for quantifying basal mRNA expression of LPA receptor subtypes in the intact spinal cord.

manufacturer's protocol. RNA was treated with DNaseI (Qiagen) to eliminate genomic DNA contamination. 1 µg of obtained RNA was primed with random hexamers (Promega) and reverse transcribed using Omniscript RT kit (Qiagen). RNase inhibitor (Roche) was added (1 U/µl final concentration) to avoid RNA degradation. Quantitative

RT-PCR analysis was performed using a MyiQ Single-Color Real-Time PCR Detection System (BIO RAD). RT-PCR reactions were performed using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) according to the manufacturer's instructions. The expression level of the target mRNA was normalized to the relative ratio of the expression of the GAPDH housekeeping gene. Primer sequences used in RT-PCR reaction are detailed in Table 4.

8.- Mass spectrometry

Mice were perfused with sterile saline and 5 mm length of uninjured or injured spinal cord centered at the impact site was harvested at 6 hours, 1, 3, 7 and 14 dpi. Samples were homogenized in 0.01% w/v butylated hydroxytoluene in PBS. After centrifugation at 15,000 g for 20 min at 4ºC, supernatants were processed for protein concentration determination using the BCA protein assay (Pierce, Rockford, IL, USA).

The volume corresponding to 300 µg of protein was taken of every sample. 200 pmol of 1-O-dodecanoyl-2-dodecanoyl phosphatidic acid was added as internal standard and water up to 100 µl. Samples were acidified with 500 µl of H2O/HCl 19:1 and lysophosphatidic acid species extracted with 500 µl of water-saturated butanol twice. Organic phase was recollected, evaporated until dryness and resuspended in 100 µl CHCl3. 50 µl of this extract was injected in a high-performance liquid chromatograph equipped with a binary pump Hitachi LaChrom Elite L-2130 binary pump and a Hitachi Autosampler L-2200 (Merck) coupled to a Bruker esquire6000 ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The column was a Supelcosil LC-Si 3 µm 150 x 3 mm column (Sigma-Aldrich) protected with a Supelguard LC-Si 20 x 3 mm guard cartridge (Sigma-Aldrich). Mobile phase was a gradient of solvent A (chloroform/methanol/32% ammonium hydroxide 75:24.5:0.5 v/v/v), and solvent B (chloroform/methanol/water/32% ammonium hydroxide 55:39:5.5:0.5 v/v/v/v/). The gradient was started at 100% solvent A; it was decreased linearly to 50% A in 2 min, maintained for 4 min, to 0% A in 6 min, and maintained

there for an additional 18 min. The flow rate was 0.5 ml min^{-1} in the chromatographic step and 0.2 ml min^{-1} entered into the electrospray interface of the mass spectrometer. Nebulizer gas was set to 30 pounds per square inch, dry gas to 8 l/min, and dry temperature to 365°C. LPA species were detected in negative ion mode with the capillary current set at +3500 v as [M-H]- ions.

9.- Primary oligodendrocyte cultures

Primary oligodendrocyte progenitor cells (OPCs) were isolated from mice cortices at 2-4 post-natal days (P2-4). After removal of the meninges, cortical tissue was warmed to 37 ºC and minced using a P1000 pipette tip. Tissue was then dissociated by incubation in papain solution (Table 5) for 20 min at 37ºC. Mixed glial culture media (MGCM; Table 5) was added to stop enzymes activity. After centrifugation at 1200 rpm (300g) for 5 min, cells were plated into T25 tissue culture flasks (one brain per flask) coated with 10 µg/ml poly-D-lysine for 1h at 37ºC, and cultured at 37ºC in an humidified incubator with 5% CO2 supplementation. Three hours after plating, floating cells were discarded by replacing the medium. Two thirds of the MGCM was replaced every 3 days with new MGCM supplemented

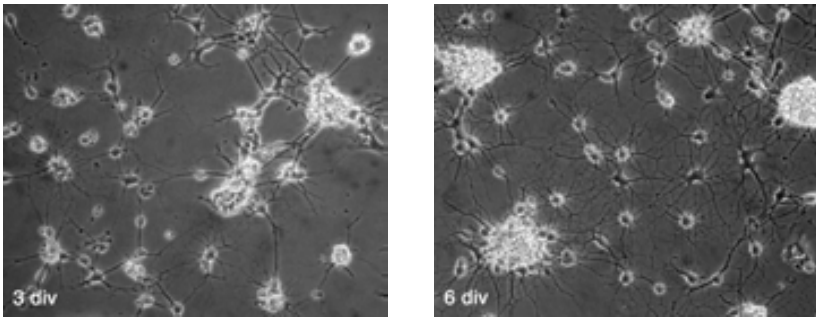

Figure 4. Primary cultured mouse oligodendrocyte progenitor cells (OPCs) after three (left) and six (right) days *in vitro* (div). Note that after three days *in vitro* OPCs have extended only a few processes. At six days *in vitro* cells have extended many processes, denoting a more advanced state of differentiation.

with 5 µg/ml insulin (Seralab). At nine days *in vitro*, flasks were shacked into the incubator at 50 rpm for 45 min, to remove any loosely adherent contaminating cell from the OPC monolayer. Then, MGCM was replaced and OPCs were harvested by shaking again the flasks into the incubator at 220 rpm for 16 hours. Collected cells were first incubated into non-coated 10 cm culture dishes for 30 min to allow non-OPC linage cells to adhere to the base of the plate. Finally, floating cells were plated into 10 µg/ml poly-D-lysine coated 24 well plates with oligodendrocytes medium (OLM; Table 5). OPC maturation in oligodendrocytes was achieved after 5-6 days *in vitro* (Figure 4). For maintenance of the culture half of the medium was changed every 2-3 days.

10.- Primary DRG neuron cultures

Dorsal root ganglia (DRG) neurons were harvested from P4 mice and placed in Gey's salt solution (Sigma) with 6 mg/ml glucose. After cleaning DRGs from connective tissue and rests of roots, they were dissociated in 1 mg/ml collagenase (Sigma), 2.5 mg/ml trypsin (Sigma) and 1 mg/ml DNAse I in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS, Sigma) for 30 min in a water bath at 37ºC, shaking kindly every 10 min. Cells were resuspended in Neurobasal A medium (Gibco) supplemented with 2% B27, 1% P/S, 2mM L-glutamine (Sigma) and 6 mg/ml glucose and plated on 24 wells plates coated with 10 µg/ml poly-D-lysine for 1h at 37ºC, washed, dried and further coated overnight with 2ug/ml laminin (Sigma). After 24 hours in culture, medium was fully changed. Cells were maintained in culture for 4-5 days *in vitro*.

11.- Primary microglial cultures and conditioned medium generation

Primary microglial cells were isolated from P4 mice cortices, and tissue dissociation and cell isolation was performed as in OPC culture. Mixed glial cultures were prepared into T25 tissue culture flasks without any coating. Cells were seeded at a density of 300.000 cells/ml in microglial

medium (MGM; Table 4), and cultured at 37ºC in an humidified incubator with 5% CO2 supplementation. Medium was replaced every 4-5 days and confluency was achieved after 10-12 days *in vitro*. At this point, present medium was removed and kept warm. Mixed cultures were incubated with a mild trypsin solution (Table 5) for 30 min at 37ºC. Mild trypsinization resulted in the detachment of the upper layer of cells, mainly astroglial cells, in one piece, leaving microglial cells perfectly adhered to the plate. MGM was added to stop enzymes activity, and incubated for 5 min in the incubator. Then, medium and floating cells layers were discarded and replaced with the previous medium, containing important factors for stabilization of the culture. Primary microglial cultures were incubated for 24 hours before starting experiments (Figure 5). For maintenance of the culture, if needed, MGM was changed every 4 days.

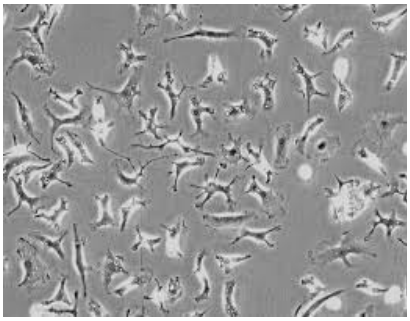

Figure 5. Primary cultured mouse microglial cells.

For conditioned medium generation, microglial primary cultures were exposed to different treatments:

- MGM with 0.025% DMSO (control)
- MGM with 1 μM LPA and 0.025% DMSO
- MGM with 1 μM LPA and 12.5 μM AM095 (dissolved in DMSO)

After 24 hours, medium was replaced by OLM and cultures were incubated for 24 hours more, to allow microglial cells continue secreting the factors promoted by the treatment. After incubation

period, microglial conditioned medium was centrifuged and frozen at -20ºC.

Papain solution – made in MEM medium (Gibco)	1.54 mg/ml papain (Worthington Biochemical)
	400 µg/ml L-cystein (Sigma)
	1 mg/ml DNase I (Roche)
Mixed glial culture medium (MGCM) – made in DMEM medium (Gibco)	10% inactivated fetal bovine serum (FBS, Sigma)
	0.33% penicillin-streptomycin (P/S, Sigma)
	1% Glutamax 100x (Gibco)
Oligodendrocytes medium (OLM) – made in DMEM medium	1% Glutamax 100x
	2% B27 (Gibco)
	0.5% FBS
	50 pg/ml recombinant mouse ciliary-neurotrophic factor (CNTF, BioTrend)
	1% OL supplement
OL supplement – made in DMEM medium	10 µl/ml N-2 supplement 100x (Gibco)
	10 mg/ml bovine serum albumin (BSA, Sigma)
	40 µg/ml 3,3',5-triiodo-L-thyronine (Sigma)
Microglia medium (MGM) – made in DMEM-F12 medium (Gibco)	10% FBS
	5% P/S
Trypsin solution – made in DMEM-F12 medium	25% trypsin-EDTA (0.25%) (Gibco)

Table 5. Media recipes for oligodendrocyte and microglial enriched primary cultures.

12.- Immunological staining of cultured cells

For immunocytochemistry, wells were fixed in 4% paraformaldehyde for 20 min, washed in 0.1 M PBS and incubated overnight with primary antibodies diluted in BB. After several washes in PBST, Alexa-conjugated secondary antibodies diluted in BB were incubated for 1 hour at room temperature. Finally, wells were washed with PBST, PBS and PB, and culture coverslips removed from the 24-well culture plates to mount over glass slides with Mowiol mounting media containing DAPI.

RESULTS

Chapter I

Activation of lysophosphatidic acid receptor type 1 (LPA_1) contributes to pathophysiology of spinal cord injury

Activation of lysophosphatidic acid receptor type 1 (LPA_1) contributes to pathophysiology of spinal cord injury

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Abstract

Lysophosphatidic acid (LPA) is an extracellular lipid mediator involved in many physiological functions, which signals through 6 known G protein-coupled receptors (GPCRs) named LPA_{1-6}. A wide range of LPA effects have been identified in the central nervous system (CNS), including neural progenitor cell physiology, astrocyte and microglia activation, neuronal cell death, axonal retraction, and developments of neuropathic pain. However, little is known about the involvement of LPA in CNS pathologies. Herein we demonstrate for the first time that LPA signaling via LPA_1 contributes to secondary damage after spinal cord injury. Our data demonstrate that LPA levels increase in the contused spinal cord parenchyma for the first fourteen days. To assess the potential contribution of LPA in the spinal cord tissue we injected LPA into the intact spinal cord. This experiment reveal that LPA induces microglia/macrophage activation and demyelination. By using a selective LPA_1 antagonist and mice lacking LPA_1, we also observed that LPA_1 activation is involved in LPA-mediated demyelination. Moreover, we show that the detrimental actions of LPA_1 are mediated by microglial cells rather than direct cytotoxic effect of LPA on

oligodendrocytes. Finally, we demonstrate that selective blockade of LPA_1 after spinal cord injury results in reduced demyelination and improvement in locomotor recovery. Overall, these results provide clear evidence that LPA-LPA_1 signaling is a novel pathway that contributes to secondary damage after spinal cord contusion in mice and suggest that LPA_1 antagonist might be useful for the treatment of acute spinal cord injury (SCI).

Introduction

Traumatic SCI causes permanent functional deficits due to the loss of axons and neurons, as well as to the death of oligodendrocytes, and the limited ability of the CNS to regenerate axons (Schwab and Bartholdi, 1996, Rowland *et al.*, 2008, David *et al.*, 2012). The pathophysiology of SCI involves two stages of tissue degeneration known as primary and secondary injury. The first results from the direct mechanical trauma to the spinal cord which is followed by a secondary wave of tissue degeneration that occurs over a period of several weeks that include inflammation and other mechanisms triggered by injury (David *et al.*, 2012). Secondary damage is thought to contribute importantly to the functional deficits seen after SCI and can be preventable. Repairing the damaged spinal cord is still a clinical challenge. Neuroprotective strategies aimed at preventing damage arising from secondary injury processes provide some hope for tissue sparing and improved functional outcome. However, there is currently no effective clinical treatment for SCI. There is therefore a need to identify factors that are involved in triggering secondary damage in SCI and to develop drug interventions to limit damage and improve functional deficits.

Lysophosphatidic acid (LPA; 2-acyl-sn-glycerol 3-phosphate) is a bioactive lipid that has a wide variety of biological activities, including cellular proliferation, prevention of apoptosis, cell migration, cytokine and chemokine secretion, platelet aggregation, smooth muscle contraction, and neurite retraction (Bot *et al.*, 2010, Choi *et al.*, 2010, Choi and Chun, 2013, David *et al.*, 2014). LPA exerts this wide variety of effects by binding to specific GPCRs, such as LPA receptors 1, 2

and 3 (LPA_{1-3}) which belong to the endothelial differentiation gene family (Edg), and the genetically more distant $LPAR_{4-6}$, which belong to the P2Y purinergic receptor family (Choi *et al.*, 2010, Choi and Chun, 2013). These receptors differ in the ability to alter intracellular Rho levels, mobilize Ca^{2+}, phosphoinositol turnover, and to activate intracellular kinases, such as MAPK and ERK (Choi *et al.*, 2010, Choi and Chun, 2013).

In vivo studies demonstrate that LPA is involved in the etiology of fetal hydrocephalus, and in the development of neuropathic pain after sciatic nerve injury and cerebral ischemia (Inoue *et al.*, 2004, Frisca *et al.*, 2012, Lin *et al.*, 2012). A recent work reveals that the administration of the B3 antibody, which binds to LPA and other lysophospholipids preventing them from interacting with their receptors, promotes functional recovery after spinal cord hemisection in mice (Goldshmit *et al.*, 2012). Although the hemisection model is not a clinical relevant model of SCI, this study provided the first insights on the potential deleterious actions of LPA in SCI. More recently, the B3 antibody was shown to promote neuroprotection after brain trauma (Crack *et al.*, 2014). Due to the wide variety of LPA receptors it is likely that LPA may exert helpful or harmful effects in the CNS depending on the receptors it signals through. There is therefore a need to know which LPA receptors contribute to neurodegeneration, and those, if any, that could mediate neuroprotection.

Here we show that LPA levels increase in the spinal cord parenchyma after contusion injury. We also provide clear evidence suggesting that LPA leads to demyelination in the spinal cord via activation of microglia LPA_1.Moreover, we finally demonstrate that selective blockade of LPA_1 after spinal cord contusion reduces functional deficits and demyelination. This study reveals for the first time an important contribution of LPA-LPA_1 signaling in secondary damage after SCI.

Materials and Methods

Surgical procedure

All surgical procedures were approved by the Universitat Autònoma de Barcelona Animal Care Committee and followed the guidelines of the European Commission on Animal Care. Adult (8-10 weeks old) female C57BL/6 mice (Charles River), LPA_1 deficient mice ($maLPA_1$) or wildtype (WT) littermates were anesthetized with ketamine (90 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.). After performing a laminectomy at the 11^{th} thoracic vertebrae, the exposed spinal cord was either intraspinally injected or contused.

Intraspinal injections were performed using a glass needle (30 µm internal diameter, Eppendorf, Hamburg, Germany) coupled to a 10 ml Hamilton syringe (Hamilton #701, Hamilton Co, Reno, NV, USA). 1 µl of saline, saline containing 5 nmoles of LPA (18:1 LPA; Sigma-Aldrich) or LPA plus a selective LPA_1 antagonist (AM095; 0.22 nmoles) (Swaney *et al.*, 2011) was injected into the dorsal column of the uninjured spinal cord. Injections were made at a perfusion speed of 2 µl/min controlled by an automatic injector (KDS 310 Plus, Kd Scientific, Holliston, MA, USA), and the tip of the needle was maintained inside the cord tissue 3 min after each injection to avoid liquid reflux.

Spinal cord contusion injuries were performed using the Infinite Horizon Impactor device (Precision Scientific Instrumentation), using a force of 50 kdynes and tissue displacement ranging between 400 and 600 µm (Klopstein *et al.*, 2012). Where indicated, AM095 or saline was administered orally at a dose of 30 mg/kg. Treatment was initiated 1 hour after SCI, and then given every 12 hours for one week.

Functional assessment

Locomotor recovery was evaluated at 1, 3, 5, 7, 10, 14, 21 and 28 dpi in an open-field test using the nine-point Basso Mouse Scale (BMS) (Basso *et al.*, 2006), which was specifically developed for locomotor testing after contusion injuries in mice. The BMS analysis of hindlimb

movements and coordination was performed by two independent assessors and the consensus score taken. In addition, at the end of the follow up (day 28 post-injury) the highest locomotion speed of the mice was evaluated on a belt of a motorized treadmill. Briefly, each mouse was allowed to explore the treadmill compartment, with the motor speed set to zero, for 5 min. Then speed was gradually increased from 0 up to 35 cm/s and stopped at the maximum speed at which each mouse was able to run. Maximum speed was that in which animals were able to perform for at least 5 seconds.

Motor Evoked Potentials

At day 28, electrophysiological tests were used to evaluate spared motor central pathways after SCI. Motor evoked potentials (MEPs) were recorded from the gastrocnemius muscle with microneedle electrodes, in response to transcranial electrical stimulation of the motor cortex by single rectangular pulses of 0.1 ms duration. Pulses were delivered through needle electrodes inserted subcutaneously, the cathode over the skull overlying the sensoriomotor cortex and the anode at the nose (Garcia-Alias *et al.*, 2003). Compound muscle action potential (CMAP) from gastrocnemius muscle was recorded for internal control of normal peripheral conduction. In this case the sciatic nerve was stimulated percutaneously by means of single pulses of 0.02 ms duration (Grass S88) delivered through a pair of needle electrodes placed at the sciatic notch. All potentials were amplified and displayed on a digital oscilloscope (Tektronix 450S)

Cell culture

Oligodendrocyte primary cultures

Primary oligodendrocyte progenitor cells (OPCs) were isolated from mouse cerebral cortex at 2-4 post-natal days (P2-4) as described previously (O'Meara *et al.*, 2011). Briefly, after removal of the meninges, cortical tissue was minced using a P1000 pipette tip and dissociated by incubating in a solution of 1.54 mg/ml papain (Worthington Biochemical), 400 μg/ml L-cystein (Sigma) and 1 mg/ml DNase

I (Roche) in MEM (Gibco) for 20 min at 37°C. Mixed glial culture medium (MGCM; 10% inactivated and filtered fetal bovine serum (FBS, Sigma), 0.33% penicillin-streptomycin (P/S, Sigma) and 1% Glutamax 100x (Gibco) in DMEM (Gibco)) was added to stop papain and DNase I activity. Cells were plated into T25 tissue culture flasks coated with 10 µg/ml poly-D-lysine for 1 hour at 37°C and cultured at 37°C in an humidified incubator with 5% CO2 supplementation. Three hours after plating, the floating cells were discarded by replacing the medium. Two thirds of the MGCM was replaced every 3 days with new MGCM supplemented with 5 µg/ml insulin (Seralab). Nine days later, OPCs were harvested by shaking at 37°C at 220 rpm overnight. The collected cells were plated into 10 µg/ml poly-D-lysine coated coverslips with DMEM (Gibco) supplemented with 1% Glutamax 100x, 2% B27 (Gibco), 0.5% FBS, 50 pg/ml recombinant mouse ciliary-neurotrophic factor (CNTF, BioTrend) and 1% OL supplement (10 µl/ml N-2 supplement 100x (Gibco), 10 mg/ml bovine serum albumin (BSA, Sigma) and 40 µg/ml 3,3',5-triiodo-L-thyronine (Sigma). OPC maturation in oligodendrocytes was achieved after 7 days *in vitro*.

Microglial primary cultures

Primary microglial cells were isolated from P4 mouse cerebral cortex as described previously (Saura *et al.*, 2003). Tissue dissociation and cell isolation was performed as described in OPC culture. Mixed glial cultures were prepared into T25 tissue culture flasks without any coating. Cells were seeded at a density of 300,000 cells/ml in DMEM-F12 (Gibco) with 10% FBS and 5% P/S, and cultured at 37°C in a humidified incubator with 5% CO2 supplementation. Medium was replaced every 4-5 days and confluence was achieved after 10-12 days *in vitro*. At this point, mixed cultures were incubated with 0.25% trypsin-EDTA (Gibco) diluted 1:4 in DMEM-F12 for 30 min at 37°C. This mild trypsinization resulted in the detachment of the upper layer of cells in one piece, and the remaining adherent microglial cells were cultured for 24 hours before stimulation. Microglial cells were stimulated with LPA (1 µM) or LPA+AM095 (12.5µM) or DMSO as control for 24 hours. Afterwards, cells were washed to remove

the LPA from the medium, and microglia was then incubated with oligodendrocyte conditioned media for 24 hours and then used for cytotoxicity assays in oligodendrocyte cell cultures.

Assessment of oligodendrocyte cell death

Oligodendrocytes were stimulated with LPA (1μM), LPA+AM095 (12.5μM) and DMSO as control, or with conditioned media of microglia cells stimulated with DMSO, LPA or LPA+AM095 for 24 hours. Afterwards, coverslips were fixed in 4% paraformaldehyde for 20 min, washed in phosphate buffered saline (PBS) and incubated overnight with rat anti-MBP (1:300, Abcam) in blockage buffer (BB; 0.3% Triton-PBS (PBST) with 5% FCS) at 4ºC. After several washes in PBS, coverslips were incubate for 1 hour at room temperature with anti-rat 594-conjugated antibody (1:500; Molecular Proves) and DAPI (Sigma), and the total number of oligodendrocytes (MBP^+ cells) were counted under fluorescence microscope (Olympus BX51).

Histology

At 4 days after intraspinal injection and 28 days after spinal cord contusion, mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) A 10 mm length of spinal cord containing the injection or the contusion site centered was harvested, post-fixed with 4% paraformaldehyde in 0.1 M PB for 1 hour and cryoprotected with 30% sucrose in 0.1 M PB at 4ºC for a minimum of 48 hours. The samples were fast-frozen at -60ºC in cryoembedding compound (Tissue-Tek® OCT, Sakura) and cut on a cryostat (Leica). Ten series of 10-μm-thick transversal sections were picked up on glass slides, so adjacent sections on the same slide were 100 μm apart. For demyelination and myelin sparing analyses, sections were stained with Luxol fast blue (LFB) (Sigma) or Fluromyelin (Molecular Probes) After graded dehydration, sections were placed in a 1 mg/ml LFB solution in 95% ethanol and 0.05% acetic acid overnight at 37ºC. Sections were then washed in 95% ethanol and distilled water before place them into a solution of 0.5 mg/ml Li2CO3 in distilled water for 1 min at room temperature . After washes in distilled water, sections were dehydrated and mounted in

DPX mounting media (Sigma). For immunofluorescence staining, sections were rehydrated in PBS and blocked with 5% FBS in PBST for 1 hour at room temperature. The sections were then incubated overnight at 4°C with primary antibodies against NeuN (for neurons; 1:200; Millipore), NF-200 (for axons; 1:1000; Millipore), GFAP (for astrocytes; 1:500; Invitrogen), Iba1 (for macrophage/microglia; 1:400; Abcam). After several washes in PBST, sections were incubated for 1 hour at room temperature with the appropriate Alexa 594- or Alexa 488-conjugated secondary antibodies (1:200; Invitrogen) and then coverslipped in Mowiol mounting media containing DAPI (1 µg/ml; Sigma). Tissue sections were viewed with Olympus BX51 microscope and images were captured using an Olympus DP50 digital camera attached to it and using the Cell^A Image acquisition software.

The epicenter of the injection or contusion injury impact was determined for each mouse spinal cord by localizing the tissue section with the greatest demyelination. The NIH ImageJ software was used to quantify the histological parameters. Demyelination after LPA injection was measured by delineating LFB non-stained white matter in the dorsal column. Myelin sparing after SCI was calculated by delineating the spared FluoroMyelin stained tissue, whereas neuronal survival was assessed by counting the number of $NeuN^+$ cells in the ventral horns.

Electron microscopy

4 and 21 days after intraspinal injection of LPA or saline mice were perfused with 0.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Five millimeter thick cross-sections of the spinal cord containing the injection site were left in the same fixative for several days followed by post-fixation in 2% osmium tetroxide for 2 hours at room temperature and then processed for embedding in Epon. 1 mm thick cross-sections of the spinal cord were stained with 1% toluidine blue for light microscopy. Then, sections were cut at 90 nm, stained with lead citrate and viewed with a Philips CM 10 electron microscope.

RNA isolation, reverse transcription and real-time PCR

Mice were perfused with sterile saline and 5 mm length of uninjured spinal cord was removed. Tissue was homogenized with QIAzol lysis reagent (Qiagen) and RNA extracted using RNeasy Lipid Tissue kit (Qiagen), according to the manufacturer's protocol. RNA was treated with DNaseI (Qiagen) to eliminate genomic DNA contamination. 1 µg of obtained RNA was primed with random hexamers (Promega) and reverse transcribed using Omniscript RT kit (Qiagen). RNase inhibitor (Roche) was added (1 U/µl final concentration) to avoid RNA degradation. Real Time (RT)-PCR analysis was performed using a MyiQ Single-Color Real-Time PCR Detection System (BIO RAD). Primer sequences are specified in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The amount of cDNA was calculated based on the threshold cycle (CT) value, and was standardized by the amount of housekeeping gene using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001).

Gene	forward primer (5′-3′)	reverse primer (5′-3′)
LPA_1	TGTCCTGGCCTATGAGAAGTTCT	TTGTCGCGGTAGGAGTAGATGA
LPA_2	CTCACTGGTCAATGCAGTGGTATAT	GAAGGCGGCGGAAGGT
LPA_3	GGGACGTTCTTCTGCCTCTTTA	GAAAGTGGAACTTCCGGTTTGT
LPA_4	GATGGAGTCGCTGTTTAAGACTGA	TGTTTGATCACTAACTTCCTCTTGGATA
LPA_5	CCGTACATGTTCATCTGGAAGAT	CAGACTAATTTCTCTTCCCACCT
GAPDH	TCAACAGCAACTCCCACTCTTCCA	ACCCTGTTGCTGTAGCCGTATTCA

Table 1. Real Time-PCR primer sequences.

Mass spectrometry analysis of LPA in the spinal cord

Mice were perfused with sterile saline and 5mm length of uninjured or injured spinal cord centered at the impact site was harvested at 6 hours, 1, 3, 7 and 14 dpi. Samples were homogenized in 0.01% w/v butylated hydroxytoluene in PBS. After centrifugation at 15,000 g for 20 min at 4°C, supernatants were processed for protein concentration

determination using the BCA protein assay (Pierce, Rockford, IL, USA).

The volume corresponding to 300 µg of protein was taken of every sample. 200 pmol of 1-O-dodecanoyl-2-dodecanoyl phosphatidic acid was added as internal standard and water up to 100 µl. Samples were acidified with 500 µl of H2O/HCl 19:1 and LPA species extracted with 500 µl of water-saturated butanol twice. Organic phase was recollected, evaporated until dryness and resuspended in 100 µl CHCl3. 50 µl of this extract was injected in a high-performance liquid chromatograph equipped with a binary pump Hitachi LaChrom Elite L-2130 binary pump and a Hitachi Autosampler L-2200 (Merck) coupled to a Bruker esquire6000 ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The column was a Supelcosil LC-Si 3 µm 150 x 3 mm column (Sigma-Aldrich) protected with a Supelguard LC-Si 20 x 3 mm guard cartridge (Sigma-Aldrich). Mobile phase was a gradient of solvent A (chloroform/methanol/32% ammonium hydroxide 75:24.5:0.5 v/v/v), and solvent B (chloroform/methanol/water/32% ammonium hydroxide 55:39:5.5:0.5 v/v/v/v). The gradient was started at 100% solvent A; it was decreased linearly to 50% A in 2 min, maintained for 4 min, to 0% A in 6 min, and maintained there for an additional 18 min. The flow rate was 0.5 ml min-1 in the chromatographic step and 0.2 ml min-1 entered into the electrospray interface of the mass spectrometer. Nebulizer gas was set to 30 pounds per square inch, dry gas to 8 l/min, and dry temperature to 365°C. LPA species were detected in negative ion mode with the capillary current set at +3500 v as $[M-H]^-$ ions.

Statistical analysis

All analyses were conducted through IBM SPSS Statistics v19. Two-tailed Student's *t* test was used for the single comparison between two groups. Maximal speed on a treadmill was analyzed using the Mantel-Cox test. Functional follow-up for BMS score and subscore, as well as histological analysis of myelin and neuronal sparing was analyzed using two-way repeated measure ANOVA. The rest of the data were analyzed using one-way or two-way ANOVA depending on

the appropriate design. *Post-hoc* comparisons were carried out only when a main effect showed statistical significance. P values for multiple comparisons were adjusted using Bonferroni's correction. Results are expressed as mean and standard error. Differences were considered significant at $p < 0.05$.

Results

LPA levels are increased injured mouse spinal cord

We first assessed whether LPA levels increased in the spinal cord following contusion injury. Using mass spectrometry we detected the presence of 3 LPA molecular variants (18:1, 18:0 and 20:1) in the uninjured spinal cord (Table 2). After contusion injury, these three LPA species were significantly elevated at all point analyzed (one-way ANOVA; *post-hoc* Bonferroni's $p < 0.01$; n = 4 per time point). Moreover, additional LPA variants (16:0, 20:4, 22:6 and 22:4) were also detected in the contused spinal cord (Table 2). Quantification of the different LPA species revealed that the total level of LPA increased very rapidly in the injured spinal cord (Figure 1A), being ~5 fold higher at 6 hours, raising to ~10 times at day 3, and remaining ~8 fold increased from 7 to 14 days post-injury as compared to intact spinal cord (one-way ANOVA; *post-hoc* Bonferroni's $p < 0.01$; n = 4 per time point). LPA could increase into the spinal cord parenchyma after injury due to plasma extravasation or to *de novo* synthesis by glial cells or neurons. We therefore analyzed the expression of the enzymes involved in the synthesis of LPA after SCI. Our data show that the mRNA levels for the three forms of phospholipase D family (PLD1-3) are significantly up-regulated after SCI (one-way ANOVA; *post-hoc* Bonferroni's $p < 0.05$; n = 3 per time point) (Figure 1B). The two main forms of the intracellular PLA_2 family ($cPLA_2$ GIVA and $iPLA_2$ GVIA) were also up-regulated at 1 and 3 days post-injury ($p < 0.05$) (Figure 1C). However, mRNA levels for autotaxin were down-regulated at day 1 and 3 after injury ($p < 0.05$) (Figure 1D). These results therefore demonstrate that LPA levels increase in the spinal cord after contusion injury and suggest that LPA is *de novo* synthesized after injury via PLD-PLA_2 pathway.

LPA leads to demyelination

To assess the potential contribution of increased levels of LPA in the spinal cord parenchyma we injected 1 µl of saline containing 5 nmoles of LPA into the dorsal column of uninjured spinal cord or saline alone as control group. We studied microglia/macrophage activation in the spinal cord parenchyma at four days after injection by measuring the presence of reactive Iba1^{+} cells. We observed the presence of activated microglia/macrophage restricted to the injection area in saline-injected mice, probably due to the mechanical injury caused by the needle insertion (Figure 2A-C). Interestingly, spinal cord sections from mice injected with LPA showed a marked activation of microglia/

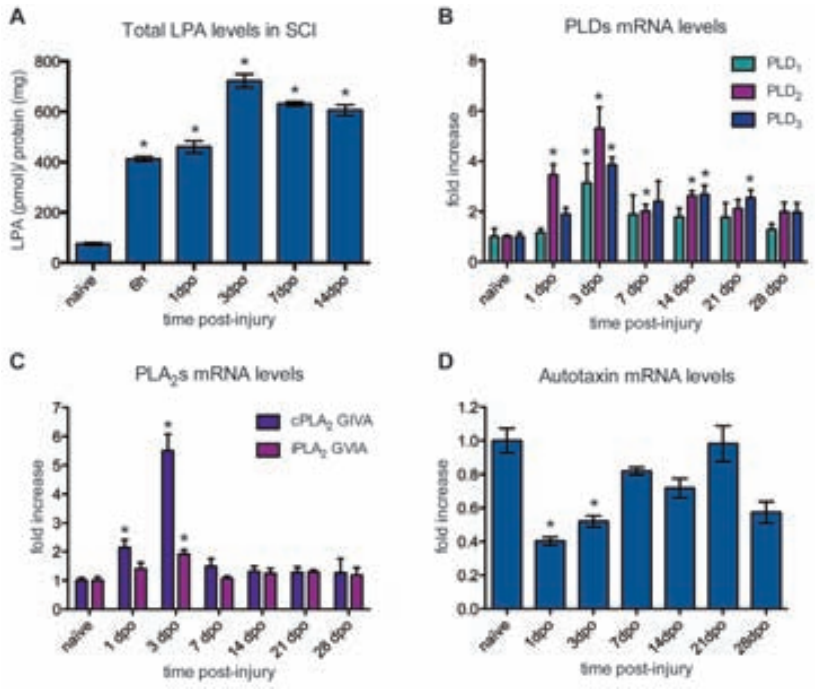

Figure 1. Increased LPA levels in the contused spinal cord and mRNA expression of LPA synthesis enzymes. (A) Mass spectrometry analyses of LPA in spinal cord tissue from uninjured and contused spinal cord at 6 hours and 1, 3, 7 and 14 days after SCI (n = 4 time point). (B-D) mRNA expression of enzymes involved in LPA synthesis in spinal cord tissue from uninjured and contused spinal cord at 1, 3, 7, 14, 21 and 28 days after SCI (n = 3 time point). * $p < 0.05$ Error bars indicate SEM.

LPA species	16:0	18:1	18:0	20:4	20:1	22:6	22:4	22:1
Intact	0	33.5±7.1	34.6±3.2	0	7.1±1.5	0	0	0
6 hours	7.7±0.3*	166.4±9.0*	79.7±4.4*	10.5±0.9*	77.8±5.8*	55.0±4.5*	15.1±0.7*	0.4±0.1
1 days	7.4±0.9*	167.8±18.6*	80.5±8.3*	13.1±1.8*	94.0±8.4*	60.3±6.1*	14.9±1.9*	0.5±0.1
3 days	13.4±1.0*	290.5±21.2*	143.3±10.5*	20.2±1.1*	144.0±12.9*	85.0±6.2*	25.5±1.7*	0.7±0.0
7 days	12.9±0.8*	255.4±7.1*	125.4±4.1*	21.9±0.7*	119.8±6.1*	73.4±2.3*	22.3±0.5*	0.7±0.0
14 days	0	307.7±35.7*	125.1±8.2*	25.7±2.0*	52.1±8.2*	82.0±7.6*	13.6±2.3*	0

*$p < 0.01$ vs intact spinal cord

Table 2. Quantification of the different LPA species in SCI.

macrophage four days following injection (Figure 2D). The presence of these inflammatory cells was not limited to the injection site, since they were also observed in more rostral regions of the dorsal column (Figure 2E). In addition, the spread of $Iba1^+$ cells along the spinal cord was increased 3 fold in LPA- as compared to saline-injected mice (Figure 2) ($p = 0.006$). We also observed mild activation of astrocytes restricted to areas close to the LPA injection (Figure 3), indicating that LPA activates more robustly microglial cells than astrocytes.

We then assessed whether activation of microglia/macrophages triggered by LPA led to demyelination. At 4 days post-injection, LFB staining revealed the presence of a small demyelinating lesion in the dorsal column of saline-injected spinal cords (Figure 4). This lesion was restricted to the area of the needle insertion (Figure 4B). We also observed a demyelinating lesion in the spinal cords injected with LPA (Figure 4D). However, the area of this lesion was significantly increased in LPA- as compared to PBS-injected mice and its extension along the spinal cord was almost 10 times larger ($p = 0.001$) (Figure 4A, B-E). To confirm that LPA led to demyelination we used electron microscopy (Figure 4F-I). This technique confirmed the presence of fibers with degenerating myelin (Figure 4G, H) as well as naked axons (Figure 4I) within the demyelinating lesion. Therefore, these results indicate that the increased levels of LPA in the spinal cord leads to inflammation

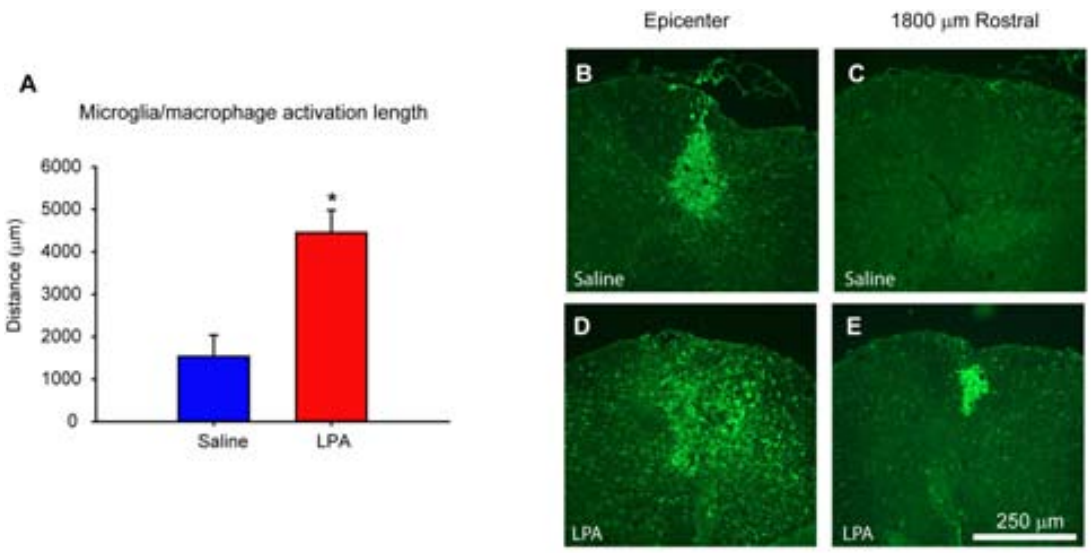

Figure 2. Intraspinal injection of LPA leads to microglia/macrophage activation (A) Quantification of microglia/macrophage activation length at 4 days after intraspinal injection of saline or LPA. (B-E) Representative images of spinal cords stained against Iba1 at the epicenter (B, D) and 1800 μm rostral to the injection site (C, E) of saline (B, C) and LPA (D, E) injected mice (n = 4 per group). * $p = 0.006$. Error bars indicate SEM. Scale bar = 250 μm.

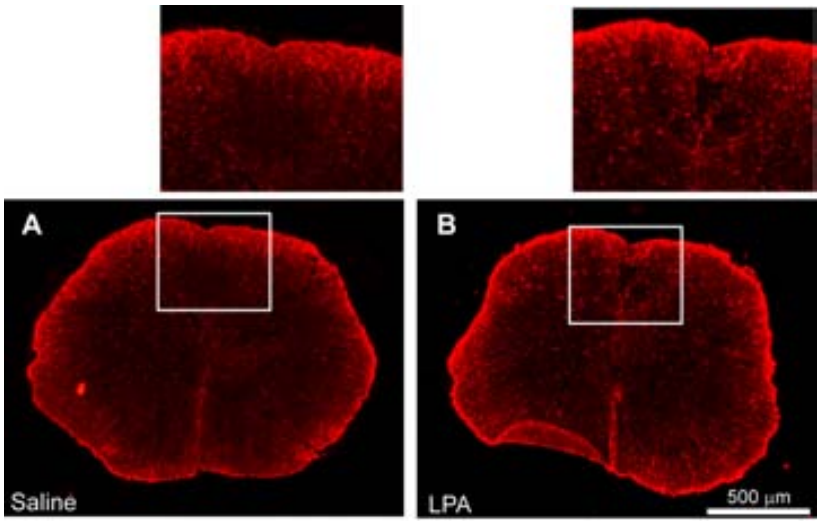

Figure 3. (A-B) Representative images of spinal cords stained against GFAP taken at the injection site in saline (B) and LPA (C) injected mice. Inserts show a higher magnification of the dorsal column. Note that in spinal cord injected with LPA show greater astrocyte reactivity.

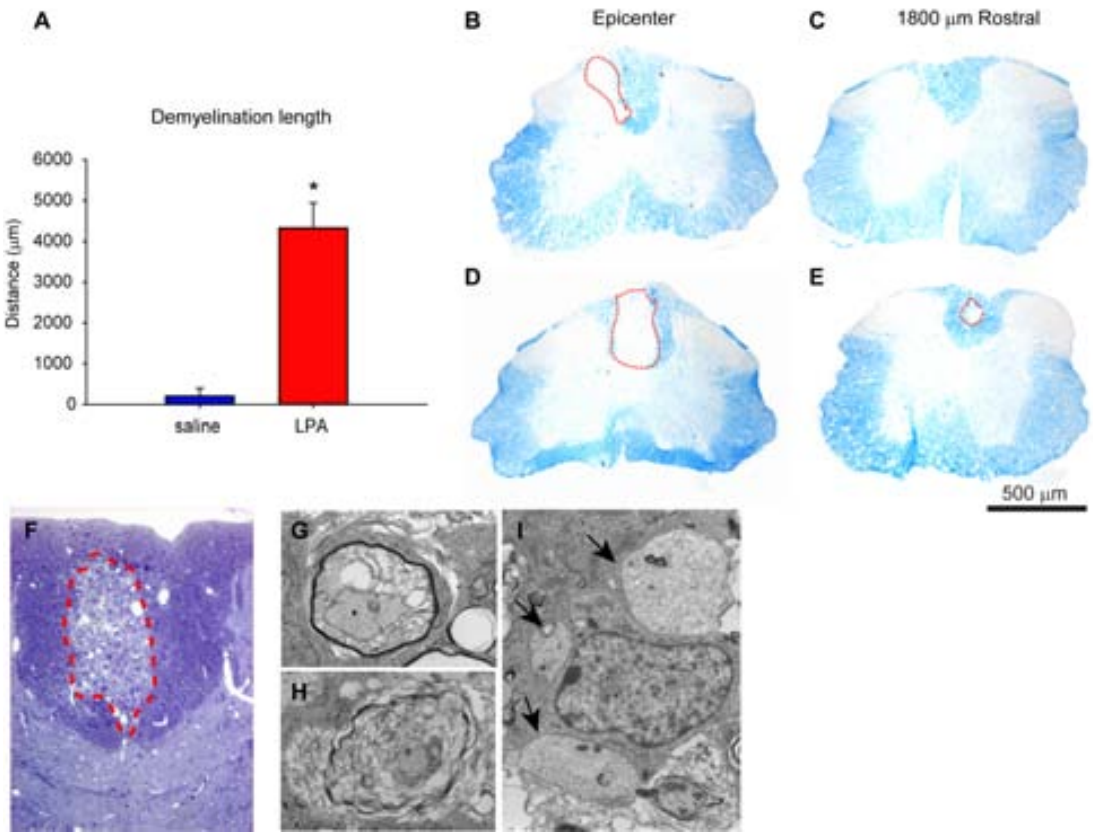

Figure 4. Intraspinal injection of LPA leads to demyelination (A) Quantification of demyelination length injury at 4 days after intraspinal injection of PBS or LPA. (B-E) Representative images of spinal cords stained with Luxol fast blue at the epicenter (B, D) and 1800 µm rostral to the injection site (C, E) in saline (B,C) and LPA (D, E) injected mice. (F) Toluidin Blue stained section from spinal cord at 4 days after intraspinal injection of LPA. (G-I) Electron microscopy images taken from the demyelinating area showing fibers with myelin breakdown (G, H) as well as demyelinated axons (I) (see arrows). * $p = 0.001$. Error bars indicate SEM. Scale bar = 500 µm.

and demyelination.

LPA mediates demyelination via LPA_1

Since LPA signals through 6 different receptors, we studied which ones were constitutively expressed in the normal spinal cord. RT-PCR analysis revealed that all 6 LPA receptors are expressed in the uninjured spinal cord, with LPA_1 being the most highly expressed (Figure 5). To assess if the demyelination triggered by intraspinal injection of LPA

was mediated via the LPA_1 we injected LPA together with AM095, a selective LPA_1 antagonist (Swaney *et al.*, 2011), into the intact spinal cord. Histological analysis revealed that four days following LPA administration, the demyelinating lesion was substantially reduced when LPA_1 was blocked (Figure 6). Quantification of the demyelinated areas revealed that mice treated with AM095 showed reduced myelin loss at the injection site and in the adjacent sections corresponding to 900 µm rostral and caudal to the epicenter (two-way ANOVA *post-hoc* Bonferroni's $p < 0.05$; n = 4 per group). We also found that spinal cord tissue sections from LPA_1 null mice ($maLPA_1$ variant) also displayed smaller demyelinating lesions (Figure 7) after intraspinal injection of LPA, further demonstrating the importance of LPA_1 signaling in LPA-induced demyelination.

Since LPA_1 is expressed in mature oligodendrocytes (Weiner *et al.*, 1998), we assessed whether LPA-induced demyelination was due to a toxic effect of LPA on oligodendrocytes. We found that LPA treatment led to a mild reduction in the viability of oligodendrocytes (~20%) at concentrations of 1 µM (Figure 8A). However, administration of AM095 failed to rescue oligodendrocyte from cell death, indicating that the LPA_1-induced demyelination is not related to a direct cytotoxic effect of LPA_1 signaling in oligodendrocytes (Figure 8B). Since LPA also triggers microglia activation when injected into the spinal cord we then assessed whether microglial LPA_1 was responsible for the LPA-induced demyelination. In agreement with previous work (Tham *et al.*, 2003) we found by RT-PCR analysis that cultured microglia express LPA_1 (data not shown). Interestingly, we observed that conditioned medium of primary microglial cells stimulated with LPA led to marked reduction (~85%) in oligodendrocyte survival (Figure 8C-F) (one-way ANOVA *post-hoc* Bonferroni's $p < 0.001$), suggesting that microglia become cytotoxic after LPA stimulation. Interestingly, oligodendrocyte cell death was reduced ~3 times when microglia LPA_1 was blocked with AM095 (Figure 8C-F) (one-way ANOVA *post-hoc* Bonferroni's $p = 0.017$), indicating that the activation of LPA_1 in microglial cells is responsible, in part, for the cytotoxic effects mediated by LPA-

stimulated microglia. These results therefore suggest that demyelination triggered by intraspinal injection of LPA is mediated, in part, by the activation of microglial LPA_1.

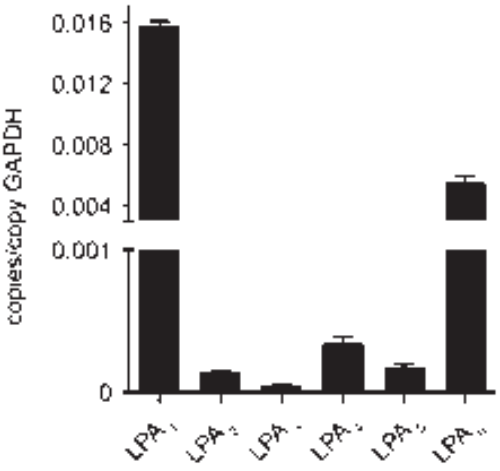

Figure 5. Expression of $LPA_{1\text{-}5}$ in the intact spinal cord parenchyma. Note that LPA_1 is the LPA receptor most highly expressed in the spinal cord. Data are presented as copies of receptor mRNA relative to copies of GAPDH mRNA (n = 4). Error bars indicate SEM.

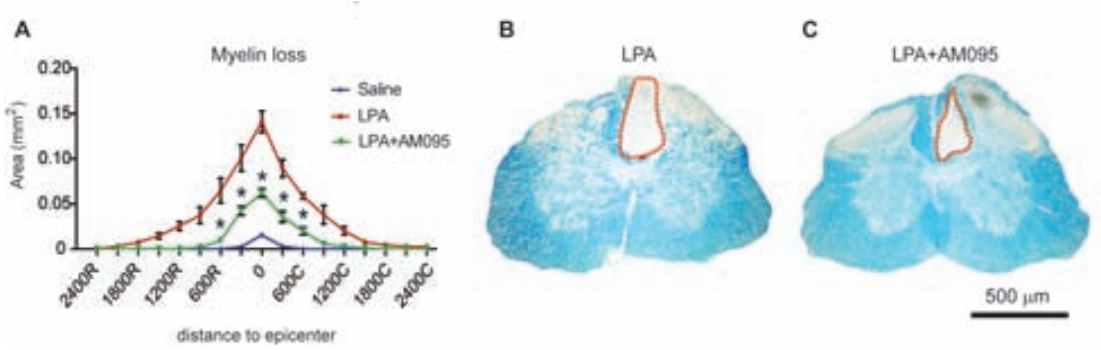

Figure 6. LPA_1 signaling mediates demyelination in the spinal cord. (A) Quantification of the demyelinating injury in the spinal cord of animals injected with saline, LPA or LPA+AM095. (B, C) Representative images of spinal cords stained with Luxol fast blue at the epicenter of the injection site in mice administered with LPA (B) or LPA+AM095 (C). Note that demyelination was marked reduced in the absence of LPA_1 activity. Scale bar = 500 µm. (*p < 0.05 vs LPA). Error bars indicate SEM.

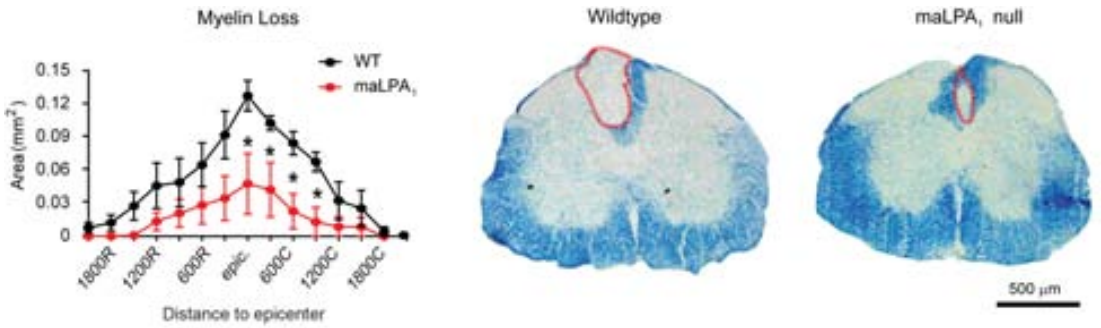

Figure 7. (A) Quantification of the demyelinating injury after intreaspinal injection of LPA in WT or $maLPA_1$ null mice. (B, C) Representative images of spinal cords stained with Luxol fast blue at the epicenter of the injection site in WT (B) and $maLPA_1$ null mice (C). Note that demyelination was reduced in the absence of LPA_1. Scale bar = 500 µm. (*p < 0.05 vs LPA). Error bars indicate SEM.

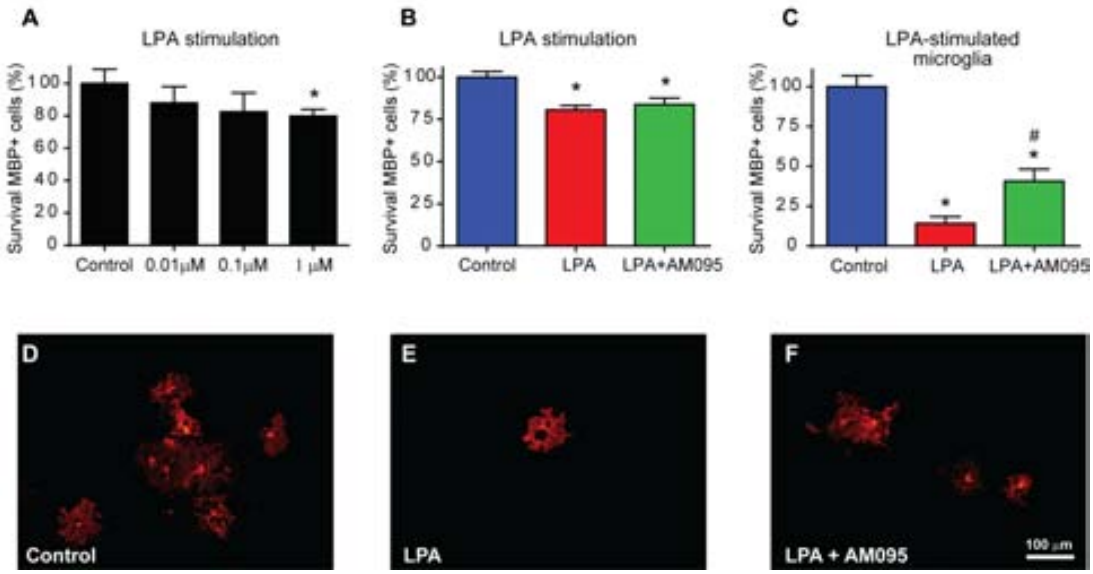

Figure 8. Microglia LPA_1 mediates oligodendrocyte cell death. (A) Effects of several concentrations of LPA on oligodendrocyte survival. (B, C) Quantification of oligodendrocyte cell death after stimulation with LPA or LPA+AM095 (B) or with microglia conditioned medium stimulated with LPA or LPA+AM095 (C). (D-E) Representative images of oligodendrocytes (MBP^+) from cultures treated with conditioned medium from untreated microglia (control), or microglia treated with LPA or LPA+AM095 stimulated microglia. Note that that microglia LPA_1 blockade significantly reduces oligodendrocyte cell death. Scale bar = 100 μm. (*$p < 0.05$ *vs* control; #$p < 0.05$ *vs* LPA). Error bars indicate SEM (n = 4 per group).

LPA_1 contributes to SCI pathophysiology

We finally assessed whether LPA-LPA_1 signaling contributes to secondary damage and functional impairment after spinal cord contusion injury in mice. Since maLPA_1 null mice showed locomotor deficits (data not shown), probably due its severe developmental brain defects (Santin *et al.*, 2009), we used AM095 to assess the role of LPA_1 in SCI. We found that oral administration of AM095 significantly reduced locomotor impairments based on the Basso Mouse Scale (BMS) assessment (Figure 9A) ($p < 0.001$; two-way RM-ANOVA; n = 8 per group). At the end of the follow up (28 days post-injury), the BMS score was improved in 1.5 points after AM095 treatment. Mice treated with saline showed occasional plantar stepping and no coordination

while AM095 showed frequent/consistent plantar stepping and most of them (87.5%) had coordination. In addition, the BMS subscore, that assess fine aspects of locomotion, was markedly improved after LPA_1 blockade (Figure 9B) ($p < 0.001$; two-way RM-ANOVA; n = 8 per group). Along this line, we also observed that mice treated with AM095 were able to run at significant higher speeds on a treadmill (Figure 9C) ($p = 0.011$; Mantel-Cox test; n = 8 per group).

We also performed electrophysiological test to assess the preservation of descending pathways of the spinal cord based on MEPs analysis. At 28 dpi, mice treated with AM095 showed significant greater MEP amplitudes for the gastrocnemius muscle compared to controls ($p = 0.018$; *t* test), reflecting an increase in spared functional descending tracts in the spinal cord after the lesion (Figure 9D,E).

We then studied whether the improvement in locomotor skills observed in mice administered with AM095 was associated with amelioration of secondary tissue damage. Histological sections of the spinal cord stained with FluoroMyelin revealed that animals treated with the LPA_1 antagonist underwent significant protection against demyelination, at the injury epicenter and in adjacent regions ($p < 0.001$; two-way ANOVA) (Figure 9F-H). However, AM095 treatment led to only minor improvement in neuronal sparing, seen only at 400 µm caudal to the injury epicenter (Figure 10). These results therefore suggest that the increased levels of LPA in the injured spinal cord contributes to demyelination and loss of function by signaling via LPA_1.

Discussion

The results of the present study provides clear evidence that the LPA-LPA_1 pathway contributes to secondary damage after SCI. Our data reveals that LPA levels increase in the spinal cord following contusion injury. We show that LPA leads to activation of microglia/macrophages and demyelination, and that such responses are mediated, in part, via LPA_1 signaling. Moreover, our *in vitro* experiments suggest that detrimental actions of LPA_1 are mediated by microglial cells. Finally,

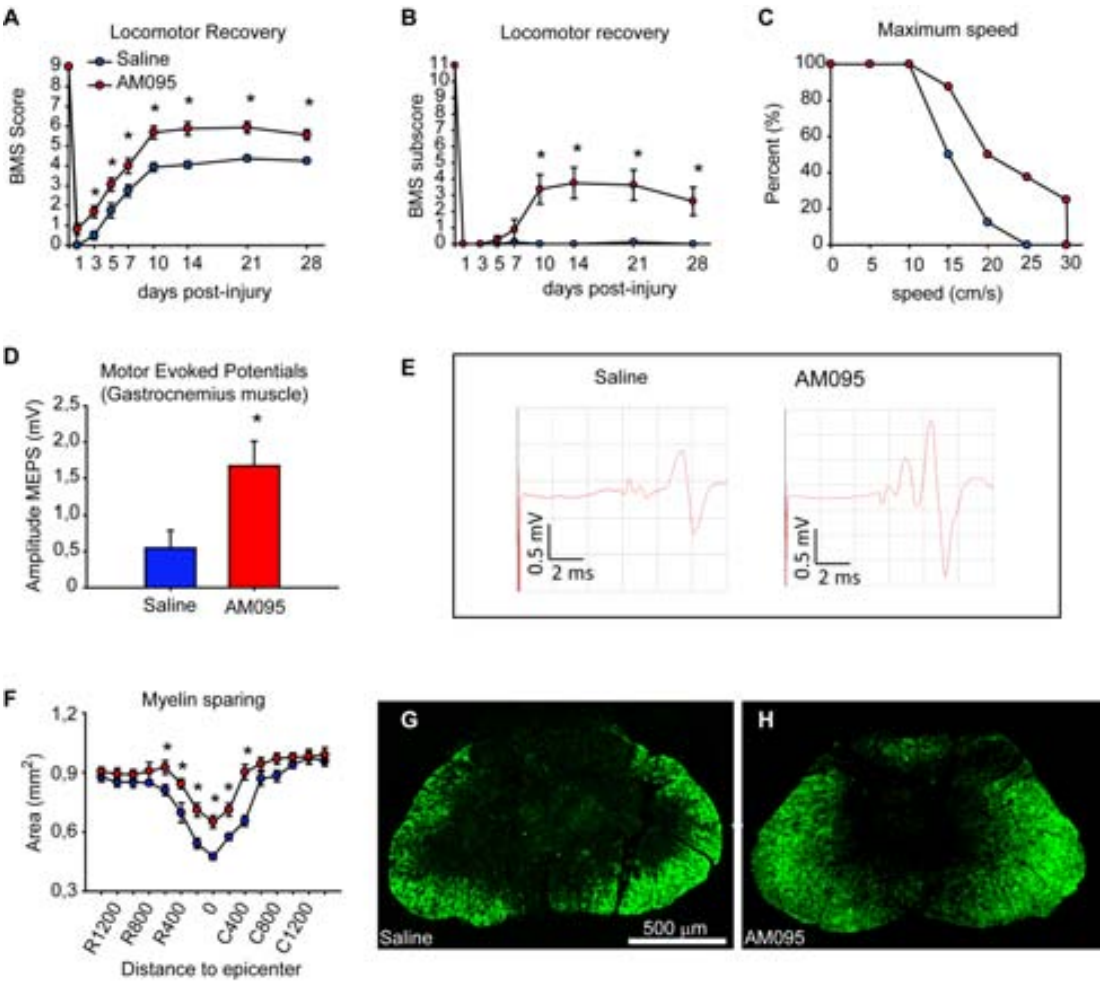

Figure 9. LPA$_1$ blockade enhances functional outcomes and reduces demyelination after SCI. (A-B) Animals treated with AM095 show significant improvement in locomotor performance as compared to saline treated mice using the (A) 9-point Basso Mouse Scale (BMS) and the (B)11-point BMS subscore, as well as (C) faster locomotion on a treadmill. (D-E) Mice treated with AM095 greater preservation of MEPs. (D) Quantification of MEPs and in the gastrocnemius muscle at 28 days after SCI and representative MEP recordings from mice treated with saline or AM095. (F) Quantification of myelin sparing at various distances rostral and caudal to the injury epicenter revels significant reduction in tissue loss in mice treated AM095 at the epicenter of the injury and in adjacent sections. (G, H) Representative micrographs showing myelin sparing at the injury epicenter in section stained against FluoroMyelin from mice treated with saline (G) or AM095 (H). (n = 8 per group). Scale bar = 500 µm. (*$p < 0.05$). Error bars indicate SEM.

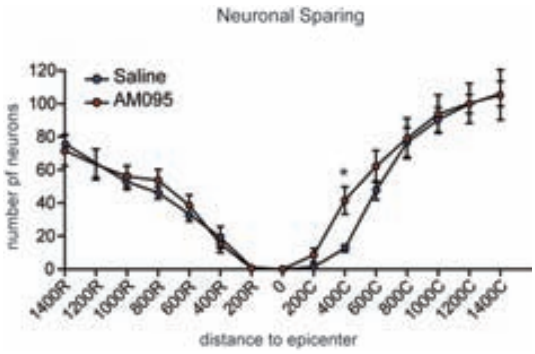

Figure 10. Quantification of ventral horn neuron survival at various distances rostral and caudal to the injury epicenter reveals in mice treated with AM095 or saline. Note that AM095 did not enhance neuronal survival except to regions located 400 µm caudal to the injury epicenter.

we demonstrate that selective inhibition of LPA_1 after SCI confers protection from demyelination and enhances locomotor skills. Overall, this work demonstrates for the first time that the LPA-LPA_1 pathway contributes to secondary damage and is detrimental to functional recovery after SCI.

Over the last few years, lysophospholipids (LPLs) have emerged as key modulators of inflammation, including in nervous system disorders (David *et al.*, 2012, Choi and Chun, 2013). The contribution of LPLs to CNS pathology comes from early experiments showing that lysophosphatidylcholine (LPC) triggers a potent demyelinating and inflammatory response in the CNS (Ousman and David, 2000, Ousman and David, 2001). More recently, another LPL named sphigosine 1-phosphate (S1P) has been demonstrated to play a key role in multiple sclerosis (Brinkmann *et al.*, 2010). Indeed, fingolimod (FTY720), a non-selective S1P receptor modulator, is the first worldwide oral treatment for relapsing forms of multiple sclerosis (Brinkmann *et al.*, 2010).

LPA is a LPL that acts as a potent signaling molecule, evoking multiple physiological responses in a wide variety of cells (Choi *et al.*, 2010, Choi and Chun, 2013). LPA is present in low concentrations in mammalian cells and tissues, however, its concentrations in the blood can range

from 0.1 μM in plasma to over 10 μM in serum. LPA is synthesized *in vivo* from membrane phospholipids by 2 pathways (Choi *et al.*, 2010, Choi and Chun, 2013). In the first route, phosphatidic acid, generated by the action of the phospholipase D (PLD), is deacylated by phospholipase A2 (PLA_2) to generate LPA (Choi *et al.*, 2010, Choi and Chun, 2013). This is the main synthesis route for LPA in host tissues. In the second pathway, LPLs generated by PLA_2 are subsequently converted to LPA by autotaxin (ATX) (Choi *et al.*, 2010, Choi and Chun, 2013). Most of the LPA in serum is generated by this second pathway (Choi *et al.*, 2010, Choi and Chun, 2013). Here we observed that LPA levels rapidly rise in the spinal cord parenchyma following injury. This is likely due to its *de novo* production in the contused spinal cord, since recent reports revealed that various forms of PLA_2 enzymes are up-regulated in SCI (Titsworth *et al.*, 2009, Lopez-Vales *et al.*, 2011, David *et al.*, 2012). However, plasma leakage due to blood vessel disruption as well as the serum production during the clotting process may also contribute to increase LPA levels in SCI. Indeed, the presence of the 20:4 LPA in the injured spinal cord, which is the major form of LPA in plasma (Scherer *et al.*, 2009), which was not found in the intact cord, may be indicative that plasma extravasation is also a source of LPA in SCI.

Independently of its origin, little is known about the effects on CNS cells when exposed to substantial levels of LPA. Several studies revealed that LPA activates astrocytes and microglia in cell culture (Choi *et al.*, 2010, Frisca *et al.*, 2012). *In vivo* studies has also shown that LPA leads to astrogliosis when injected into the brain (Sorensen *et al.*, 2003). More recently, two studies revealed that the administration of the B3 antibody, which binds to LPA and other LPLs, mediates protection after spinal cord hemisection and traumatic brain injury (Goldshmit *et al.*, 2012, Crack *et al.*, 2014), suggesting a detrimental role of LPA in CNS injuries. However, little is known about the mechanisms underlying the detrimental actions of LPA in the injured nervous system. Here we demonstrate that LPA triggers the activation microglia/macrophage when injected into the intact spinal cord. Although we also noticed

mild astrogliosis in the spinal cord after LPA injection, the effects of the LPA were markedly more pronounced in microglia/macrophages, suggesting that microglial cells are more susceptible to become activated in the presence of LPA. Importantly, we also observed that intraspinal injection of LPA also led to the development of a demyelinating lesion in the spinal cord. This is of crucial importance since myelin loss contributes to functional impairments after SCI, and in other neural conditions, such as multiple sclerosis.

As previously mentioned, LPA exerts its effects through 6 LPA receptors (LPA_{1-6}). However, since very high concentration of LPA (10 μM) are needed to activate LPA_6 (Choi and Chun, 2013), the effects of LPA in the spinal cord are likely due by signaling via LPA_{1-5}. Our RT-PCR results revealed that the LPA_1 is the mostly highly expressed LPA receptor in the spinal cord, suggesting that the detrimental actions of LPA are likely to be mediated by binding to LPA_1. Along this line, we demonstrate that maLPA_1 knockout mice as well as mice treated with AM095, a selective LPA_1 inhibitor (Swaney *et al.*, 2011), showed reduced myelin loss when LPA is injected into the intact spinal cord, revealing that that LPA_1 signaling is involved in the LPA-demyelinating lesion. A previous work also revealed a pivotal role of LPA_1 in triggering myelin breakdown of the dorsal roots after sciatic nerve injury (Inoue *et al.*, 2004), suggesting that LPA_1 mediates demyelination, not only in the CNS, but also in the PNS.

LPA_1 is found on oligodendrocytes (Weiner *et al.*, 1998) and its expression appears shortly before maturation/myelination (Garcia-Diaz *et al.*, 2014). Although LPA_1 signaling plays a key role in the regulation of oligodendrocyte differentiation and myelination in the CNS (Garcia-Diaz *et al.*, 2014), little is known about the effects of LPA on mature oligodendrocytes. A previous work, revealed that LPA did not induce oligodendrocyte cell death (Stankoff *et al.*, 2002). We show that LPA exerts mild toxicity in oligodendrocytes at doses ≥ 1 μM. However this effect is not mediated via LPA_1, since AM095 failed to rescue cell death. Hence, LPA-mediated demyelination is likely to be mediated by

the activation of LPA_1 in other cell types, rather than to activation of LPA_1 in oligodendrocytes. Indeed, we show that intraspinal injection of LPA leads to activation of microglia/macrophages, which express LPA_1 (Tham *et al.*, 2003). Importantly, conditioned medium of microglial cell stimulated with LPA mediates marked oligodendrocyte cell death, indicating that microglial become cytotoxic upon LPA stimulation. Interestingly, selective blockade of LPA_1 in microglial cells increase oligodendrocyte cell survival, revealing that the detrimental actions of LPA-stimulated microglia are mediated via LPA_1 signaling. This result suggest that microglia LPA_1 is involved in the development of the demyelinating injury triggered by LPA injection into the spinal cord. However, LPA_1 inhibition did not completely suppress the harmful effects of LPA-stimulated microglia, suggesting that other microglia LPA receptors may contribute to mediate oligodendrocyte cell death. We also show that myelin loss was not completely abrogated in the maLPA_1 null mice or in mice administered with AM095, indicating that other LPA receptors or other non-LPA mediated mechanisms also contribute to demyelination.

We provide clear evidence that the increased LPA levels in the contused spinal cord leads to demyelination by signaling via LPA_1. Our data reveals that administration of AM095 reduces locomotor impairments after spinal cord contusion injury. In addition, we also show that mice treated with the LPA_1 antagonist display reduced myelin loss, emphasizing the importance of LPA_1 in triggering demyelination in a clinical relevant model of SCI. Overall, our data demonstrate for the first time that LPA signaling through its receptor LPA_1 contributes to secondary damage in SCI. Our results suggest that LPA_1 could be a novel therapeutic target for the treatment of acute SCI, but also for other CNS conditions in which demyelination contributes to the pathology.

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Chapter II

Lysophosphatidic acid receptor type 2 (LPA_2) mediates secondary damage following spinal cord injury in mice

Lysophosphatidic acid receptor type 2 (LPA_2) mediates secondary damage following spinal cord injury in mice

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Abstract

Lysophosphatidic acid (LPA) is a pleiotropic extracellular lipid mediator with many physiological functions, that signals through 6 known G-protein coupled receptors (GPCRs) named LPA_{1-6}. In chapter I, we reported that LPA plays an important role in the pathophysiology of spinal cord injury (SCI) by signaling through LPA_1. Here, we aimed at studying whether LPA_2 contributes to secondary damage after SCI. We show that LPA_2 mRNA expression is up-regulated in the spinal cord parenchyma after contusion injury. We also demonstrate that demyelination lesion triggered by intraspinal administration of LPA is markedly reduced in the lack of LPA_2. Moreover, we reveal that LPA_2 deficient mice showed better functional recovery, as well as greater myelin and neuronal sparing after spinal cord contusion injury. We finally show that microglia LPA_2 is responsible for oligodendrocyte cell death whereas activation of neuronal LPA_2 mediates neuronal loss. Overall, our results provide clear evidence that LPA signaling via LPA_2 triggers secondary damage and functional impairments after SCI. Targeting LPA_2 could therefore lead to the development of a novel approach to mediate neuroprotection in acute SCI.

Introduction

Traumatic SCI is a major cause of disability that results in a functional decline due to disruption of axonal pathways and death of neurons and oligodendrocytes (Dumont *et al.*, 2001; Schwab and Bartholdi, 1996). There are two waves of tissue degeneration after SCI, which are known as a primary and secondary injury. The first is caused by the initial mechanical damage to the spinal cord parenchyma, whereas the latest is due to the activation of several cellular and molecular events that take place in the spinal cord tissue from hours to weeks after trauma (Dumont *et al.*, 2001; Schwab and Bartholdi, 1996). Primary injury cannot be avoided, however, the mechanisms participating in secondary injury can be substantially abrogated or even blocked, providing some options to minimize secondary degeneration and functional loss. Hence, it is important to identify the factors that contribute to secondary damage, in order to develop an effective therapy to reduce functional deficits after SCI.

LPA is an extracellular lipid mediator with a wide range of biological functions. LPA receptors are expressed in almost all cells of the nervous system, where it has many effects (Choi and Chun, 2013; Choi *et al.*, 2010). Some studies showed that LPA induces neuronal cell death (Holtsberg *et al.*, 1998), axonal retraction (Tigyi *et al.*, 1996) (Tigyi *et al.*, 1996), inhibition of oligodendrocyte maturation (Dawson *et al.*, 2003), and proliferation of astrocytes and mouse microglial cells (Shano *et al.*, 2008; Sorensen *et al.*, 2003). Moreover, LPA has been related with some nervous system pathologies as fetal hydrocephalus (Yung *et al.*, 2014), psychiatric diseases (Harrison *et al.*, 2003; Roberts *et al.*, 2005), and neuropathic pain (Halder *et al.*, 2013; Inoue *et al.*, 2004; Ma *et al.*, 2009). However, little information is currently available on the role of LPA in nervous system trauma or disorders. Two recent works showed that preventing LPA interaction with its receptors by means of the administration of B3, an antibody that binds to LPA, reduces tissue damage after spinal cord hemisection injury in mice (Goldshmit *et al.*, 2012) and brain trauma (Crack *et al.*, 2014). However, considering

the pleiotropic effects of LPA and the variety of receptors with which it interacts, it is possible that LPA could exert helpful or harmful effects in SCI depending on the receptor it signals through, as previously observed with other lipid mediators, such as prostaglandins (Kawano *et al.*, 2006; Kerr *et al.*, 2008; Liang *et al.*, 2011; Redensek *et al.*, 2011). There is therefore a need to dissect out the specific contribution of each LPA receptor in SCI in order to specifically block those that mediate degeneration without altering the activity of those that may mediate protection or neutral effects.

In chapter I we demonstrated that LPA by binding to microglia LPA receptor 1 (LPA_1) contributes to demyelination and functional deficits after SCI. Herein, we aimed to examine the potential involvement of LPA_2 in the pathophysiology of the SCI. Our results demonstrate that LPA_2 also plays a major role to SCI pathophysiology.

Material and methods

Surgical procedure

All surgical procedures were approved by the Universitat Autònoma de Barcelona Animal Care Committee and followed the guidelines of the European Commission on Animal Care. Adult (8-10 weeks old) LPA_2 deficient female mice in a 129/SvJ-C57BL/6 background or wildtype littermates (WT) were anesthetized with ketamine:xylazine (90:10 mg/kg, i.m.). After performing a laminectomy at the 11^{th} thoracic vertebrae, the exposed spinal cord was either injected or contused.

Intraspinal injections were performed using a glass needle (30 µm internal diameter, Eppendorf, Hamburg, Germany) coupled to a 10 ml Hamilton syringe (Hamilton #701, Hamilton Co, Reno, NV, USA). 1 µl of saline containing 5 nmoles of 18:1 LPA (Sigma-Aldrich) or sterile saline alone was injected in the dorsal funiculi of intact spinal cords. Injections were made at a perfusion speed of 2 µl/min controlled by an automatic injector (KDS 310 Plus, Kd Scientific, Holliston, MA, USA), and the tip of the needle was maintained inside the cord tissue 3 min after each injection to avoid liquid reflux.

Spinal cord contusion injuries were performed using the Infinite Horizon Impactor device (Precision Scientific Instrumentation), using a force of 50 kdynes and tissue displacement ranging between 400 and 600 μm.

Functional assessment

Locomotor recovery was evaluated at 1, 3, 5, 7, 10, 14, 21 and 28 days post-injury (dpi) in an open-field test using the nine-point Basso Mouse Scale (BMS) (Basso *et al.*, 2006), which was specifically developed for locomotor testing after contusion injuries in mice. The BMS analysis of hindlimb movements and coordination was performed by two independent assessors and the consensus score taken.

The highest locomotion speed of the mice was tested at day 28 post-injury. Animals were placed on a belt of a motorized treadmill (DigiGaitTM Imaging System, Mouse Specifics, Boston, MA). Each mouse was allowed to explore the treadmill compartment, with the motor speed set to zero, for 5 min. Then speed was gradually increased from 0 up to 35 cm/s and stopped at the maximum speed at which each mouse was able to run. Maximum speed was that in which animals were able to perform for at least 5 seconds.

Electrophysiological analysis

Electrophysiological tests were used to evaluate spared motor central pathways after SCI. Motor evoked potentials (MEPs) were recorded from the tibialis anterior and gastrocnemius muscles with microneedle electrodes, in response to transcranial electrical stimulation of the motor cortex by single rectangular pulses of 0.1 ms duration. Pulses were delivered through needle electrodes inserted subcutaneously, the cathode over the skull, overlaying the sensoriomotor cortex, and the anode at the nose (García-Alías *et al.*, 2003). Compound Muscle Action Potential (CMAP) M waves from tibialis anterior and gastrocnemius muscles were recorded for internal control of peripheral normal conduction. In this case the sciatic nerve was stimulated percutaneously by means of single pulses of 0.02 ms duration (Grass S88), delivered through a pair of needle electrodes placed at the sciatic notch (García-Alías *et al.*, 2003).

Histology

Mice were deeply anaesthetized using Dolethal (pentobarbital sodium; Vétoquinol E. V. S. A.) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4 days post-injection or at 28 days post-contusion. 7 mm length of spinal cord containing the injection or the lesion site centered was harvested, post-fixed for 1 hour in 4% paraformaldehyde in 0.1 M PB and cryoprotected with 30% sucrose in 0.1 M PB at 4ºC, for a minimum of 48 hours. Spinal cords were fast-frozen at -60ºC in cryoembedding compound (Tissue-Tek® OCT, Sakura) and cut on a cryostat (Leica). Ten series of 10 µm-thick transversal sections were picked up on glass slides, so adjacent sections on the same slide were 100 µm apart. For assessing demyelination after intraspinal injection, tissue sections were gradually dehydrated and placed in a 1 mg/ml Luxol fast blue (LFB) solution in 95% ethanol and 0.05% acetic acid overnight at 37ºC. Then, sections were placed into a solution of 0.5 mg/ml Li2CO3 in distilled water for 1 min at room temperature, washed, dehydrated and mounted in DPX mounting media (Sigma).

For histological analysis after SCI, sections were stained using FluoroMyelin Green fluorescent myelin stain (Invitrogen) for assessing myelin loss. Briefly, tissue sections were rehydrated in PBS and incubated with FluoroMyelin 1:300 in PBS for 20 min at room temperature. Then sections were washed and mounted in Mowiol mounting media containing DAPI (1 µg/ml; Sigma). For assessing neuronal sparing, tissue sections were rehydrated in PBS and blocked with blocking buffer (BB; 5% FBS in 0.3% Triton-PBS (PBST)) for 1 hour at room temperature. Sections were then incubated overnight at 4ºC with mouse anti-NeuN biotin conjugated (1:100, Chemicon). After several washes in PBST, sections were incubated for 1 hour at room temperature with Alexa 594-conjugated streptavidin (1:500, Invitrogen) diluted in BB. Finally, sections were washed with PBST, PBS and PB and coverslipped in Mowiol mounting media containing DAPI.

Tissue sections were viewed with Olympus BX51 microscope and images were captured using an Olympus DP50 digital camera attached to it and using the Cell^A Image acquisition software.

The epicenter of the injection or contusion injury impact was determined for each mouse spinal cord by localizing the tissue section with the greatest demyelination. The NIH ImageJ software was used to quantify the histological parameters. Demyelination after LPA injection was measured by delineating LFB non-stained white matter in the dorsal column. Myelin sparing after SCI was calculated by delineating the spared FluoroMyelin stained tissue, whereas neuronal survival was assessed by counting the number of $NeuN^+$ cells in the ventral horns.

RNA isolation, reverse transcription and real-time PCR

For spinal cord mRNA extraction, mice were perfused with sterile saline and 5 mm length of uninjured or injured spinal cord centered at the impact site was harvested at 1, 3, 7, 14, 21 and 28 dpi. Tissue was homogenized with QIAzol lysis reagent (Qiagen) and RNA extracted using RNeasy Lipid Tissue kit (Qiagen), according to the manufacturer's protocol. RNA was treated with DNaseI (Qiagen) to eliminate genomic DNA contamination. 1 µg of obtained RNA was primed with random hexamers (Promega) and reverse transcribed using Omniscript RT kit (Qiagen). RNase inhibitor (Roche) was added (1 U/µl final concentration) to avoid RNA degradation. Real Time (RT)-PCR analysis was performed using a MyiQ Single-Color Real-Time PCR Detection System (BIO RAD). The GAPDH housekeeping gene was selected as the reference gene. RT-PCR reactions were performed using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) according to the manufacturer's instructions. Primer sequences included the following: LPA_2 forward 5'- CTCACTGGTCAATGCAGTGGTATAT-3', LPA_2 reverse 5'- GAAGGCGGCGGAAGGT-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene: GAPDH forward 5'- TCAACAGCAACTCCCACTCTTCCA-3',GAPDH reverse

5'- ACCCTGTTGCTGTAGCCGTATTCA-3'. The amount of cDNA was calculated based on the threshold cycle (CT) value, and was standardized by the amount of housekeeping gene using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001).

Animal genotyping

PCR analysis was used to detect the presence of WT and *lpar2* mutated allele. For tissue sampling, the tip of the tail was cut at the time of weaning the mice. Genomic DNA extraction was carried out by using the ArchivePure DNA Purification System (5PRIME), as described by the manufacturer. Allele amplification was performed by PCR reaction, using the Taq DNA Polymerase kit (Invitrogen), and PCR products were analyzed by standard electrophoresis in 1% agarose gels. Primers sequences were the following: LPA_2 forward 5′- AGTGTGCTGGTATTGCTGACCA -3′, LPA_2 reverse 5′- CTCTCGGTAGCGGGGATGG -3′, LPA_2 mutated 5′- CAGCTGGGGCTCGACTAGAGGAT -3′. Product sizes for WT and targeted alleles were 576 bp and 328 bp, respectively.

Cell cultures

Oligodendrocyte primary cultures

Primary oligodendrocyte progenitor cells (OPCs) were isolated from WT littermate of LPA_2 deficient mice cortices at 2-4 post-natal days (P2-4) as described previously (O'Meara *et al*., 2011). Briefly, after removal of the meninges, cortical tissue was minced and dissociated by incubating in a solution of 1.54 mg/ml papain (Worthington Biochemical), 400 µg/ml L-cystein (Sigma) and 1 mg/ml DNase I (Roche) in MEM (Gibco) for 20 min at 37ºC. Mixed glial culture media (MGCM; 10% inactivated and filtered fetal bovine serum (FBS, Sigma), 0.33% penicillin-streptomycin (P/S, Sigma) and 1% Glutamax 100x (Gibco) in DMEM (Gibco)) was added to stop papain and DNase I activity. Cells were plated into T25 tissue culture flasks coated with 10 µg/ml poly-D-lysine (Sigma) for 1 hour at 37ºC and cultured at 37ºC in an humidified incubator with 5% CO2 supplementation. 3

hours after plating, the floating cells were discarded by replacing the medium. Two thirds of the MGCM was replaced every 3 days with new MGCM supplemented with 5 µg/ml insulin (Seralab). 9 days later, OPCs were harvested by shaking at 37ºC at 220 rpm overnight. The collected cells were plated into 10 µg/ml poly-D-lysine coated 24 well plates with DMEM (Gibco) supplemented with 1% Glutamax 100x, 2% B27 (Gibco), 0.5% FBS, 50 pg/ml recombinant mouse ciliary-neurotrophic factor (CNTF, BioTrend) and 1% OL supplement (10 µl/ml N-2 supplement 100x (Gibco), 10 mg/ml bovine serum albumin (BSA, Sigma) and 40 µg/ml 3,3',5-triiodo-L-thyronine (Sigma)). OPC maturation in oligodendrocytes was achieved after 7 days *in vitro* and for maintenance of the culture half of the medium was changed every 2-3 days.

Dorsal root ganglia (DRG) primary cultures

Dorsal root ganglia (DRG) neurons were harvested from WT or LPA_2 deficient P4 mice and placed in Gey's salt solution (Sigma) with 6 mg/ml glucose. After cleaning DRGs from connective tissue and rests of roots, they were dissociated in 1 mg/ml collagenase (Sigma), 2.5 mg/ml trypsin (Sigma) and 1 mg/ml DNAse I in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS, Sigma) for 30 min in a water bath at 37ºC, shaking kindly every 10 min. Cells were resuspended in Neurobasal A medium (Gibco) supplemented with 2% B27, 1% P/S, 2mM L-glutamine (Sigma) and 6 mg/ml glucose and plated on 24 wells plates coated with 10 µg/ml poly-D-lysine for 1 hour at 37ºC, washed, dried and further coated overnight with 2ug/ml laminin (Sigma). After 24 hours in culture, medium was fully changed. Cells were maintained in culture for 4-5 days *in vitro*.

Microglial cells

Primary microglial cells were isolated from LPA_2 null P4 mice or WT littermates and cortices were removed as described previously (Saura *et al.*, 2003). Tissue dissociation and cell isolation was performed as in OPC cultures. However, mixed glial cultures were prepared into T25 tissue culture flasks with no coating. Cells were seeded at a density of

300.000 cells/ml in DMEM-F12 (Gibco) with 10% FBS and 5% P/S (MG medium), and cultured at 37ºC in a humidified incubator with 5% CO2 supplementation. Medium was replaced every 4-5 days and confluency was achieved after 10-12 days *in vitro*. At this point, mixed cultures were incubated with 0.25% trypsin-EDTA (Gibco) diluted 1:4 in DMEM-F12 for 30 min at 37ºC. This mild trypsinization resulted in the detachment of the upper layer of cells in one piece. The remaining adhered microglial cells were cultured for 24 hours before starting experiments.

For the generation of microglial conditioned medium (MCM), WT and LPA_2 null microglial cells were incubated for 24 hours in new medium with DMSO (control), 1 μM LPA (LPA) or with 1 μM LPA together with 12.5 μM of the LPA_1 antagonist AM095 (LPA+AM095). Then, this culture medium was replaced with new oligodendrocyte or DRG neuronal specific culture medium, leading microglial cells to continue releasing factors induced by the treatment for 24 hours. Afterwards, the conditioned medium (MCM) was centrifuged and frozen and applied to oligodendrocytes and dorsal root ganglia neuronal cells for assessment of cell death

Assessment of cell death in oligodendrocytes and neurons

To assess whether oligodendrocyte and neuronal LPA_2 signaling leads to cell death, WT and LPA_2 null mice DGR neuronal cells and oligodendrocytes and were exposed to 1 μM of LPA for 12 and 24 hours, respectively. To assess the involvement of microglia LPA_2 on cell death, WT DRG neuronal cells and oligodendrocytes were exposed for 12 and 24 hours, respectively, to control-, LPA- or LPA+AM095-MCM obtained from WT and LPA_2 null mice.

Afterwards, DRG neuronal cells were washed in PBS and cells were then stained with Annexin V Alexa 488-conjugated (Invitrogen). Cells were washed in cold PBS and incubated in Annexin V solution (50 μl/ml in 10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4) for 15 min at room temperature. Neuronal survival was determined by quantifying the Annexin V^+ area of each culture well.

Oligodendrocytes were fixed in 4% paraformaldehyde for 20 min,

washed in 0.1 M PBS and incubated overnight with rat anti-MBP antibody (1:200, Abcam) diluted in BB. After several washes in PBST, cells were incubated with anti-rat Alexa 594-conjugated secondary antibody diluted in BB for 1 hour at room temperature, and then mounted in Mowiol mounting media containing DAPI. Oligodendrocyte survival was determined by counting the number of nucleated MBP^+ cells.

Statistical analysis

All analyses were conducted through IBM SPSS Statistics v19. Two-tailed Student's *t* test was used for the single comparison between two groups. Functional follow-ups for BMS score and subscore as well as histopathological data was analyzed using two-way repeated measure (RM)-ANOVA with *post-hoc* Bonferroni's test. The rest of the data were analyzed using one-way ANOVA with *post-hoc* Bonferroni's test. *p* values for multiple comparisons were adjusted using Bonferroni's correction. Results are expressed as mean ± SEM. Differences were considered significant at $p < 0.05$.

Results

Expression of LPA_2 in the injured and uninjured mouse spinal cord

We first assessed the changes in mRNA levels of LPA_2 in the spinal cord parenchyma after contusion injury by using RT-PCR. Our data revealed that LPA_2 was marked up-regulated at 1 and 3 dpi, being the latest time point when mRNA levels reached the peak (one way ANOVA $p = 0.022$ and $p < 0.001$, respectively; n = 3 per group). After 1 week post-injury, we found that mRNA levels for LPA_2 in the injured spinal cord were two fold increased and remained a steady level up to day 28, however, no statistical significance was found (Figure 1).

Intraspinal injection of LPA into the intact spinal cord of LPA_2 deficient mice

In chapter 1 we found that LPA leads to demyelination when injected into the uninjured spinal cord by signaling via LPA_1. However, LPA_1

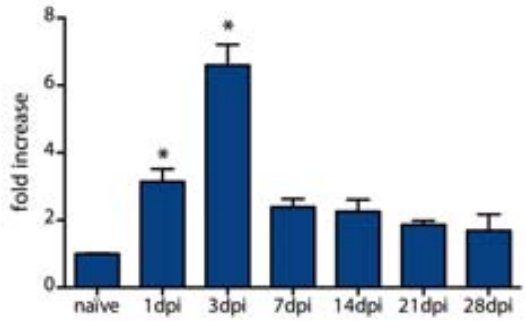

Figure 1. LPA_2 mRNA expression is up-regulated in the injured spinal cord. Real Time-PCR quantification of LPA_2 mRNA levels from spinal cords harvested at different time points after contusion injury. Error bars indicate SEM (*$p < 0.05$).

blockade or gene deletion did not completely reverse LPA-triggered demyelination. This data suggest that there are other LPA receptors involved in LPA-mediated demyelination. To test whether LPA_2 signaling also contributes to demyelination, we injected 5 nmoles of LPA or sterile saline (control) into the dorsal funiculi of intact spinal cords from WT and LPA_2 deficient mice. We observed that the LPA-triggered demyelination was substantially reduced at the epicenter of the injection and in caudal regions in the lack of LPA_2 ($p < 0.05$; two-way ANOVA; n = 4 WT+saline, n = 4 LPA_2 null+saline; n = 8 WT+LPA; n = 7 LPA_2 null+LPA) (Figure 2A-C). This data indicates that LPA_2 signaling contributes to the demyelination lesion triggered by LPA.

LPA_2 plays a harmful role in spinal cord injury

Since LPA_2 contributes to LPA-mediated demyelination, we then examined whether LPA_2 signaling leads to tissue damage and functional deficits after SCI. We found that LPA_2 deficient mice had significant improvement in locomotor skills according to BMS scores ($p < 0.05$; two way RM-ANOVA; n = 12 for WT and 11 for LPA_2 deficient mice). *Post-hoc* Bonferroni's test analysis revealed significant differences between LPA_2 null and WT littermate mice starting from day 10 post-injury and remaining enhanced until the end of the follow up (Figure 3A). At 28 dpi only 50% of WT mice were able to perform

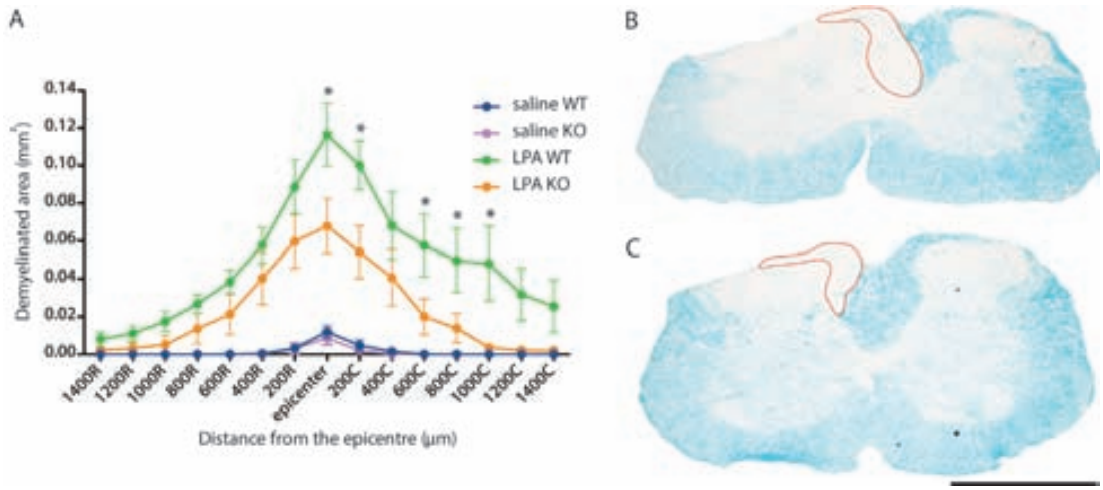

Figure 2. Lack of LPA_2 reduces LPA-triggered demyelination. (A) Quantification of myelin loss at various distances rostral and caudal to the injury epicenter. (B, C) Representative micrographs showing myelin loss at the injury epicenter in WT (B) and LPA_2 deficient (C) mice spinal cords injected with 5 nmols of LPA. (*$p < 0.05$). Error bars indicate SEM. Scale bar = 500 µm.

occasional stepping, in at least one hindlimb (score 3.58 ± 0.17). In contrast, 100% of LPA_2 null mice showed occasional stepping both hindlimbs, and 55% of the mice were able to perform frequent stepping in at least with one of the hindlimbs (score 4.41 ± 0.13). Moreover, forced locomotion on a treadmill revealed that LPA_2 null mice were able to run faster than WT littermate mice ($p < 0.001$; *t* test; n = 8 per group) (Figure 3B).

We also performed electrophysiological evaluation (MEPs) to assess the preservation of motor descending pathways. At day 28, LPA_2 null mice showed higher MEP amplitudes for the tibialis anterior and gastrocnemius muscles as compared to WT littermates ($p = 0.01$ and 0.02, respectively; *t* test) (Figure 3C-D). No differences were found in CMAP amplitudes (data not shown).

Histolopathological assessment revealed that the locomotor improvement observed in LPA_2 deficient mice was associated with amelioration of secondary tissue damage. Histological sections of the

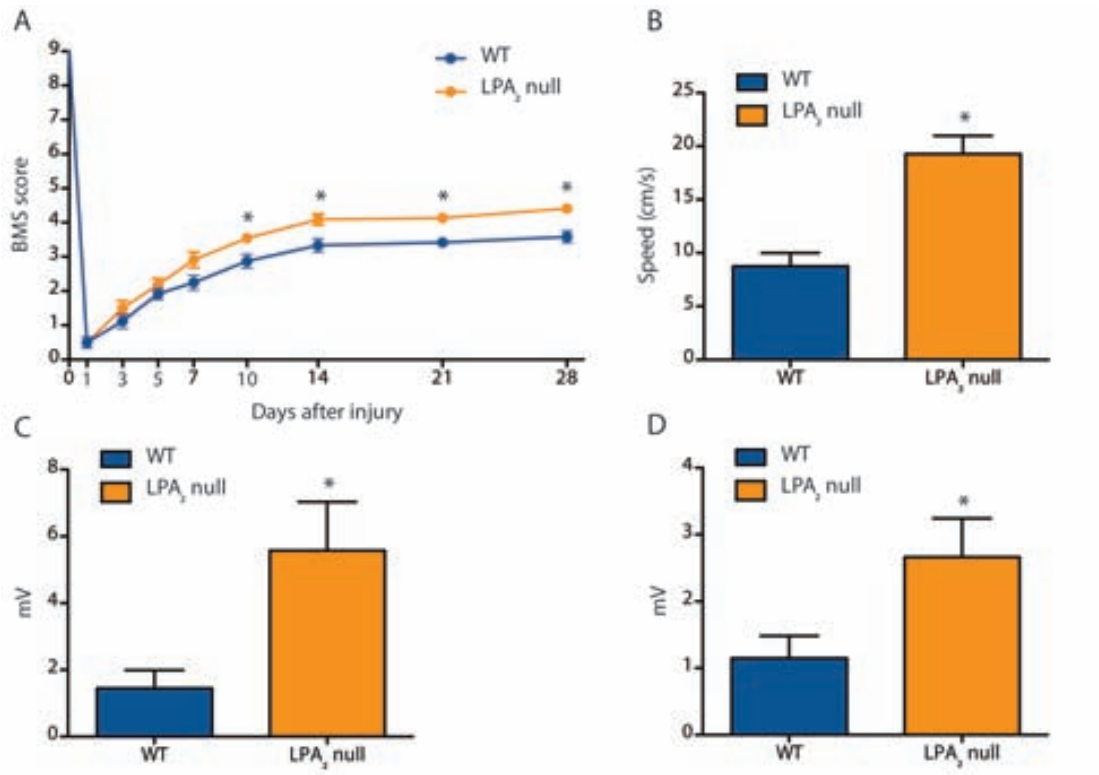

Figure 3. Lack of LPA_2 promotes functional recovery after spinal cord injury. (A) Locomotor recovery after spinal cord contusion injury in WT and LPA_2 null mice using the 9-point Basso Mouse Scale. The lack of LPA_2 led to significant improvement in locomotor performance compared to WT mice. (B) The ability to run at a constant speed was evaluated at 28 dpi by placing mice onto the belt of a motorized treadmill. Note that mice lacking LPA_2 were able to run at faster speeds. (C, D) Preservation of descending axonal tracts after spinal cord injury was evaluated by registering the motor evoked potentials (MEPs) from tibialis anterior and gastrocnemius muscles. Mice lacking LPA_2 showed higher MEP amplitudes for both muscles. (*$p < 0.05$). Error bars indicate SEM.

spinal cord stained with FluroMyelin revealed that LPA_2 deficient mice had reduced myelin loss at the injury epicenter and in adjacent rostral regions ($p < 0.001$; two-way ANOVA) (Figure 4A-C). In addition, quantification of $NeuN^+$ cells in the ventral horns of the spinal cord revealed greater neuronal survival at rostral and caudal regions from the lesion site ($p < 0.001$; two-way ANOVA) (Figure 4D-F). This data therefore suggest that LPA_2 contributes to myelin and neuronal loss after SCI, leading, consequently, to functional deficits.

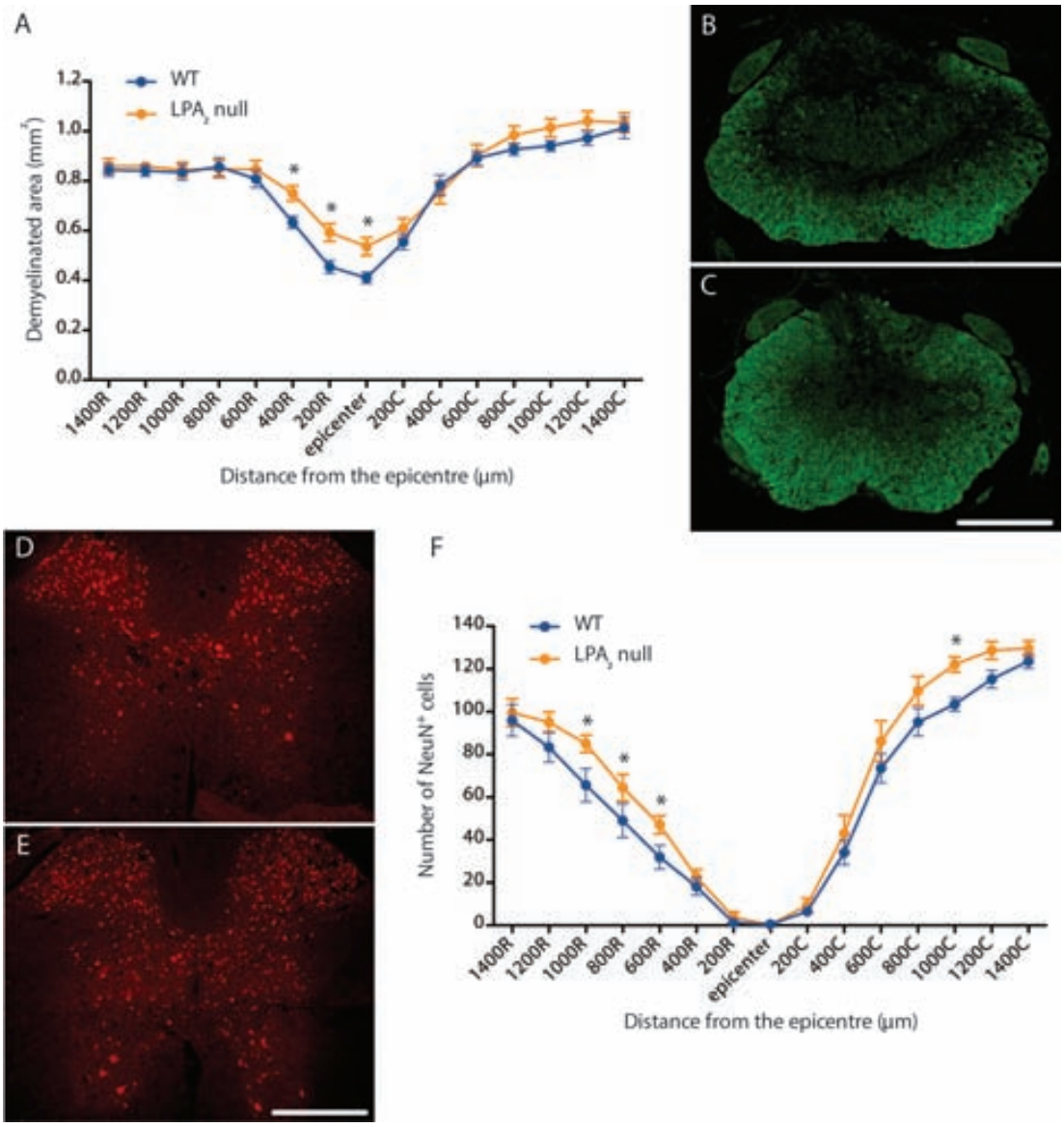

Figure 4. The lack of LPA_2 results in reduced secondary damage after spinal cord injury. (A) Quantification of myelin sparing at various distances rostral and caudal to the injury epicenter. (B, C) Representative micrographs of spinal cord tissue at the injury showing myelin sparing at the injury epicenter in WT (B) and LPA_2 deficient (C) mice. (D) Quantification of ventral horn neuron survival at various distances rostral and caudal to the injury epicenter. (E, F) Representative micrographs show sparing of ventral horn neurons at 600 µm rostral to the injury epicenter in WT (E) and LPA_2 null (F) mice spinal cords. (*$p < 0.05$). Error bars indicate SEM. Scale bar = 500 µm in B, C; 250 µm in D, E.

LPA_2 signaling induces oligodendrocyte cell death in vitro

Primary oligodendrocyte cultures were used to study the potential involvement of LPA_2 in oligodendrocyte cell death. First, cultured oligodendrocytes from WT and LPA_2 deficient mice were exposed to 1 μM LPA for 24 hours. We observed that LPA led to mild (~25%) although significant reduction in the number of MBP^+ cells in both groups (one way ANOVA $p < 0.05$ n = 8 per group). These results indicate that LPA triggers oligodendrocyte cell death, which is not mediated via activation of oligodendrocyte LPA_2 (Figure 5A).

In chapter I we reported that LPA-stimulated microglia secrete some unknown cytotoxic molecules that induce over 80% oligodendrocyte cell death, and that such effect was partially mediated via microglia LPA_1. We therefore assessed whether microglia LPA_2 also contributed to cytotoxicity by exposing oligodendrocytes for 24 hours to MCM from WT and LPA_2 deficient mice. Similarly to observed in chapter I, LPA-MCM from WT mice led to 85.80 ± 4.16% reduction in the number of MBP^+ cells ($p < 0.001$, two-way ANOVA, n = 8 per group) (Figure 5B, E), and oligodendrocytes were protected from death when microglia LPA_1 was blocked ($p < 0.05$, n = 8 per group) (Figure 5B, G). Interestingly, we found that oligodendrocyte survival was markedly increased when exposed to LPA-MCM from LPA_2 deficient mice (74.87 ± 5.12% *post-hoc* Bonferroni's $p < 0.001$ vs. WT LPA-MCM, n = 8 per group) (Figure 5B, F). These results indicate that activation of microglia LPA_2 exerts cytotoxic effects on oligodendrocytes. Highlight, that microglia LPA_2 seems to trigger greater toxicity than microglia LPA_1, since the number of oligodendrocytes were almost two fold increased upon incubation with LPA-stimulated microglia conditioned medium lacking LPA_2 as compared to that lacking LPA_1 activity. Besides, our results revealed that lack of LPA_1 and LPA_2 signaling in microglial cells completely suppressed the toxic effects of LPA-stimulated microglia on oligodendrocytes (n = 8 per group) (Figure 5B, H), indicating that the harmful effects of microglial cells stimulated with LPA are mediated via LPA_1 and LPA_2 signaling by independent pathways (Figure 5B, H).

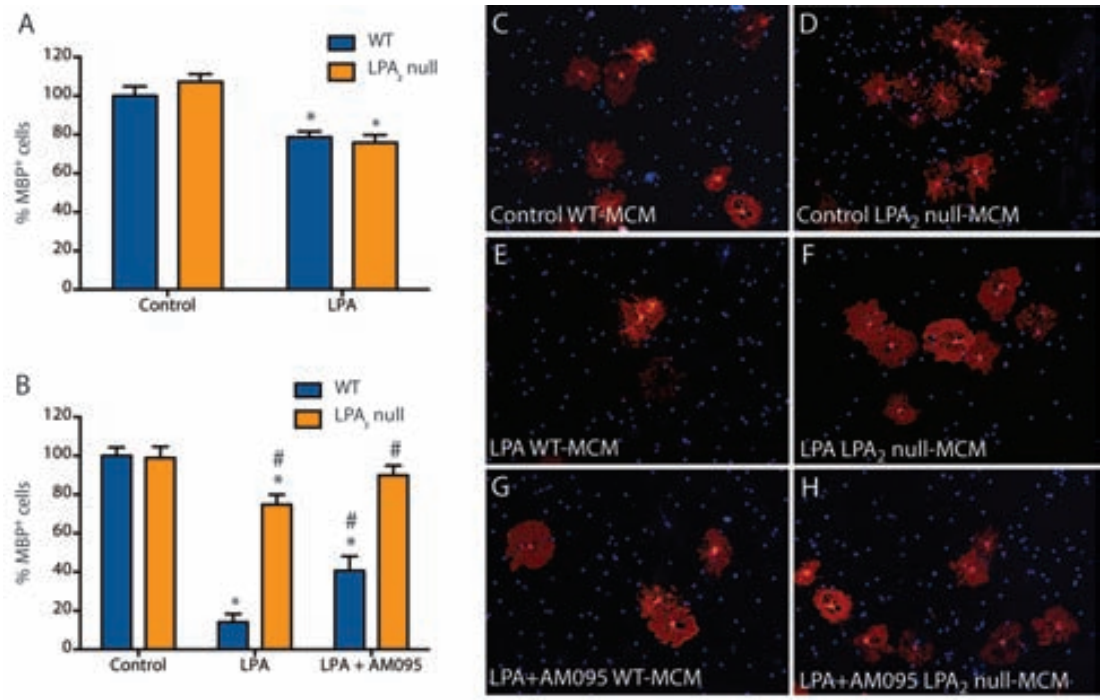

Figure 5. LPA induced oligodendrocyte cell death *in vitro* through LPA_2 signaling on microglial cells. (A) Quantification of MBP^+ cells on primary cultured WT and LPA_2 null oligodendrocytes exposed to 1 μM LPA for 24 hours. (B) Quantification of MBP^+ cells on primary cultured WT oligodendrocytes exposed for 24 hours to LPA-stimulated microglial-conditioned medium (MCM) from WT or LPA_2 deficient mice treated with or without the LPA_1 antagonist AM095. (C-H) Representative micrographs showing MBP^+ cells exposed to WT (C, E, G) or LPA_2 null (D, F, H) MCM in control (C, D), LPA (E, F) or LPA+AM095 (G,H) conditions (*$p < 0.05$ vs. control; #$p < 0.05$ *vs*. LPA).

LPA_2 signaling induces DRG neuronal cell death in vitro

Since we observed that the lack of LPA_2 activity led to greater neuronal sparing after SCI, we assessed whether neuronal LPA_2 is responsible for inducing cell death. We observed, by using Annexin V staining, that LPA stimulation triggered that apoptosis of DRG neuronal cells ($p < 0.001$, two-way ANOVA; n = 8 per group) (Figure 6A-D). Interestingly, LPA-mediated apoptosis in DRG neurons was reverted in the lack of LPA_2 ($p < 0.001$ two-way ANOVA; n = 8 per group) (Figure 6A-E). These results suggest that neuronal LPA_2 signaling is responsible for the direct cytotoxic effects of LPA on DRG neurons.

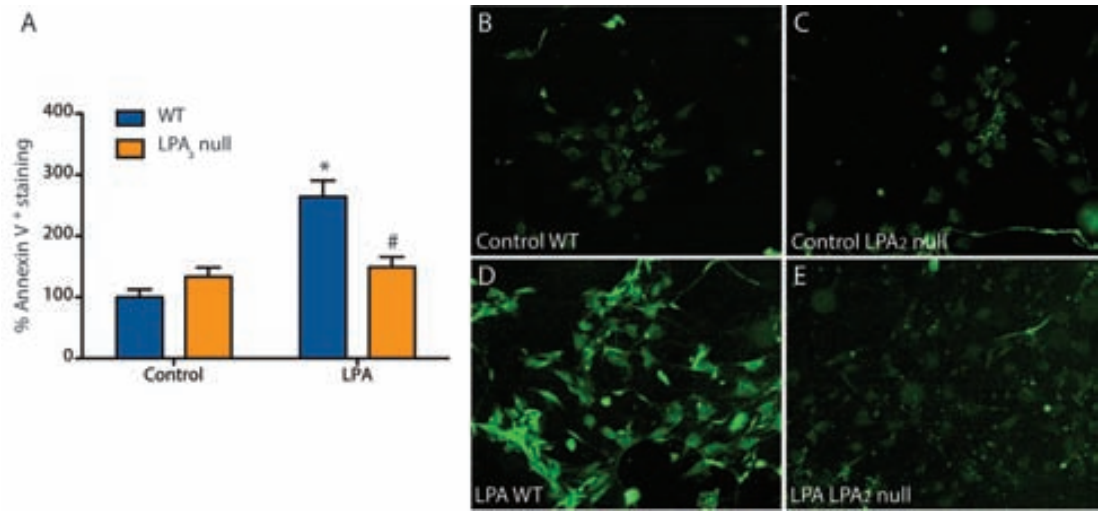

Figure 6. LPA induce DRG neuronal cell death *in vitro* through LPA_2 signaling. (A) Quantification of Annexin V^+ stained area on WT and LPA_2 null DRG neurons exposed to 1 μM LPA for 12 hours. (B-E) Representative micrographs showing annexin V staining on WT (B, D) and LPA_2 null (C, E) DRG neurons exposed to control media (B, C) or LPA (D, E). (*$p < 0.05$ vs. control; #$p < 0.05$ vs. LPA).

Discussion

In the present work we studied whether LPA_2 exerts harmful functions after SCI. We show that LPA_2 mRNA expression is up-regulated in the spinal cord following contusion injury beginning at 1 day post-injury and peaking at day 3. We demonstrate that LPA_2 activation is involved in triggering demyelination after intraspinal injection of LPA into the intact spinal cord. Moreover, we also reveal that lack of LPA_2 leads to better functional recovery and to reduced myelin and neuronal loss after SCI. Finally, we show through *in vitro* experiments that LPA_2 signaling in neurons leads to cell death whereas microglia LPA_2 induces the release of unknown factors that mediate toxicity in oligodendrocytes. These results provide clear evidence for the first time that LPA_2 contributes to secondary damage after SCI.

SCI is frequently companied by necrosis and apoptosis of neurons and oligodendrocytes, resulting in demyelination of axonal pathways

(Brosius Lutz and Barres, 2014; Mekhail *et al.*, 2012). Oligodendrocytes and neurons undergo cell death during the firsts days following SCI (Mekhail *et al.*, 2012), and it is induced by several mechanisms that occur in the spinal cord parenchyma as a consequence of the injury, such as the formation of reactive oxygen species, release of a myriad of inflammatory components, increase in Ca^{2+} influx, accumulation of high levels of glutamate in the extracellular milieu, and hypoxia, among others (Brosius Lutz and Barres, 2014; David *et al.*, 2012; Dumont *et al.*, 2001; Mekhail *et al.*, 2012).

Two recent works revealed that LPA may play an important role in secondary damage after central nervous system (CNS) injury (Crack *et al.*, 2014; Goldshmit *et al.*, 2012). These studies showed that the administration of the B3 antibody, which binds to LPA, and to lesser extend to other lysophospholipids, reduced tissue loss after spinal cord hemisection and brain trauma (Crack *et al.*, 2014; Goldshmit *et al.*, 2012). However, we have not observed any functional improvement when mice were treated with the B3 antibody after spinal cord contusion injury, a relevant clinical model of SCI (data not shown). This is likely due to the low penetrance of the antibody into the injured spinal cord and to the difficulty of the antibodies to block lipid compounds when they are not bounded to circulating albumin or other proteins. Nevertheless, in chapter I of the present thesis, we demonstrated that LPA contributes to secondary damage after SCI by signaling, at least through microglia LPA_1. However, whether other LPA receptors are involved in inducing degenerative processes after central nervous system trauma remains still unknown.

LPA_2, together with LPA_1 and LPA_3, belong to the endothelial differentiation gene LPA receptor family (Choi and Chun, 2013; Choi *et al.*, 2010). LPA_2 shows high homology to LPA_1 since it shares 60% amino acid similarity(Choi and Chun, 2013; Choi *et al.*, 2010). LPA_2 transcripts are expressed at high levels in the testis and leukocytes, whereas some other tissues, including the CNS, display moderate levels (An *et al.*, 1998; Choi and Chun, 2013). Herein we show LPA_2

is expressed in the spinal cord parenchyma and that its levels increase markedly for the first 3 days, suggesting that LPA_2 might be contributing to physiopathology of SCI.

LPA_2 couples with the same three types of G_α proteins as does LPA_1: $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$ (Choi and Chun, 2013). LPA_2 deficient mice do not show any phenotypical abnormality, and have normal prenatal and postnatal viability and expected Mendelian birth ratios (Contos *et al.*, 2002). Little is known about the functional role of LPA_2 in host tissues. LPA_2 activation is generally associated with cell survival and cell migration (Deng *et al.*, 2002; Goetzl *et al.*, 1999; Zheng *et al.*, 2000). Indeed, LPA_2 is involved in cancer, where its activity has been associated with invasion and metastasis of several types of cancer cells (Lee and Yun, 2010; Shida *et al.*, 2004). LPA_2 is also involved in some aspects of nervous system development and function (Choi and Chun, 2013; Kingsbury *et al.*, 2003). Activation of LPA_2 leads to synthesis of myelin P0 protein in cultured Schwann cells, implicating LPA_2 signaling in the myelination of the peripheral nervous system (Weiner *et al.*, 2001). However, it remains unknown whether LPA_2 plays a helpful of detrimental function after CNS disorders, including SCI.

In chapter I we revealed that intraspinal injection of LPA in the dorsal column of intact spinal cord leads to the formation of a demyelinating lesion. Moreover, we also showed that LPA-mediated demyelination was triggered by LPA_1 activation. However, we noticed that LPA_1 blockade, or gene deletion, did not completely avoid myelin loss after LPA injection, suggesting that other LPA receptors are involved in LPA-mediated demyelination. Here, we show that despite the low constitutive expression of LPA_2 in the spinal cord, LPA_2 deficient mice showed reduced demyelinating lesion after intraspinal injection of LPA, suggesting that LPA_2, together to LPA_1 signaling, contributes to LPA-mediated demyelination. This results might indicate that the production of LPA in the spinal cord parenchyma following injury lead to secondary damage. In agreement with our hypothesis, we found that mice lacking LPA_2 underwent greater functional recovery after contusion lesion of the spinal

cord. Interestingly, histopathological analysis reveal that LPA_2 deficient mice had greater neuronal and myelin sparing, and thus, directly relating LPA_2 signaling in oligodendrocytes and neuronal cell death.

In chapter I we found that LPA exerts mild, but significant, cytotoxicity on oligodendrocytes. However, we show herein that this is effect is not related to LPA_2 signaling since the lack of LPA_2 in oligodendrocytes did not confer protection upon LPA stimulation. Microglial cell expressed constitutively LPA_2 (data not shown). Interestingly, the lack of LPA_2 in microglial cells reduced in ~60% the toxicity in oligodendrocytes, suggesting a major contribution of microglia LPA_2 to oligodendrocyte cell death. Highlight, the loss of LPA_1 and LPA_2 activity in microglial cells stimulated with LPA did not cause oligodendrocyte cell death. These results indicate that microglial cells release unknown cytotoxic factors upon LPA stimulation via LPA_1 and LPA_2 activation.

A part from demyelination, we also observed that lack of LPA_2 confers neuronal protection after SCI. A previous *in vitro* study revealed that hippocampal neuronal cells, as well as P12 cells, undergo cell death upon LPA stimulation (Holtsberg *et al.*, 1998). In agreement with these observations we also found that LPA led to similar effect on DRG neuronal cells, indicating a direct cytotoxic effect of LPA on neurons. Interestingly, LPA_2 seems to play an important role in neuronal cell death, since that lack of LPA_2 in DGR neurons did not result in LPA-mediated cell death. Further studies are needed to investigate whether activation of microglia LPA_2 or even astrocyte LPA_2 leads to neuronal cell death. However, due to time constrains I was unable to perform these experiments in the laboratory, but these will be conducted during the next months.

Overall, the results of the present chapter provide clear and novel evidence that LPA_2 contributes to secondary damage in SCI. Since LPA_1 and LPA_2 signaling seems to exert harmful additive effects on cells death, our data suggest that selective blockade of LPA_1 and LPA_2 could led to a novel combinatory drug approach to reduce functional deficits in patients with acute SCI.

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Chapter III

Lysophosphatidic acid receptor type 3 (LPA_3) does not contribute to secondary damage after spinal cord injury

Lysophosphatidic acid receptor type 3 (LPA_3) does not contribute to secondary damage after spinal cord injury

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Abstract

Lysophosphatidic acid (LPA) is a molecular messenger that regulate many physiological functions in a myriad of cells by binding to 6 G protein-coupled receptors (GPCRs) known as LPA_{1-6}. In chapter I and II we show that LPA_1 and LPA_2 contribute to secondary damage after spinal cord injury (SCI). In the present study we aimed at assessing whether LPA_3 is involved in triggering tissue degeneration and functional deficits after spinal cord contusion in mice. We found, by doing Real Time-PCR analysis, that LPA_3 mRNA levels markedly up-regulated in the spinal cord parenchyma at 1 day post-injury. By using LPA_3 deficient mice we demonstrate that the lack of LPA_3 does not confer protection from functional deficits or myelin and neuronal loss. Similarly, we show that administration of a selective LPA_3 agonist does not improve locomotor skills after spinal cord contusion. Overall, this study therefore demonstrate that LPA_3 is not involved in the physiopathology of SCI.

Introduction

Injury to the spinal cord results in immediate (primary) damage followed by a secondary phase of tissue damage that occurs over a period of several weeks (Schwab and Bartholdi, 1996). There are multiple mechanisms that trigger secondary injury, however, a large number of studies suggest that the inflammatory response that occurs after SCI is one of the main contributors to secondary damage (David, López-Vales, *et al.*, 2012; Hawthorne and Popovich, 2011). Endogenous glial cells (microglia and astrocytes) and peripheral immune cells play a key role during the course of the inflammatory response after SCI (David, López-Vales, *et al.*, 2012; Hawthorne and Popovich, 2011). Although these cells are needed for efficient clearance of cellular and myelin debris and tissue healing, they also release several factors such as cytokines, free radicals, proteases, eicosanoids that cause damage to neurons, glia, axons and myelin (David, López-Vales, *et al.*, 2012; Hawthorne and Popovich, 2011). In addition, these toxic mediators can further activate glial cells and macrophages in a positive feedback loop, and thus, exacerbate secondary damage.

Lysophosphatidic acid (LPA; 2-acyl-sn-glycerol 3-phosphate) is a bioactive lipid that is found in high concentrations in serum, where it is produced by platelets in μM concentrations (Rivera and Chun, 2008) (Choi and Chun, 2013). LPA was originally described as a key intermediate in de novo lipid synthesis, but it has emerged as an intra- and extracellular phospholipid messenger with a wide variety of biological activities, including cellular proliferation, prevention of apoptosis, cell migration, cytokine and chemokine secretion, platelet aggregation, smooth muscle contraction, and neurite retraction (Rivera and Chun, 2008) (Choi and Chun, 2013). LPA exerts these variety of effects through specific GPCRs, such as LPA receptor 1, 2 and 3 (LPA_{1-3}) which belong to the Endothelial differentiation gene family (Edg), and the genetically more distant LPA_4 (GPR23 or P2Y9), LPA_5 (GPR92) and LPA_6 (P2Y5), which belong to the non-Edg LPA receptors (Rivera and Chun, 2008) (Choi and Chun, 2013).

In chapter I of the present thesis, we found that LPA is largely produced in the spinal cord parenchyma after contusion injury, contributing to tissue damage and functional disabilities of the mice. In the same chapter, together with the results from chapter II, we demonstrate that the harmful effects of LPA are mediate via microglia LPA_1 and LPA_2. However, it is still unknown whether LPA_3 also contributes to the physiopathology of SCI. Due to the wide variability of LPA receptors, it is important to dissect out the selective contribution of each LPA receptor in SCI. It is important to block specifically those LPA receptors that play detrimental actions after SCI without altering the activity of those that may trigger neutral or even helpful effects. This could lead to the development of a combinatory pharmacological approach to promote neuroprotection and reduce functional loss after SCI. In this work, we provide clear evidence that demonstrate that LPA_3 does not contribute to secondary injury after SCI.

Material and methods

Surgical procedure

All surgical procedures were approved by the Universitat Autònoma de Barcelona Animal Care Committee and followed the guidelines of the European Commission on Animal Care.

Adult (8-10 weeks old) female wildtype (WT) or LPA_3 deficient mice in a Balb/C background, and C57BL/6 female mice were anesthetized with ketamine:xylazine (90:10 mg/kg, i.m.). After performing a laminectomy at the 11^{th} thoracic vertebrae, the exposed spinal cord was contused. Spinal cord contusion injuries were performed using the Infinite Horizon Impactor device (Precision Scientific Instrumentation), using a force of 50 kdynes and tissue displacement ranging between 400 and 600 μm.

Stimulation of LPA_3 was done by administrating a selective LPA_3 agonist named OMPT (*(2S)-3-[(hydroxymercaptophosphinyl)oxy]-2-methoxypropyl ester, 9Z-octadecenoic acid, triethyl ammonium*) (Cayman Chemical). OMPT was intravenously administered diluted

in sterile saline at concentrations of 0.01, 0.1 and 1 mg/kg. Mice were administered 1 hour after SCI and once a day for one week.

Functional assessment

Locomotor recovery was evaluated at 1, 3, 5, 7, 10, 14, 21 and 28 days post-injury (dpi) in an open-field test using the nine-point Basso Mouse Scale (BMS) (Basso *et al.*, 2006), which was specifically developed for locomotor testing after contusion injuries in mice. The BMS analysis of hindlimb movements and coordination was performed by two independent assessors and the consensus score taken. The final score is presented as mean ± SEM.

The ability to run at a constant speed was analyzed at 28 dpi and maximum speed taken. Animals were placed on a belt of a motorized treadmill (DigiGaitTM Imaging System, Mouse Specifics, Boston, MA). Each mouse was allowed to explore the treadmill compartment, with the motor speed set to zero, for 5 min. Then speed was gradually increased from 0 up to 35 cm/s and stopped at the maximum speed at which each mouse was able to run. Maximum speed was that in which animals were able to perform for at least 5 seconds.

Histology

28 days after contusion injury, mice were deeply anaesthetized using Dolethal (pentobarbital sodium; Vétoquinol E. V. S. A.) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). 7 mm length of spinal cord containing the lesion site centered was harvested, post-fixed for 1 hour in 4% paraformaldehyde in 0.1 M PB and cryoprotected with 30% sucrose in 0.1 M PB at 4ºC, for a minimum of 48 hours. Spinal cords were fast-frozen at -60ºC in cryoembedding compound (Tissue-Tek® OCT, Sakura) and cut on a cryostat (Leica). Ten series of 10 µm-thick transversal sections were picked up on glass slides, so adjacent sections on the same slide were 100 µm apart.

For assessment of myelin sparing, tissue sections were stained with FluoroMyelin Green fluorescent myelin stain (Invitrogen). Briefly,

spinal cord tissue sections were rehydrated in PBS and incubated with FluoroMyelin (1:300) in PBS for 20 min at room temperature. Then, sections were washed several times in PBS and mounted in Mowiol mounting media containing DAPI (1 μg/ml; Sigma).

For neuronal sparing tissue sections were immunstained against NeuN. Spinal cord tissue sections were rehydrated in 0.1% Triton PBS (PBST), and blocked with 5% FBS in PBST (blocking buffer, BB) for 1 hour at room temperature. Sections were then incubated overnight at 4ºC with mouse anti-NeuN biotin conjugated (1:100, Chemicon). After several washes in PBST, sections were incubated for 1 hour at room temperature with Alexa 594-conjugated streptavidin (1:500, Invitrogen) diluted in BB. Finally, sections were washed with PBST, PBS and PB and coverslipped in Mowiol mounting media containing DAPI. Tissue sections were viewed with Olympus BX51 microscope and images were captured using an Olympus DP50 digital camera attached to it and using the Cell^A Image acquisition software.

The epicenter of the injury was determined for each mouse spinal cord by localizing the tissue section with the greatest demyelination. Myelin sparing after SCI was calculated by delineating the spared Fluoromyelin stained tissue, whereas neuronal survival was assessed by counting the number of $NeuN^+$ cells in the ventral horns. All quantifications were done at the epicenter and in sections every 200 μm to 1400 μm rostral and caudal from the epicenter, using the ImageJ software.

Genotyping PCR

PCR analysis was used to detect the presence of WT and *lpar3* mutated allele. For tissue sampling, the tip of the tail was cut at the time of weaning the mice. Genomic DNA extraction was carried out by using the ArchivePure DNA Purification System (5PRIME), as described by the manufacturer. Allele amplification was performed by PCR reaction, using the Taq DNA Polymerase kit (Invitrogen), and PCR products were analyzed by standard electrophoresis in 5% agarose gels. Primers sequences were the following: LPA_3

forward 5′-GAAGAAATCCGCAGCAGCTAA-3′, LPA_3 reverse 5′-TGACAAGCGCATGGACTTTTTC-3′, LPA_3 mutated 5′-AGCGCCTCCCCTACCCGGTAGAAT-3′. Product sizes for WT and targeted alleles were 216 bp and 170 bp, respectively.

RNA isolation, reverse transcription and real-time PCR

For spinal cord mRNA extraction, C57BL/6 mice were perfused with sterile saline and 5 mm length of uninjured or injured spinal cord centered at the impact site was harvested at 1, 3, 7, 14, 21 and 28 dpi. Tissue was homogenized with QIAzol lysis reagent (Qiagen) and RNA extracted using RNeasy Lipid Tissue kit (Qiagen), according to the manufacturer's protocol. RNA was treated with DNaseI (Qiagen) to eliminate genomic DNA contamination. 1 µg of obtained RNA was primed with random hexamers (Promega) and reverse transcribed using Omniscript RT kit (Qiagen). RNase inhibitor (Roche) was added (1 U/µl final concentration) to avoid RNA degradation. Real Time (RT)-PCR analysis was performed using a MyiQ Single-Color Real-Time PCR Detection System (BIO RAD). The GAPDH housekeeping gene was selected as the reference gene. RT-PCR reactions were performed using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) according to the manufacturer's instructions. Primer sequences were the following: LPA_3 forward 5'-GGGACGTTCTTCTGCCTCTTTA-3'; LPA_3 reverse 5'-GAAAGTGGAACTTCCGGTTTGT-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene: GAPDH forward 5'- TCAACAGCAACTCCCACTCTTCCA-3'; GAPDH reverse 5'- ACCCTGTTGCTGTAGCCGTATTCA-3'.

The amount of cDNA was calculated based on the threshold cycle (CT) value, and was standardized by the amount of housekeeping gene using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001).

Statistical analysis

All analyses were conducted through IBM SPSS Statistics v19. LPA_3 expression in the contused spinal cord by RT-PCR was analyzed

using one way ANOVA with *post-hoc* Bonferroni's test. BMS score and histological parameters were analyzed using two-way repeated measure (RM)-ANOVA with *post-hoc* Bonferroni's test. Maximal speed on a treadmill was analyzed by using t-test. Results are expressed as mean ± SEM. Differences were considered significant at $p < 0.05$.

Results

Expression of LPA_3 in the injured and uninjured mouse spinal cord

We assessed mRNA levels of LPA_3 after SCI using RT-PCR. The results showed that mRNA levels for LPA_3 were 6.8 ± 0.75 fold increased at 24 hours of injury (*post-hoc* Bonferroni's $p = 0.001$; one-way ANOVA; n = 3 per group) (Figure 1). This rapid raise in the LPA_3 mRNA levels suggests a role of this receptor in the pathophysiology of SCI.

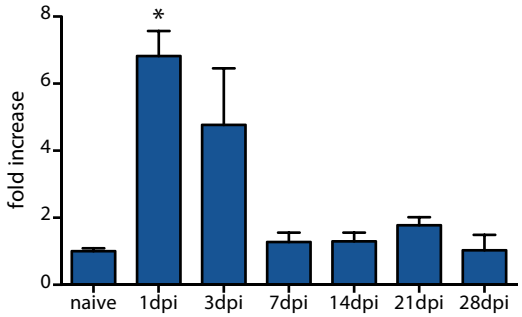

Figure 1. LPA_3 mRNA expression is up-regulated in the injured spinal cord. LPA_3 mRNA levels quantified by Real Time-PCR in the spinal cord harvested at different time points after SCI. Note the marked up-regulation in LPA_3 mRNA levels at 24 hours post-injury (*$p < 0.05$).

The lack of LPA_3 does not improve functional recovery after SCI

To study the potential role of LPA_3 in SCI, we induced contusion injuries in mice deficient for LPA_3. Our results reveal that LPA_3 deficient mice did not result in enhancement of motor skills assessed by the BMS score (two way RM-ANOVA; n = 10 for WT and n = 9 for

LPA_3 null mice) (Figure 2A). Similarly, evaluation of the faster speed after forced locomotion on a treadmill did not reveal any significant improvement in mice lacking LPA_3 signaling (Figure 2B) (*t* test). In line with functional results, histological analyses revealed that the lack of LPA_3 did not confer protection from myelin or neuronal loss (Figure 2 C-H).

Treatment with OMPT does not promote functional recovery after spinal cord injury.

Since the lack of LPA_3 did not reduced secondary damage after SCI, we then evaluated whether activation of LPA_3 signaling exerted some beneficial actions on functional recovery. In order to conduct these experiments, we administered OMPT, a selective LPA_3 agonist, to C57BL/6 mice beginning at 1 hour after injury and once a day for one week. We first carried out a pilot experiment in which animals were intravenously administered with several concentrations of 0.01, 0.1 and 1 mg/kg OMPT or saline. Although we did not observe significant differences in locomotor skill upon OMPT administration (two way RM-ANOVA; n = 4 per group), the groups treated with the dose of 0.1 and 1 mg/kg showed worst functional recovery as compared to the saline-treated mice (Figure 3A). Since the dose of 0.1 and 1 mg/kg seemed to exert similar effects, we chose the lower dose for conducting the definitive functional experiments. Similarly, but in a larger group of mice, we showed that the administration of the LPA_3 agonist did not promote functional outcomes (two way RM-ANOVA; n = 8 for saline and n = 9 for OMPT) (Figure 3B). Moreover, mice treated with OMPT did not display greater locomotor speeds after forced locomotion on a treadmill (data not shown).

Discussion

In chapter I and II, we showed that LPA by signaling via LPA_1 and LPA_2 contributes to secondary damage after SCI. In the present chapter we reveal that LPA_3 does not contribute to functional deficits and tissue damage after SCI. We show that LPA_3 mRNA levels are up-

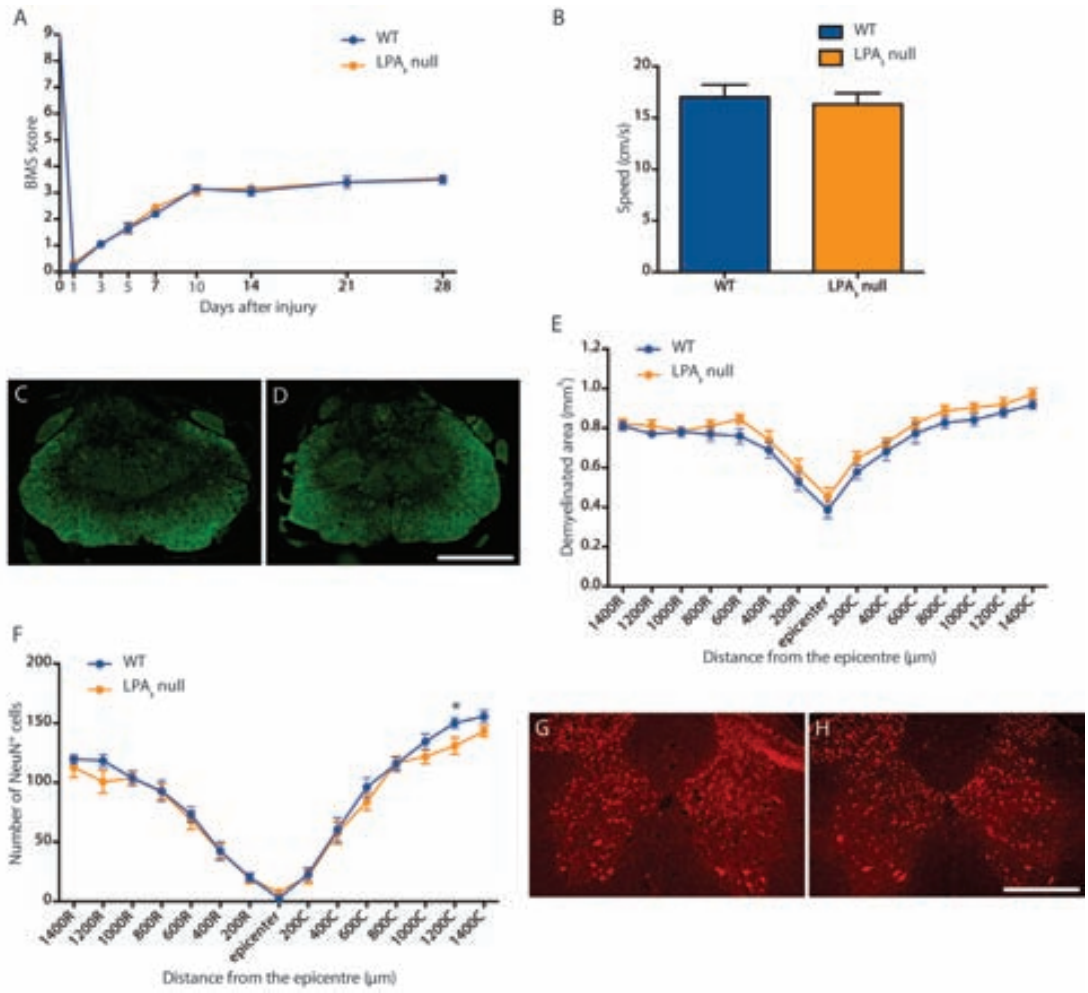

Figure 2. The lack of LPA_3 does not promote any functional improvement after SCI. (A) Locomotor recovery after spinal cord contusion injury in WT and LPA_3 null mice using the 9-point Basso Mouse Scale. The lack of LPA_3 did not promote any improvement in locomotor performance compared to WT mice. (B) The ability to run at a constant speed was evaluated at 28 dpi by placing mice onto the belt of a motorized treadmill. Mice lacking LPA_3 were not able to run at faster speeds compared to WT. (E) Quantification of myelin sparing at various distances rostral and caudal to the injury epicenter. (C, D) Representative micrographs of spinal cord tissue showing myelin sparing at the injury epicenter in WT (C) and LPA_3 deficient (D) mice. (F) Quantification of ventral horn neuron survival at various distances rostral and caudal to the injury epicenter. (G, H) Representative micrographs show sparing of ventral horn neurons at 600 µm rostral to the injury epicenter in WT (G) and LPA_3 null (H) mice spinal cords. (*$p < 0.05$). Error bars indicate SEM. Scale bar = 500 µm in C, D; 250 µm in G, H.

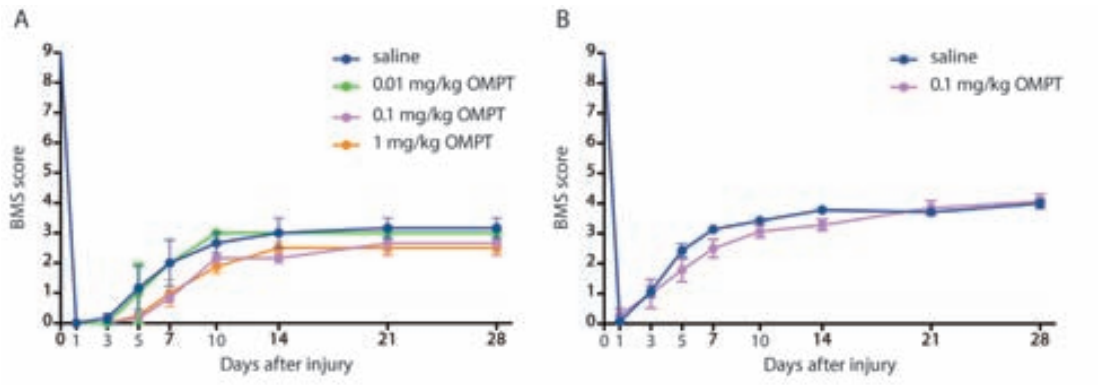

Figure 3. Administration of OMPT does not promote any functional recovery after SCI. (A) Locomotor recovery was assessed after spinal cord contusion injury in a dose-response pilot experiment for OMPT dose determination using the 9-point Basso Mouse Scale. Doses of 0.01, 0.1 and 1 mg/kg were intravenously administered. Mice administered with the higher OMPT doses (1 and 0.1 mg/kg) show a tendency to a worse locomotor performance compared to control mice. (B) Locomotor recovery was assessed after spinal cord contusion injury in animals administered with the selected 0.1 mg/kg OMPT dose. Treatment does not show significant differences in locomotor performance compared to control mice.

regulated ~6 times in the contused spinal cord, suggesting that this LPA receptor might be involved in detrimental or restorative processes after SCI. Surprisingly, we reveal that mice lacking LPA_3 does not show enhancement in motor skills or in myelin/neuronal sparing. Similarly, we show that administration of a LPA_3 has no significant effect on functional deficits and tissue damage. Overall, our results indicates that LPA_3 is not a central contribution of secondary damage in SCI.

LPA_3 is the third member of the Edg family LPA receptors, sharing ~50% identical in amino acid sequence to LPA_1 and LPA_2. Like both, LPA_1 and LPA_2, LPA_3 can couple with $G_{\alpha i/o}$ and $G_{\alpha q/11}$ to induce phosphatidylcholine (PLC) activation, Ca^{2+} mobilization, adenyl cyclase inhibition and activation, and MAPK activation (Choi and Chun, 2013). LPA_3 expression is found in the human heart, testis, prostate, pancreas, lung, ovary, and brain (Choi *et al.*, 2010; Im *et al.*, 2000; Ye *et al.*, 2005) and is most prominent in the mouse testis, kidney, lung, small intestine, heart, stomach, spleen, brain, and thymus. Despite the

fact that LPA_3 is expressed in several regions of the brain, including frontal cortex, hippocampus, and amygdala (Im *et al.*, 2000; Ye *et al.*, 2005), no phenotypes related to LPA_3 loss in the nervous system have been reported to date.

$LPA_3^{-/-}$ mutant mice, however, have delayed embryo implantation, embryo crowding, and reduced litter size that were traced to maternal effects, based on transfer of wild-type embryos into $LPA_3^{-/-}$ dams that failed to implant normally (Ye *et al.*, 2005). These defects are remarkably similar to the phenotypes of mice lacking COX-2, the limiting enzyme in the production of prostaglandins. Indeed, exogenous administration of prostaglandins to $LPA_3^{-/-}$ dams rescues the delayed implantation and reduced litter sizes, suggesting that LPA_3-mediated signaling appears to be upstream of prostaglandin synthesis in this system (Ye *et al.*, 2005). Similarly, mice lacking the cytosolic form of phospholipase A2 group IV ($cPLA_2$ GIV), which is upstream of COX-2 signaling, show similar defects (Bonventre *et al.*, 1997), indicating a link between LPA_3, $cPLA_2$ GIV and COX-2.

PLA_2s and COX-2 are key enzymes that regulate several physiological functions, including the onset, progression and resolution of the inflammatory response (David, Greenhalgh, *et al.*, 2012). Several works have shown that various forms of PLA_2 and COX-2 are involved in activating degenerative and repair processes in SCI and other neural conditions (David, Greenhalgh, *et al.*, 2012; López-Vales *et al.*, 2011). In chapter I and II we showed that LPA plays a key role in inflammation, driving microglial cells towards a cytotoxic phenotype via signaling via LPA_1 and LPA_2, and contributes to secondary damage in SCI. However, whether LPA_3 also contributes to tissue degeneration in SCI remains unknown.

Herein, we show that LPA_3 levels are marked increased in the spinal cord parenchyma at day 1 post-injury. The cell source of LPA_3 in the spinal cord is currently unknown since all the commercial antibodies for LPA_3 we tested showed unspecific binding in spinal tissue sections and homogenates harvested from LPA_3 knockout mice (data not

shown). However, several *in vitro* studies reveal that astrocytes and microglia cells express LPA_3 at the mRNA level (Rao *et al.*, 2003; Tham *et al.*, 2003). Moreover, microglial cells upregulate LPA_3 upon stimulation with lipopolysaccharide (Tham *et al.*, 2003). These works, together with its relation to PLA_2-COX-2 pathway as well as to our RT-PCR data from contused spinal cords, suggest that LPA_3 signaling could exert a major function in secondary damage SCI.

Little is known about the role of LPA_3 in human pathologies. A previous study suggests that LPA_3 mediates tissue damage in renal ischemia, since administration of OMPT, a LPA_3 agonist, resulted greater degree of injury (Okusa *et al.*, 2003). However, it remains unknown whether LPA_3 exerts harmful effects in neural conditions. A recent work shows that intrathecal LPA triggers the production of LPA in the central nervous system via signaling LPA_3, indicating a feed-forward LPA production (Ma *et al.*, 2009). This data suggest that the lack of LPA_3 may lead to reduce LPA levels in the injured spinal cord, and consequently, mitigate tissue damage and functional deficits. Another work shows that LPA_3 is involved in neuronal branching, and thus, suggesting a role for this receptor in neuronal plasticity and functional recovery. Herein we show for the first time that LPA_3 is not involved in secondary damage in SCI.

Overall, the results of the present paper suggest that unlike LPA_1 and LPA_2, LPA_3 is not involved in the course of secondary damage after SCI. Although a previous work showed that blockade of LPA mediate functional recovery after SCI, the data of the present work suggest that there is a need to selectively block the LPA_1 and LPA_2 receptors in SCI, in order to not interfere with the physiological functions of LPA_3 in host tissues.

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DISCUSSION

Discussion

Inflammatory response is an important component of the secondary damage events that occur after spinal cord injury (SCI) (Donnelly and Popovich, 2008). This complex and poorly understood response is initiated in the spinal cord parenchyma during the first hours after injury and persist active for several months or even years, contributing to tissue damage and functional loss (Fleming *et al.*, 2006; Norenberg *et al.*, 2004). In this context, lysophospholipids have emerged as a novel class of lipids mediators that regulate important aspects of the inflammatory response (David *et al.*, 2012; Sevastou *et al.*, 2013).

Lysophospholipids are membrane-derived phospholipids that can originate from homeostatic lipid metabolism or as a response to stimulus-induced cellular activation (Sevastou *et al.*, 2013). They are classified in lysoglycerophospholipids and lysosphingolipids, being LPA and lysophosphatidylcholine (LPC) the most representative for the first, and sphingosine 1-phosphate for the second (Sevastou *et al.*, 2013).

LPC is believed to play a crucial role in the inflammatory processes in the central nervous system (CNS), since intraspinal injection of LPC triggers a rapid induction in the expression of cytokines and chemokines, accompanied by a robust activation of macrophages/microglia and influx of T cells, (Ghasemlou *et al.*, 2007; Ousman and David, 2000; 2001). Moreover, LPC promotes and stabilizes macrophage polarization towards an M1 proinflammatory phenotype (Qin *et al.*, 2014), which are known to mediate cytotoxic actions in the CNS (Kigerl *et al.*, 2009; Kroner *et al.*, 2014).

A part from inflammation, LPC has been widely described as a potent demyelination agent, which has been extensively used for the study of demyelination and remyelination responses in the central

and peripheral nervous system (Blakemore *et al.*, 1977; Hall, 1973; Harrison, 1985; Kotter *et al.*, 2001; Ousman and David, 2001). These evidences therefore highlight the importance of lysophospholipids to course of the neuroinflammatory response, and suggest that one potential therapeutic intervention to avoid the detrimental actions of lysophospholipids in neural disorders is to target the enzymes responsible of their synthesis.

Lysophospholipids are generated by the action of the phospholipase family enzymes, being phospholipase A2 (PLA_2) the most important member. PLA_2 hydrolyzes oxidized LPC resulting in the production of the majority of lysophospholipids (Qin *et al.*, 2014). Moreover, this hydrolysis also results in the production of fatty acids, such as arachidonic acid, which can also be metabolized into proinflammatory eicosanoids. Eicosanoids, as prostaglandins, leukotrienes and thromboxanes, are potent mediators of inflammation by increasing vascular permeability and inducing chemotaxis of immune cells (Dennis *et al.*, 1991; Funk, 2001; Murakami *et al.*, 1997). There are several types of PLA_2 enzymes, which include secreted ($sPLA_2$) and cytosolic forms. Cytosolic PLA_2 are also divided in calcium-dependent ($cPLA_2$) and independent ($iPLA_2$) PLA_2. More than one third of the mammalian PLA_2 enzymes belong to the $sPLA_2$ family, and mouse and human genomes encode for 11 to 12 $sPLA_2$ enzymes. The different $sPLA_2$ isoforms has specific tissue and cellular location, as well as enzymatic properties, suggesting a range of distinct biological roles including inflammatory pathologies as asthma (Henderson *et al.*, 2007) or inflammatory arthritis (Boilard *et al.*, 2010), cardiovascular diseases (Leite *et al.*, 2009), metabolic disorders such as adipogenesis and obesity (Li *et al.*, 2010), male fertility (Sato *et al.*, 2010), and nervous system pathologies, such as multiple sclerosis (Kalyvas *et al.*, 2009; Marusic *et al.*, 2005; 2008), SCI (López-Vales *et al.*, 2011) brain injury (Bonventre *et al.*, 1997; Tabuchi *et al.*, 2003) and peripheral nerve injury (De *et al.*, 2003; López-Vales *et al.*, 2008).

PLA_2 activity and protein levels are increased after SCI, with PLA_2

expression localized in neurons and oligodendrocytes (Liu *et al.*, 2006). Further investigation demonstrates that different PLA_2s subtypes play differing roles in SCI, with some of them mediating protection after injury, such as $cPLA_2$, whereas others, such as $sPLA_2$ and $iPLA_2$, exerting detrimental actions (López-Vales *et al.*, 2011). The divergent effects of the different PLA_2 enzymes in SCI is probably due to the myriad of metabolites they generate. Fatty acids and lysophospholipids released by the action of PLA_2 give rise to over two-dozen bioactive lipid mediators, which are classified into three classes. The first includes metabolites of arachidonic acid, which, as mentioned before, are referred to as eicosanoids. The second comprehends lyosophospholipids such as LPC, platelet-activating factor, LPA, sphingosine 1-phosphate, endocannabinoids, and LPA, the form we focused on this thesis. Additionally, a third class of metabolites includes the newly identified group of dual anti-inflammatory and pro-resolution mediators derived from omega-3 polyunsaturated fatty acids, which bind to specific receptors that activate cellular programs that shut off inflammation (David *et al.*, 2012). Since PLA_2s generate both pro-inflammatory mediators and several lipids that are important to regulate and resolve inflammation, inhibition of PLA_2s enzymes may block the synthesis of molecules that exert harmful and helpful effects in the physiopathology of SCI, and thus, lead to weak therapeutic effects. Therefore there is a need to block selectively those lipid mediators generated by PLA_2s that mediate harmful actions in CNS conditions.

LPA is a PLA_2-derived bioactive phospholipid, generated from phosphatidic acid, or from LPC by the hydrolysis of the choline head by autotaxin. LPA acts through six GPCRs (LPA_{1-6}), which belong to the endothelial gene differentiation (Edg) family LPA receptors (LPA_{1-3}), and to the non-Edg family LPA receptors (LPA_{4-6}). In the present thesis we showed that all of them are expressed constitutively in the spinal cord parenchyma.

Edg family LPA receptors share high homology to the sphingosine-1 phospate (S1P) receptor, which plays an important role in

demyelination. Indeed, fingolimod—known in the scientific literature as FTY720 and commercially as Gilenya™ (Novartis AG, Basel)—is the first oral treatment for relapsing forms of multiple sclerosis. Now approved worldwide, fingolimod is phosphorylated to become a non-selective S1P receptor modulator and represents the first compound targeting lysophospholipid receptors that has become a human medicine (Brinkmann *et al.*, 2010). Nowadays, there is emerging data demonstrating that LPA is also involved in the course of several pathological conditions. Nevertheless, whether LPA receptors are involved in triggering tissue damage after CNS injuries or diseases is currently not fully known. A recent work reveals that the administration of the B3 antibody, which binds to LPA and other lysophospholipids preventing them from interacting with their receptors, promotes functional recovery after spinal cord hemisection in mice (Goldshmit *et al.*, 2012). Despite the hemisection model is not a clinical relevant model of SCI, this study provided the first insights on the potential deleterious actions of LPA in SCI. More recently, the B3 antibody also showed to promote neuroprotection after brain trauma (Crack *et al.*, 2014). Due to the wide variety of LPA receptors it is likely that LPA may exert helpful or harmful effects in the CNS depending on the receptors it signals through, as it has been already observed with other lipid mediators, such as prostaglandins (Kawano *et al.*, 2006; Kerr *et al.*, 2008; Liang *et al.*, 2011; McCullough *et al.*, 2004; Redensek *et al.*, 2011). Indeed, we tested the efficacy of the B3 antibody after spinal cord contusion injury and did not observed any improvement in functional outcomes at the end of the follow up (data not shown). There is therefore a need to know which LPA receptors contribute to secondary damage after SCI, and those, if any, that mediate neuroprotection.

In this thesis we studied the individual contribution of each of the Edg family LPA receptors ($LPA_{1\text{-}3}$) in the pathophysiology of the SCI, and investigated the mechanisms that underlie their harmful effects. We demonstrate that LPA plays an important role in promoting demyelination and neuronal cell death after spinal cord contusion

injury, and consequently functional impairments.

Oligodendrocytes are particularly susceptible to cell death after SCI. They undergo necrosis and apoptosis acutely after injury, but large number of oligodendrocytes also suffer apoptosis at chronic stages of SCI, and at distant regions from the injury epicenter (Profyris *et al.*, 2004).

The death of oligodendrocytes causes myelin loss. Although myelin was first thought to be mainly a fundamental structure to facilitate speed of axonal conduction, it also provide trophic support to axons that are metabolically isolated and support their integrity (Nave, 2010). This is manifested by the fact that axonal durability after SCI depends on the presence of myelin, as myelinated axons have been found to be more vulnerable than unmyelinated ones (Maxwell, 1996). Demyelination that occurs after SCI significantly contributes to functional impairments, and thus, the study of the molecular mechanisms that cause myelin loss after injury has emerged as a key research field. Therapeutic interventions aimed at reducing oligodendrocyte death and/or enhancing remyelination after SCI may lead to improvement in neurological outcomes and may become of tremendous value to individuals living with SCI. In this line, rolipram, an inhibitor of phosphodiesterase 4, an enzyme that hydrolyze cAMP and that is expressed in oligodendrocytes, has been found to significantly attenuate oligodendrocyte cell death after SCI (Whitaker *et al.*, 2008). PTEN also promoted neural cell survival and oligodendrocyte-mediated myelination of axons (Walker and Xu, 2014). However, neuropsin, a serine protease expressed by oligodendrocytes after injury, has been found to promote oligodendrocyte death, demyelination and axonal degeneration (Terayama *et al.*, 2007).

In the present thesis, we reveal that LPA is a new factor involved in demyelinating events in the CNS. We showed that LPA levels increase very rapidly in the spinal cord parenchyma after injury and that remained at high levels for at least 2 weeks. The increase in LPA levels during the first hours post-injury is likely due to serum production,

since PLA_2 enzymes are not up-regulated until 24 hours following trauma. However, from 1 day to day 14, the last time point evaluated, LPA synthesis is likely produced by neural and infiltrating cells in the spinal cord by actions of PLD and PLA_2 activity, since these enzymes are up-regulated after SCI, whereas autotaxin is downregulated.

Raised LPA levels after SCI may therefore signal on the distinct cells and generate a variety of physiological responses depending on the LPA receptor it signals through. Interestingly, we found that a substantial increase in LPA levels in the spinal cord leads to demyelination as revealed our experiments using intraspinal injection of LPA. These findings suggest that the production of LPA in the spinal cord parenchyma following contusion injury may lead to demyelination. We also showed, using *in vitro* experiments, that LPA exerts only mild toxicity on oligodendrocytes, suggesting that LPA-mediated demyelination is likely mediated by the activation of LPA receptors in glial cells, rather to a direct effect of LPA in oligodendrocytes.

Inflammation is one of the main contributors to oligodendrocyte cell death after SCI. Microglial cells under *in vitro* conditions, as well as *in vivo* microglia cells sorted from uninjured and injured spinal cord, express LPA_1 and LPA_2. Interestingly, we found that intraspinal injection of LPA into the intact spinal cord parenchyma led to the development of an inflammatory response. This effect was especially evident in microglia, but also in astrocytes although to a lesser extent. Thus, this data suggest that the stimulation of LPA receptors in microglia induces their activation. Interestingly, we found that the conditioned medium of microglia stimulated with LPA led to remarkable induction in oligodendrocyte cell death. This would indicate that microglia release some cytotoxic mediators when exposed to LPA. In the search of the LPA receptors that are involved in these harmful effects, we found that microglia LPA_1 and LPA_2 signaling is responsible for oligodendrocyte cell death, suggesting that activation of microglia LPA_1 and LPA_2 may trigger demyelination in the spinal cord parenchyma. These observations were further validated *in*

vivo, since demyelination was significantly reduced when LPA was injected into the intact spinal cord of mice lacking LPA_1 signaling (by using a selective LPA_1 antagonist or LPA_1 deficient mice) as well as in mice deficient for LPA_2. Furthermore, LPA_1 blockade or LPA_2 deletion also resulted in significant reduction of myelin loss after spinal cord contusion injury. Altogether, our data provides clear evidence demonstrating that the increase in LPA levels after SCI activates LPA_1 and LPA_2 in microglial cells, leading to demyelination and functional loss.

Although LPA_1 and LPA_2 can couple to the same G proteins, it is likely that they couple to different G proteins if found in the same cell. If so, this would imply that LPA may activate, via LPA_1 and LPA_2, divergent intracellular pathways to mediate cytotoxicity. Actually, we observed that the simultaneous blockade of LPA_1 and LPA_2 signaling in microglial cells completely prevented LPA-triggered oligodendrocyte cell death, indicating that microglia LPA_1 and LPA_2 mediate toxicity through independent intracellular cascades. These results are of high relevance because suggest that the development of a combined therapy targeting both, LPA_1 and LPA_2, may lead to synergic effect in promoting myelin preservation after SCI and thus further improve functional recovery. This study has not been yet carried out but will be conducted in the laboratory in the future.

The harmful mediators released by microglia cells upon activation of LPA_1 and LPA_2 are not known yet. Several studies reveal that cytokines produced by microglial cells, such as TNF-α and IL-1β, are potent promoters of oligodendrocyte cell death (D'Souza *et al.*, 1995; Takahashi *et al.*, 2003). We studied the protein levels of 25 cytokines in the conditioned medium of LPA-primed microglia. We observed that LPA induced mild production (increase < 2 fold) in just a few number of cytokines (data not shown). However, the lack of LPA_1 or LPA_2 signaling did not altered cytokine expression (data not shown). Other works also reveal that microglia mediates oligodendrocyte cell death through the production of nitric oxide (Merrill *et al.*, 1993).

However, the levels of nitrate/nitrite in the conditioned medium of LPA stimulated microglia were significantly reduced (data not shown). Therefore, our preliminary analyses indicate that the toxic factors released by microglia upon LPA stimulation are neither cytokines nor nitric oxide. Due to time constrains I was unable to identify such cytotoxic mediators, however, this will be further investigated in our laboratory.

A part from oligodendrocyte loss, death of neuronal cell also occurs after SCI, leading to disruption of neural circuits, axonal pathways and consequently, to functional loss (Profyris *et al.*, 2004). LPA has been previously found to induce cell death in primary hippocampal neurons and in the PC12 cell line. High concentrations of LPA (50 μM) causes neuronal cell death by necrosis, while lower doses of LPA (0.1 and 1 μM) induces apoptosis (Steiner *et al.*, 2000). Recent experiments *in vivo* has also shown that LPA leads to cell death after spinal cord hemisection or brain trauma (Crack *et al.*, 2014; Goldshmit *et al.*, 2012). In the present thesis, we found that the lack of LPA_2 reduced neuronal loss after SCI. We also observed that DRG neurons underwent apoptosis when exposed to LPA, and the lack of LPA_2 prevented the activation of programmed cell death. These results therefore indicate that the increase of LPA levels in the contused spinal cord tissue activates neuronal LPA_2 and induce cell death. We do not discard that LPA_2 signaling in microglial cells may also contribute to neuronal loss after SCI, and this will be assessed in the laboratory.

LPA_3, the third member of the Edg family LPA receptors, couple to the same G proteins and share high homology in aminoacid sequence to LPA_1 and LPA_2. Strikingly, we found that LPA_3, in contrast to LPA_1 and LPA_2, is not involved in triggering secondary damage after SCI. This data therefore indicates that although LPA contributes actively to the physiopathology of SCI, not all the LPA receptors exert noxious actions. Indeed, we do not discard the possibility that some of the LPA receptors belonging to the non-Edg family could even mediate protection. This would reinforce our idea about the importance of

generating an adjusted therapy in which only the LPA receptors that mediate tissue damage are blocked, whereas the activity of those that are not involved in the pathophysiology of SCI is maintained. There is therefore a need to elucidate the role for the non-Edg family receptors (LPA_{4-6}) in SCI, which will be assessed in the laboratory.

Overall, this thesis reveal that the increase in LPA levels in the contused spinal cord contributes to demyelination and neuronal loss by signaling through microglia LPA_1 and LPA_2 and neuronal LPA_2. Since microglia activation, as well as myelin and neuronal loss, are key events that occurs in several CNS disorders, targeting LPA receptors may lead to the development of a new pharmacological approach to treat a wide range of neural conditions.

CONCLUSIONS

Conclusions

Chapter I:

- LPA levels are increased in the spinal cord parenchyma after contusion injury.
- Injection of LPA into the intact spinal cord leads to inflammation and demyelination.
- LPA_1 is the LPA receptor expressed at higher constitutive levels in the spinal cord.
- Gene deletion or pharmacology inhibition of LPA_1 partially reverts demyelination after intraspinal injection of LPA.
- Administration of AM095, a LPA_1 antagonist, reduces functional deficits and myelin loss after spinal cord contusion injury.
- *In vitro* exposure of cultured oligodendrocytes to LPA leads to mild induction of cell death. However, pharmacological blockade of oligodendrocyte LPA_1 does not revert cell death.
- *In vitro* exposure of cultured oligodendrocytes to conditioned medium of LPA-stimulated microglial leads to marked reduction in oligodendrocyte survival. However, this effect is partially prevented when microglia LPA_1 is inhibited.

Chapter II:

- mRNA levels of LPA_2 are up-regulated in the spinal cord after contusion injury.
- The lack of LPA_2 signaling partially prevents demyelination when LPA is injected into the uninjured spinal cord.

- LPA_2 deficient mice display reduced functional deficits as well as greater myelin and neuronal sparing after spinal cord contusion injury.
- *In vitro* exposure of cultured LPA_2 deficient oligodendrocytes to LPA does not revert cell death.
- Oligodendrocyte cell death is markedly prevented when exposed to conditioned medium of LPA-stimulated LPA_2 deficient microglia. Moreover, oligodendrocyte cell death is fully avoided when exposed to conditioned medium of LPA-stimulating microglia lacking LPA_1 and LPA_2 signaling.
- *In vitro* stimulation of cultured DRG neurons to LPA leads to apoptosis, which is prevented in the lack of LPA_2 signaling.

Chapter III:

- mRNA expression of LPA_3 is up-regulated in the spinal cord after contusion injury.
- The lack of LPA_3 or its stimulation has no effects on functional recovery and tissue damage after spinal cord contusion injury.

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ABBREVIATIONS

List of abbreviations

AIS	ASIA Impairment Scale
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AM095	*2-[4-[4-[3-methyl-4-[[(1R)-1-phenylethoxy] carbonylamino]-1,2-oxazol-5-yl]phenyl] phenyl]acetic acid*
ASIA	American Spinal Injury Association
ATP	Adenosine triphosphate
ATX	Autotaxin
BB	Blocking buffer
BDNF	Brain-derived neurothophic factor
BMS	Basso Mouse Scale
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CMAP	Compound Muscle Action Potential
CNS	Central Nervous System
CNTF	Ciliary neurotpophic factor
COX	Cyclooxygenase
DGK	Diacylglycerol kinase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DRG	Dorsal root ganglion
Edg	Endothlial differentiation gene
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular-signal-Regulated Kinase

FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial-cell derived neurothophic factor
GFAP	Glial Fibrillary Acidic Protein
GPAT	Glycerophosphate acyltransferase
GPCR	G protein-coupled receptor
Gr-1	Myeloid differentiation antigen
GTPase	Guanosine triphosphatase
G3P	Glycerol-3-phosphate
HEPES	*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*
Iba1	Ionized calcium binding adaptor molecule 1
IFN-γ	Interferon-γ
IL	Interleukin
KO	Knock Out
LFB	Luxol Fast Blue
LPA	Lysophosphatidic acid
LPA_1	Lysophosphatidic acid receptor 1
LPA_2	Lysophosphatidic acid receptor 2
LPA_3	Lysophosphatidic acid receptor 3
LPA_4	Lysophosphatidic acid receptor 4
LPA_5	Lysophosphatidic acid receptor 5
LPA_6	Lysophosphatidic acid receptor 6
LPC	Lysophosphatidylcholine
LPL	Lysophospholipid
MAPK	Mitogen-Activated Protein Kinase
MBP	Myelin Basic Protein
MEM	Minimum Essential Media

MEP	Motor Evoked Potential
MGCM	Mixed Glial Culture Medium
mRNA	Messenger RNA
NASCIS	National Acute Spinal Cord Injury Studies
NeuN	Neuronal Nuclei
NGF	Nerve Growth Factor
NMDA	N-methyl-D-aspartate
NT-3	Neurotrophin-3
OMPT	*(2S)-3-[(hydroxymercaptophosphinyl)oxy]-2-methoxypropyl ester, 9Z-octadecenoic acid, triethyl ammonium salt*
OPC	Oligodendrocyte progenitor cell
PA	Phosphatidic acid
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
PBST	Triton-PBS
PL	Phospholipid
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PNS	Peripheral Nervous System
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
ROS	Reactive Oxygen Species
SCI	Spinal Cord Injury
S1P	Sphingosine 1-phosphate

TNF-α	Tumor Necrosis Factor-α
TGF-β	Transforming Growth Factor-β
WT	Wildtype