Universitat Autònoma de Barcelona

Role of lysophosphatidic acid receptors in spinal cord injury physiopathology

Clara López Serrano

Role of lysophosphatidic acid receptors in spinal cord injury physiopathology

ACADEMIC DISSERTATION

To obtain the degree of PhD in Neuroscience by the

Universitat Autònoma de Barcelona

Clara López Serrano

PhD student

Dr. Rubèn López Vales

Thesis director

Dr. Xavier Navarro Acebes

Tutor

Group of Neuroplasticity and Regeneration

Institut de Neurociènces, 2017

Index

Summary

The spinal cord is an extremely vital part of the central nervous system (CNS) and, although it is well protected by the spinal column, it can be damaged, resulting in serious consequences. Spinal cord injury (SCI) leads to a disruption of the neuronal networks that are involved in many physiological functions. Since CNS axons of adult mammals do not regenerate following the lesion, and dead neurons and glial cells are not successfully replaced, this results in an irreversible functional loss in patients suffering from SCI.

The pathophysiology of SCI involves two degenerative stages, known as primary and secondary injury. The first one results from the direct mechanical trauma to the spinal cord, directly causing cell death, damage to axons, and loss of myelin. This is followed by a secondary wave of tissue degeneration that can extend for several weeks, in which inflammation plays a crucial role. Although regeneration of damaged axons and replacement of lost neurons and glial cells are important goals for the restoration of the injured spinal cord, minimizing 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. Since inflammation is a major contribution to secondary damage in SCI, targeting the detrimental actions of this physiological response could result in the development of novel approaches for the treatment of this pathology.

Lysophosphatidic acid (LPA) is an extracellular bioactive lipid with many physiological functions. It signals through six known G-protein coupled receptors (LPA_{1-6}), which are classified into two families: Endothelial differentiation family gene (Edg) LPA receptors (LPA_{1-3}) and Non-Edg family gene LPA receptors (LPA_{4-6}). LPA synthesis is carried out by two different pathways: (i) by the action of the enzyme named autotaxin (ATX), which is the main responsible in synthesis of this lipid in plasma, and (ii) by action of the phospholipase A_2 (PLA_2) family enzymes, which are the main route of LPA synthesis in tissues.

LPA is a key trigger of secondary damage after SCI, since its increased levels in the spinal cord parenchyma following injury leads to demyelination. Indeed, the lack of LPA_1 and LPA_2 signalling after SCI enhances functional recovery and myelin sparing. In this thesis, we show that activation of microglial LPA_1 and LPA_2 leads to oligodendrocyte cell death. We reveal that the cytotoxic actions underlying by microglial cells stimulated with LPA are mediated by the release of purines and the subsequent activation of P_2X_7 in oligodendrocytes. We also show that, unlike LPA_1 and LPA_2, LPA_4 and LPA_5 receptors do not contribute to SCI physiopathology.

In the present thesis, we also show that pharmacological inhibition of ATX does not have any effect in functional outcomes and secondary tissue damage after SCI, suggesting that this enzyme is unlikely to be involved in the production of LPA in the spinal cord parenchyma after injury. We also demonstrate that combinatory targeting of LPA_1 and LPA_2 does not results in additive effects in SCI. Overall, the results shown here suggest that pharmacological inhibition of LPA_1, and preferably LPA_2, may open a new therapeutic avenue for the treatment of SCI.

Abbreviations

- **AMPA** – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
- **ATP** – Adenosine Triphosphate
- **ATX** – Autotaxin
- **BBB** – Brain blood barrier
- **BDNF** – Brain-derived neurotrophic factor
- **CNS** – Central Nervous System
- **DGK** – Diacylglycerol kinase
- **DRG** – Dorsal root ganglion
- **EAE** – Experimental autoimmune encephalomyelitis
- **Edg** – Endothelial differentiation gene family
- **GPCR** – G protein-coupled receptors
- **LCAT** – Lecithincholesterol acyltransferase
- **LPA** – Lysophosphatidic acid
- **LPAR** – Lysophosphatidic acid receptor
- **LPC** – Lysophosphatidylcoline
- **LPL** – Lysophospholipids
- **LysoPLD** – Lysophospholipase D
- **MBP** – Myelin Basic Protein
- **NMDA** – N-methyl-D-aspartate
- **PA** – Phosphatidic Acid
- **PC** – Phosphatidylcholine
- **PL** – Phospholipid
- **PNS** – Peripheral Nervous System
- **PS** – Phosphatidylserine
- **PTEN** – Phosphatase and tensin homolog
- **ROS** – Reactive Oxygen Species
- **S1P** – Sphingosine-1-phosphate
- **SCI** – Spinal Cord Injury

Introduction

1. Spinal cord: description and basic anatomy

The spinal cord is an extremely vital part of the central nervous system (CNS). Functionally, it is in charge of controlling the voluntary muscles of the limbs and trunk, and receiving 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 is encased by the vertebral column, lying within the ventral canal. The spinal nerves of the lumbar and sacral levels must therefore span the distance from their exit level in the spinal cord to the level of the spinal foramen, so they can leave the spinal cord and distribute to the skin, muscles, and internal organs. The lower the spinal level, the longer this distance, and the longer the spinal nerve will travel in the spinal canal (Selzer, 2010).

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 called the *cauda equine* (Watson et al., 2008).

The column is composed of individual bones called *vertebrae*. Each vertebra is separated from the next by a cushion of fibrous, connective tissue called an intervertebral disc. The spinal cord stretches from the base of the skull to about 3 inches above the top of the hips; the vertebral column extends to below the top of the hips, all the way to the middle of the buttocks. Thus, the vertebral column and the spinal canal inside it are longer than the spinal cord itself (Selzer, 2010).

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 (Watson et al., 2008).

The spinal cord is composed of grey and white matter. The inner core of grey matter contains neurons. Surrounding the grey matter is the white matter, in the shape of an H, which contains axons that carry messages between neurons at different levels of the spinal cord, and between the spinal cord and the brain (Selzer, 2010).

1.1 Organization of the grey matter

The grey 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 grey matter. Only in the thoracic and upper lumbar spinal cord there are two small lateral projections of the intermediate grey matter, the intermediolateral horns, which contain the cells of origin of the autonomic nervous system (Watson et al., 2008).

The neuronal bodies in the grey matter are organized in ten successive layers, from dorsal to ventral, called the ***Rexed laminae*** (Fig. 1). Laminae I-VI are located in the dorsal horns, laminae VII-IX are in the lateral and ventral grey 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* (Watson et al., 2008).

- ***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 function 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 send 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. It 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 grey matter".

1.2 Organization of the white matter

The white matter consists mostly of longitudinally running axons and glial cells. 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 propriospinal 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 (Fig. 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 information 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, 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 (Armand, 1982).

- **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 (Armand, 1982).

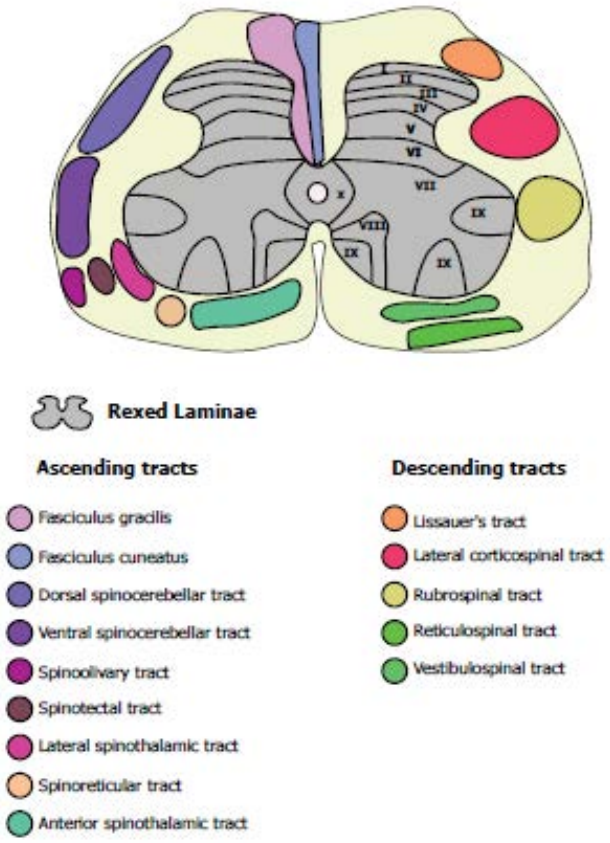


Figure 1. Schematic diagram of the organization of the grey and white matter of the spinal cord.

2. Spinal Cord Injury: description, causes and epidemiology

Spinal cord Injury (SCI) is an impairment that affects conduction of sensory and motor signals across the site of lesion due to damage to the neural elements within the spinal canal caused by an external force (Kirshblum et al., 2011).

The first spinal cord injuries were documented more than three 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 a SCI, despite the loss of functionality below the lesion site, the appearance of secondary complications, and the consequent reduction in quality of life (Rossignol et al., 2007).

2.1 Etiology and epidemiology of the SCI

SCI is one of the most prevalent and disabling conditions in the world. Although reliable information on the epidemiology for traumatic SCI is unavailable for many countries, it is clear that incidence, prevalence, and injury etiology vary considerably from region to region (Burns and Connell, 2012), depending, for example, on geographical and cultural differences, population characteristics, inclusion criteria and differences in data collection (Ackery et al., 2004).

The 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 (Wen-Ta et al., 2016). 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%). 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).

2.2 Level and severity of the spinal cord injury

The spinal cord can be damaged in different ways and, when this happens, 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 nature and extent of spinal cord injuries vary widely, depending on the level of the injury (cervical, thoracic, lumbar or sacral), its severity (partial or complete), and the type of the injury (contusion, compression, transection, etc.) and will dictate its functional impact and prognosis.

Functional deficits observed after SCI depend on whether the lesion is complete or incomplete. Incomplete injuries are characterized by the remaining of some sensory and motor function due to the percentage of motor and sensory fibers preserved. On the other hand, complete injuries lead with a total loss of function, despite there is still the presence of some spared fibers within the cord tissue. Indeed, complete transection injuries are not very common in humans.

Depending on the level of injury, SCI can be differentiated in cervical, thoracic and lumbosacral injuries (Creasey et al., 1997) (Fig. 2).

- **Cervical injuries** are the most frequent and severe injuries, leading with tetraplegia. But, depending on the specific location, limited function may be retained. A patient with **high-cervical injury** (C1-C4) may not be able to breathe on his own, cough or control bladder and bowel movements. Even speaking may be impaired or reduced. However, in the case of an injury located at **low cervical level** (C5-C8), breathing and speaking may not be affected, and allows a limited use of arms and limited wrist control but a complete hand function.

- **Thoracic injuries** result in paraplegia. Injuries in **high segments** (T1-T5), whereas arms and hands are usually normal, it results in the inability to control abdominal muscles and trunk stability. Moreover, lesions above T6 level can result in autonomic dysreflexia. On the other hand, in injuries at **thoracic low levels** (T9-T12) there is a fair to good ability to control and balance trunk while in the seated position and limited or absent control of bowel or bladder function. People with T1-T12 paraplegia have nerve sensation and function of all their upper extremities. They can become functionally independent, feeding and grooming themselves and cooking and doing light housework. These patients can transfer independently and manage bladder and bowel function. Finally, people with this type of injury can handle a wheelchair quite well and drives especially adaptive vehicles.

- **Lumbosacral injuries** lead to decreased control of the legs and hips, urinary system, and anus. There is usually little or no voluntary control of bowel or bladder function and sexual function is associated with the sacral spinal segments. People with sacral or lumbar paraplegia can be functionally independent in all their self-care and mobility needs. They can learn to skillfully handle a manual wheelchair and can drive especially equipped vehicles.

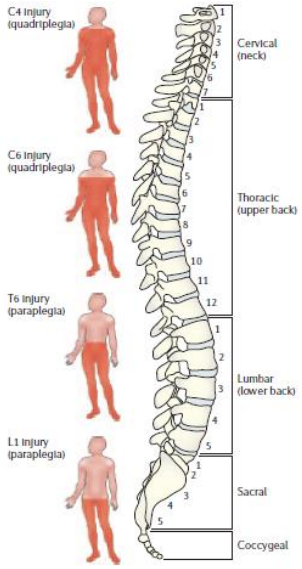


Figure 2. Extent of injury of specific spinal segments (from Thuret et al., 2006).

Much confusion surrounds the terminology associated with spinal cord injury levels, severity, and classification. In an effort to systematize the classification of spinal cord injuries, in 1992, the American Spinal Injury Association (**ASIA**) developed a uniform way to classify injuries according to the level and extension: The ASIA International Standards for Neurological Classification of the Spinal Cord Injury. For this classification, it is used the ASIA Impairment Scale (**AIS**), which is based on the examination of neurological functions to asses on a scale of 5 points (AIS A to E) any improvement or deterioration throughout the course of the injury (Kirshblum et al., 2011, 2014):

- **AIS A** indicates a "complete" spinal cord injury where no motor or sensory function is preserved in the sacral segments S4-S5.

- **AIS B** indicates an "incomplete" spinal cord injury where sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-S5.

AIS levels A and B classification depend entirely on a single observation, but for levels C and D, the usefulness of lower limb function was added as quantitative criteria. However, these criteria ignored arm and hand function in patients with cervical injury. To get around this problem, it was stipulated that a patient would be an AIS C if more than half of the muscles evaluated had a grade of less than 3, which indicates active movements with full range of motion against gravity. If not, the person was assigned to level D.

- **AIS C** indicates an "incomplete" spinal cord injury where motor function is preserved below the neurological level, and more than half of key muscles below the neurological level have a muscle grade less than 3.

- **AIS D** also indicates "incomplete" lesion where motor function is preserved below the neurological level, and at least half of key muscles below the neurological level has muscle grade of three or more.

- **AIS E** indicates that motor and sensory functions are normal but even so it is possible to have a spinal cord injury with neurological deficits.

Generally, the spinal cord normally goes into what is called **spinal shock** after it has been damaged. It is a transitory state in which all the spinal functions and reflexes are abolished. As a consequence, the true extent of many incomplete injuries is not 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.

2.3 Pathophysiology of the SCI

There are many causes that can lead to SCI, such as tumor growth, infections and other disorders. However, the mechanical damage is the most common form of acute SCI in humans (Silva et al., 2014). The pathophysiology of acute SCI involves two different neurodegenerative phases with distinct events in each phase:

- The **primary injury** is characterized by the mechanical trauma in where bone or disk displacement within the spinal column compresses and contusions the spinal cord. This primary injury is restricted to the site of the impact itself and disrupts axons, blood vessels, membranes and myelin leading hemorrhage, axonal and neural necrosis and to an important edema at the impact site (Mothe and Tator, 2013). This is an uncontrollable and unpredictable injury, so little can be done to reduce or avoid the events of the primary phase. From hours to days after the initial

trauma, a range of secondary cellular and molecular events takes place, constituting the secondary phase.

- The **secondary phase** features a continuation of some events from the acute phase such as ionic imbalance or edema, excitotoxicity, free radical production, inflammation, apoptosis, lipid peroxidation and the release of extracellular matrix components to eventually form a glial scar that represents a physical and chemical barrier for axonal regeneration and remyelination. Fortunately, unlike the primary injury, the secondary injury events can be prevented, representing an important target in developing therapeutic strategies for treatment of SCI (Silva et al., 2014). A brief description of the different secondary injury events involved in SCI are summarized below.

Vascular dysfunction and ischemia

Immediately after injury to the spinal cord, vasospasms of the superficial vessels and intraparenchymal hemorrhage are produced. Hemorrhage is initially localized in the highly vascularized and most vulnerable central grey matter, and this damage impairs tissue perfusion. Moreover, right after the mechanical trauma, the disruption of the 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 hypoglycaemia (Tator and Fehlings, 1991; Mautes et al., 2000). Moreover, systemic responses resulting from the loss of autoregulation, as post-traumatic hypotension, bradycardia, and decreased cardiac output, exacerbate the ischemic damage (Guha and 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 (Nelson et al., 1977; Armand, 1982). Although this situation is transitory, the reperfusion implies the production of reactive oxygen species (ROS), contributing also to the secondary lesion (Basu et al., 2001; Chiu et al., 2010). 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 and 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 and Xiong, 2013).

Excitotoxicity and ionic imbalance

During the secondary injury phase, there is also a disruption of cell membranes, which results in massive release of glutamate into the extracellular space, together with a global alteration of ion equilibrium. These 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 event results in sodium influx and further depolarization (Doble, 1999; Kirshblum et al., 2011) Then, passive influx of chloride occurs in order to maintain ionic equilibrium (Rothman, 1985; Silver and Miller, 2004; Yiu and He, 2006; Rowland et al., 2008), and this entire ion imbalance induces changes in the osmotic gradient, causing the entry of water. Finally, cellular lysis releases cell contents to the extracellular medium (Doble, 1999; Profyris et al., 2004). In addition, 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 (Doble, 1999; Ondarza et al., 2003) 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, such as protein kinase C and phospholipase A2 (PLA2), which will disrupt cell function and attack cell and organelles membranes (Tator and Fehlings, 1991; Doble, 1999; Mautes et al., 2000).

Oxidative stress and lipid peroxidation

Compared to other organs, the CNS is particularly susceptible to oxidative stress and free radical damage due to its active oxygen metabolism and low anti-oxidant capacity (Li et al., 2010). In fact, there are several 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 and 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 is 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 (Carlson et al., 1998; Dumont et al., 2001). Other 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 These ROS can modulate the action of several proteins and enzymes and oxidize amino acid side chains, causing fragmentation of proteins. 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).

Cell death, apoptosis and loss of oligodendrocytes

Both necrotic and apoptotic events contribute to cell death after SCI. Primary injury results in initial necrosis affecting both, the grey and white matter, and induces swelling of neuronal bodies and glial cells, disruption of organelles and release of the intracellular contents through the ruptured membrane (Hausmann, 2003; Profyris et al., 2004). On the other hand, programmed cell death known as "apoptosis" also occurs in the lesion core, preferably in the grey matter, over the first hours and days after injury and accompanies necrosis in damaging multiple types of cells. By the end of the first week, the level of apoptosis in the grey matter decreases, and a second wave of apoptotic cell death is predominant conducted in the white matter, affecting mainly to oligodendrocytes. This event lasts for several weeks leading to persistent demyelination (Profyris et al., 2004; Mekhail et al., 2012). The loss of myelin sheaths results in impairment of axonal transmission and over time may lead to degeneration of demyelinated axons (Blakemore et al., 1977).

Neuroinflammation

Inflammation is a basic pathological process produced in response to the breaching of the tissue integrity, and it is strictly necessary for wound healing. However, peculiarities of CNS tissue contribute to its characteristics during inflammation, which follows a different course compared with other organs. The presence of a very selective barrier, the blood-brain barrier, makes changes in vascular permeability more dramatically, because of the normally low permeability. In addition, 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, such as mast cells and dendritic cells (Hausmann, 2003). A 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 contributes 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 hematogenous macrophages from the circulation (David and Kroner, 2011). These cytokines, together with a number of other vasoactive substances released by glia and leukocytes (ROS, kinins, histamines, nitric oxide, elastase, etc.), enhance vascular permeability (Donnelly and Popovich, 2008a). 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 and Popovich, 2008a). Once there, neutrophils produce ROS, eicosanoids and proteases that can cause neuronal and glial toxicity. A number of studies have reported improvement in functional outcomes and tissue protection after depleting or neutralizing neutrophils (Taoka et al., 1997; Gris et al., 2004; Lee et al., 2011). However, they also support recovery through their ability to phagocytize cellular debris. In fact, a study shows 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 and Popovich, 2008a). Both microglia and macrophage 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 and years after the injury (Fleming et al., 2006).

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 (Araque and

Navarrete, 2010). A few hours after the SCI, they become activated, lasting in this state for months or even years (Hausmann, 2003). Astrocytes also secrete pro-inflammatory cytokines, contributing to the persistence of this inflammatory response. However, they are also very important for wound healing, as they are the responsible of filling the empty spaces caused by cell death (Mekhail et al., 2012). 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 are in charge of cleaning rests of dead cells and myelin debris. They are also known to produce some neuroprotective molecules, such as TGF-β1, that acts as immunosuppressant, promotes axonal growth and limits oligodendrocyte cytotoxicity (Merrill et al., 1993; Alvarez-Palomo et al., 2015). 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, induce them to produce pro-inflammatory cytokines, while IL-4 drives macrophages towards an anti-inflammatory phenotype, also known as M2, which promotes tissue healing a repair (David and Kroner, 2011). Our laboratory has recently shown that the presence of anti-inflammatory macrophages in the injured spinal cord is scarce due to the lack of IL-4 expression. Indeed, administration of IL-4 into the lesioned spinal cord redirects macrophages towards a M2-like phenotype and promotes functional recovery (Francos-Quijorna et al., 2016).

Although immune cells can play both, detrimental and beneficial actions after SCI, the harmful effects they mediate in the injured CNS are much greater than the beneficial ones. Indeed, inflammation is considered to be one of the main factors that contribute to secondary damage, and thus, efforts are being done to modulate its evolution in order to limit secondary damage and functional deficits (Fig. 3).

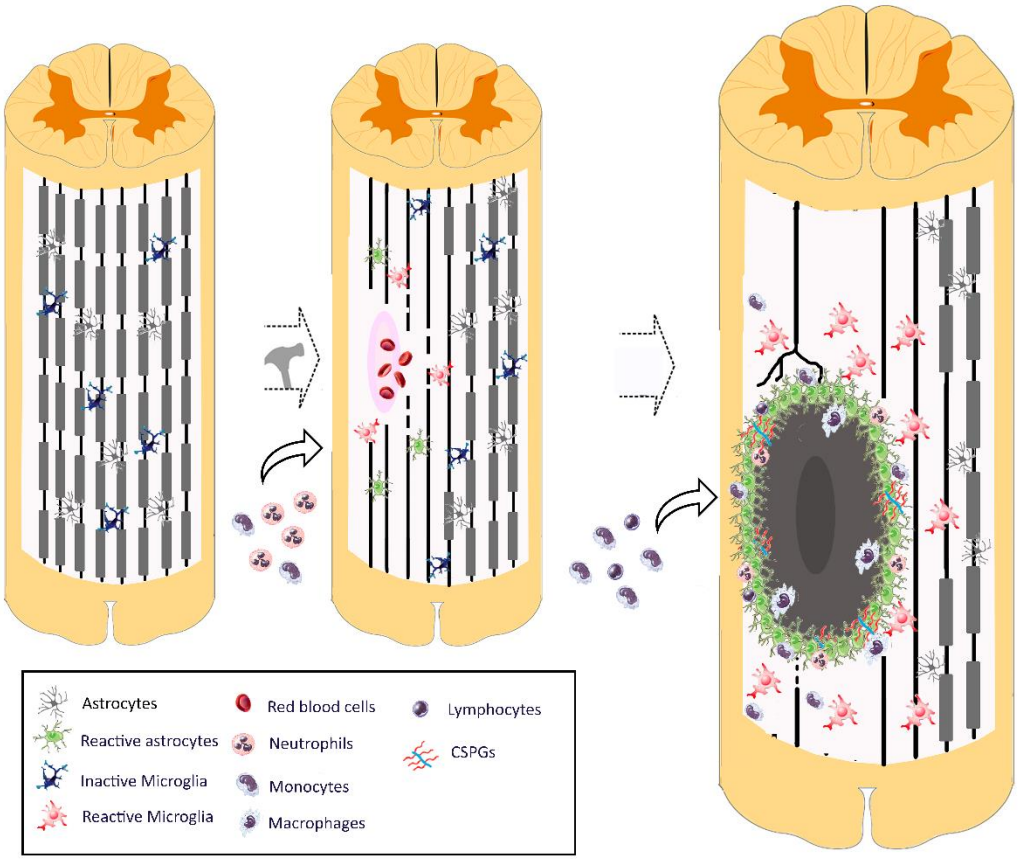


Figure 3. Pathophysiology of the SCI.

2.4 Pharmacological approaches for the treatment of the spinal cord injury

Unlike the peripheral nervous system (PNS), the CNS of adult mammals has limited abilities for spontaneous self-repair, which makes the 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 et al., 2002), the insufficient neurogenesis to replace dead neurons (Yang et al., 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 a better candidate to be prevented or reduced. That is why several pharmacological compounds targeting detrimental processes of secondary injury have been clinically proven during the last two decades (Baptiste and Fehlings, 2006). Some of these therapies are the treatment with methylprednidolone, which has 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 and Fehlings, 2001). NMDA and non-NMDA glutamate receptor antagonists improve tissue sparing and prevent loss of neurons and glia, targeting excitotoxicity (Baptiste and Fehlings, 2006). Minocycline, a synthetic tetracycline derivate

antibiotic, also attenuates secondary injury by counteracting excitotoxicity and reducing apoptosis and inflammation (Tikka and 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, but they have not been clinically proved yet (Fu et al., 2007). Rho is a small GTPase protein that mediates many of the growth-inhibitory effects in the mature CNS (Yiu and He, 2006). Cethrin (BA-210), another Rho inhibitor, has been tested in clinical trials in patients with SCI (Baptiste and 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 (Bregman et al., 1995; GrandPre et al., 2000).

Although all these drugs have been demonstrated to promote beneficial effects in experimental models of SCI, so far there are no pharmacological strategies of proved 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, 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). This has led to abandon the application of high-dose methylprednisolone after acute SCI over the last years.

Despite the lack of current therapies for SCI in clinical use, 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-center phase III future clinical trial.

3. Lysophosphatidic Acid

Lysophosphatidic acid (LPA) is the simplest phospholipid: it is composed of a phosphate, a glycerol and a fatty acid in its structure (Aoki et al., 2008). 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 and involved in many cellular processes including cellular proliferation, prevention of apoptosis, cell migration, cytokine and chemokine secretion, platelet aggregation, smooth muscle contraction, and neurite retraction. LPA also induces transformation of smooth muscle cells (Aoki et al., 2008). Moreover, LPA has critical roles in brain development, neuropathic pain, embryo implantation, hair growth, blood vessel formation, and inflammation (Okudaira et al., 2010). Most of the LPA actions seem to be mediated by 6 known G protein-coupled receptors (GPCR) specific to LPA (Aoki et al., 2008).

3.1 Lysophosphatidic Acid synthesis

LPA is a metabolite in the biosynthesis of membrane phospholipids and is ubiquitously present in all examined tissues. The generic term LPA (mono-acyl-*sn*-glycerol-3-phosphate) often refers to 18:1 oleoyl-LPA (1-acyl-2-hydroxy-sn-glycero-3-phosphate), reflecting its widespread use. However, many other chemical forms of LPA with different acyl chain lengths, saturation, and position also exist (Yung et al., 2015).

So far, the molecular mechanisms of LPA production have been poorly understood. LPA is produced in various conditions, both in cells and in biological fluids, where multiple synthetic reactions take place (Okudaira et al., 2010). Therefore, 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, whereas extracellular LPA is involved in many biological functions.

There are two major pathways for LPA production. In the first pathway, LPA is produced from lysophospholipids (LPLs) by a plasma enzyme, autotaxin (ATX), whereas in the second pathway, phosphatidic acid (PA) is first generated from phospholipids or diacylgycerol and then deacylated by PLA_1 or PLA_2 (Aoki et al., 2008).

- **LPA production mediated by autotaxin.** In this pathway, LPLs are first produced by at least two mechanisms. In **activated platelets**, LPLs are produced by secretory-type PLA_2 ($sPLA_2$-IIA) and phosphatidylserine-specific PLA_1 (PS-PLA_1). In **plasma**,

lysophosphatidylcholine (LPC) is produced from phosphatidylcholine (PC) by lecithincholesterol acyltransferase (LCAT) and PLA_1-like enzymes. LPLs and LPC thus generated are subsequently converted by ATX by its lysophospholipase D (LysoPLD) activity (Aoki et al., 2008) (Fig. 4).

Regarding ATX, this enzyme is abundantly present in blood and it is implicated in metastatic and invasive potential of tumour cells, as well as in LPA production. Indeed, recent studies have shown that ATX and LPA levels are strongly correlated (Nakamura et al., 2007; Watanabe N1, Ikeda H, Nakamura K, Ohkawa R, Kume Y, Aoki J, Hama K, Okudaira S, Tanaka M, Tomiya T, Yanase M, Tejima K, Nishikawa T, Arai M, Arai H, Omata M, Fujiwara K, 2007). In addition, in ATX-depleted serum and plasma, LPA production is completely absent (Tsuda et al., 2006). Thus, ATX is considered to be responsible for LPA production, at least, in blood. In this thesis, we will show the involvement of ATX in the LPA production after spinal cord injury.

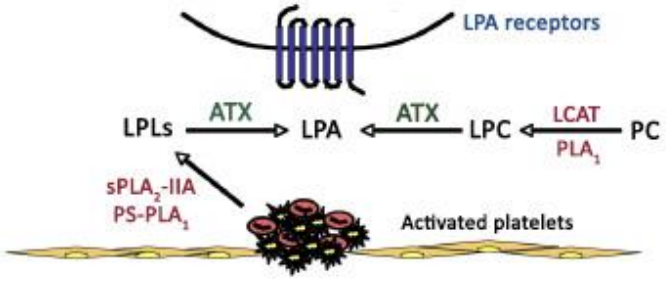


Figure 4. Action of ATX through LPA production (Adapted from Aoki et al., 2008).

- **LPA production mediated by phospholipases.** In this pathway, there is a breakdown of PA generated by either phospholipase D (PLD) or diacylglycerol kinase (DGK) from diacylglycerol (DAG), and is mediated by PLA_1 or PLA_2-type enzymes. Because PA is probably located in the cell membrane, this reaction may occur in cells or on the plasma membrane (Aoki et al., 2008) (Fig. 5).

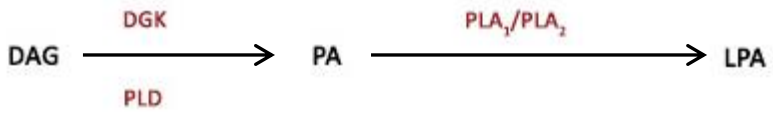


Figure 5. Action of phospholipases through LPA production.

As it is previously mentioned, platelets constitute the main source of LPA production in plasma. However, several cellular types have been documented to produce LPA, including fibroblasts, neurons, astrocytes, erythrocytes, adipocytes and cancerous cells.

3.2 Lysophosphatidic Acid receptors

In the last century, LPLs have been known to have biological activity, but these effects were long thought to be the result of nonspecific detergent-like disruptions of the plasma membrane. However, these studies were performed at very high, non-physiological concentrations. Nowadays, it is known that the effects of LPA at physiological concentrations are mediated by 6 high-affinity cognate receptors (LPA_1-LPA_6) and perhaps by additional recently proposed or as yet unidentified receptors (Choi et al., 2010).

LPA and sphingosine-1-phosphate (S1P) signal through G protein-coupled receptors (GPCRs), which are widely expressed and regulate important cellular functions. $S1P_1$ to $S1P_6$ and LPA_1 to LPA_3 are encoded by the **endothelial differentiation gene family** (**Edg**), whereas LPA_4, LPA_5 and LPA_6 belong to a **purino-receptor** or **non-Edg family**. Both families are activated by LPA (Ohuchi et al., 2008). These LPA receptors regulate a broad range of cellular functions: cell proliferation, survival, migration, cytoskeletal architecture, and adhesion. They also trigger a variety of biological activities, including vascular/nervous system development, reproduction, angiogenesis, immunity/transplantation, asthma, autoimmune diseases, cancer, cardiovascular diseases, hearing loss, and pain transmission (Choi 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 (Fig. 6):

- **Lysophosphatidic acid receptor 1 (LPA_1)** was the first high-affinity receptor identified for LPA. The mammalian *LPAR1* gene encodes an approximately 41-kDa protein consisting of 364 amino acids with 7 putative transmembrane domains. In mice, the open reading frame is encoded on two of five exons with a conserved intron, which is shared with *Lpar2* and *Lpar3,* and interrupts transmembrane domain 6. *Lpar1* has been observed in both human and adult mice, clearly in brain, uterus, testis, lung, small intestine, heart, stomach, kidney, spleen, thymus, placenta,

and skeletal muscle (Choi et al., 2010). Through the activation of three types of G proteins ($G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$), LPA_1 exerts its effects largely through activation of MAPK, PLC, AkT, and Rho pathways, cytoskeletal changes, intracellular Ca^{2+} mobilization, changes in cell-cell contact regulated by serum-response elements, and adenylyl cyclase inhibition (Ohuchi et al., 2008; Llona-Minguez et al., 2015). 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 (Choi and Chun, 2013). LPA_1 plays a fundamental role in the pathogenesis of cancer, driving cell motility and metastasis, and LPA_1 is also involved in neuropathic pain: elevated ATX-mediated hydrolysis of LPC leads to higher levels of LPA, which regulates levels of pain-related molecules through LPA_1 (Llona-Minguez et al., 2015). Using *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. 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).

- **Lysophosphatidic acid receptor 2 (LPA_2)** has a limited expression in humans, compared to LPA_1. It has a high expression in the testes and leukocytes and moderate expression in the prostate, spleen, thymus, and pancreas. In cancer cells, aberrant expression of *LPAR2* has been reported in several cases, suggesting a tumor-promoting role for LPA_2 (Llona-Minguez et al., 2015). In humans, *LPAR2* encodes a protein that has a predicted aminoacid sequence of 348 residues, yielding a calculated molecular mass of around 39 kDa (Choi et al., 2010). LPA_2 activates $G\alpha_{i/o}$, $G\alpha_{11/q}$, and $G\alpha_{12/13Ya}$, which convey signals through downstream molecules that include Ras, mitogen-activated protein kinase, phosphatidylinositol 3-kinase, Rac, PLC, diacylglycerol, and Rho (Choi et al., 2010). Activation of LPA_2 signaling is generally associated with such processes as cells survival and cell migration. Consequently, LPA_2 signaling has become a potential factor for cancer metastasis. Interestingly, several reports have provided evidence for the interaction of LPA_2 signaling with other pathways, like interactions with focal adhesion molecule TRIP6 and several PDZ proteins, and zinc finger proteins are also reported to interact

directly with the carboxyl-terminal tail of LPA_2. These studies provide evidence that LPA_2 signaling has cross-regulation between classical G protein signaling cascades and other signaling pathways to regulate the efficiency and specificity of signal transduction (Choi et al., 2010). Contrary to *Lpar1*$^{-/-}$, *Lpar2*$^{-/-}$ mice are phenotypically normal, with normal prenatal and postnatal viability (Chun, 2011). In this thesis, we have used this mouse line to study the contribution of the LPA_2 activation in the demyelination process.

- **Lysophosphatidic acid receptor 3 (LPA_3)** in humans encodes a 40-kDa GPCR that is around 50% identical to mouse LPA_1 and LPA_2 in amino acid sequence. It is expressed in human heart, testis, prostate, pancreas, lung, ovary, and brain, being abundant in mouse testis, kidney, lung, small intestine, heart, stomach, spleen, brain, thymus and uterus. LPA_3 can couple with $G\alpha_{i/0}$ and $G\alpha_q$ to mediate LPA-induced PLC activation, Ca^{2+} mobilization, adenylyl cyclase inhibition, and mitogen-activated protein kinase activation (Choi et al., 2010). 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., 2015). *Lpa3*$^{-/-}$ mice are variable and normal, but as LPA_3 has an important function during embryo implantation, this leads to a very reduced litter size (Choi and Chun, 2013).

- **Lysophosphatidic acid receptor 4 (LPA_4)** was the first identified non-Edg LPA receptor. It is structurally distinct from classical LPA receptors (it shares only 20-24% sequence homology to LPA_{1-3}), more closely related to P2Y purinergic receptors, so it is also known as p2y9 receptor. The gene *LPAR4* in humans is located in chromosome X, and the molecular mass is around 42 kDa. LPA_4 has a specific binding affinity to 18:1-LPA and structural analogues of LPA, but not to other lysophospholipids (Choi et al., 2010). In humans, *LPAR4* is ubiquitously expressed and specifically abundant in the ovary, whereas *Lpar4* mRNA in mice is expressed in heart, skin, thymus, ovary, developing brain, and embryonic fibroblasts (Choi et al., 2010). LPA induces morphological changes, such as cell rounding and stress fiber formation through the $G\alpha_{12/13}$ and Rho/Rho-kinase pathways in LPA_4-overexpressing cells. In addition, LPA induces intracellular cAMP accumulation through $G\alpha_s$, and Ca^{2+} mobilization through $G\alpha_{q/11}$ and $G\alpha_i$ (Lee et al., 2007). $LPA_4^{-/-}$ mice display no apparent abnormalities (Choi et al., 2010), so this mouse line has been used in this thesis to study the role of LPA_4 pathophysiology in spinal cord injury.

- **Lysophosphatidic acid receptor 5 (LPA_5)** shares 35% homology with LPA_4, but it is still more different to classic LPA receptors. Human *LPAR5* encodes a 41 kDa protein consisting of 372 amino acids. It also belongs to the rhodopsin-GPCR family. *Lpar5* is broadly expressed in murine tissues such as embryonic brain, small intestine, skin, spleen, stomach, thymus, lung, heart, liver, and embryonic stem cells (Choi et al., 2010). LPA induces neurite retraction and stress fiber formation in LPA_5-expressing cells by coupling to $G\alpha_{12/13}$ and increases intracellular calcium levels by activation of $G\alpha_q$. Moreover, LPA increases cAMP levels and inositol phosphate production through LPA_5 activation (Rieske et al., 2007). Other lipid-derived ligands different than LPA have been recently reported for LPA_5, such as N-arachinodylglycin and farnesyl-pyrophosphate (Choi et al., 2010). $LPA_5^{-/-}$ mice do not show abnormalities, so this mouse line has been used in this thesis to study the role of LPA_5 pathophysiology in spinal cord injury.

- **Lysophosphatidic acid receptor 6 (LPA_6)** is the most recently identified LPA receptor (Yanagida et al., 2009). Also known as p2y5 receptor, it shares the highest sequence homology with p2y9/LPA_4 among all GPCRs. LPA_6-overexpressed cells showed activation of the G13-Rho signaling pathway when activated with LPA. LPA_6 seems to be involved in regulation of cell morphology, endothelial permeability and other processes such as human hair growth (Yanagida et al., 2009). Since LPA_6 mice or antagonist are not currently available, the contribution of this receptor to secondary injury after SCI has not been studied.

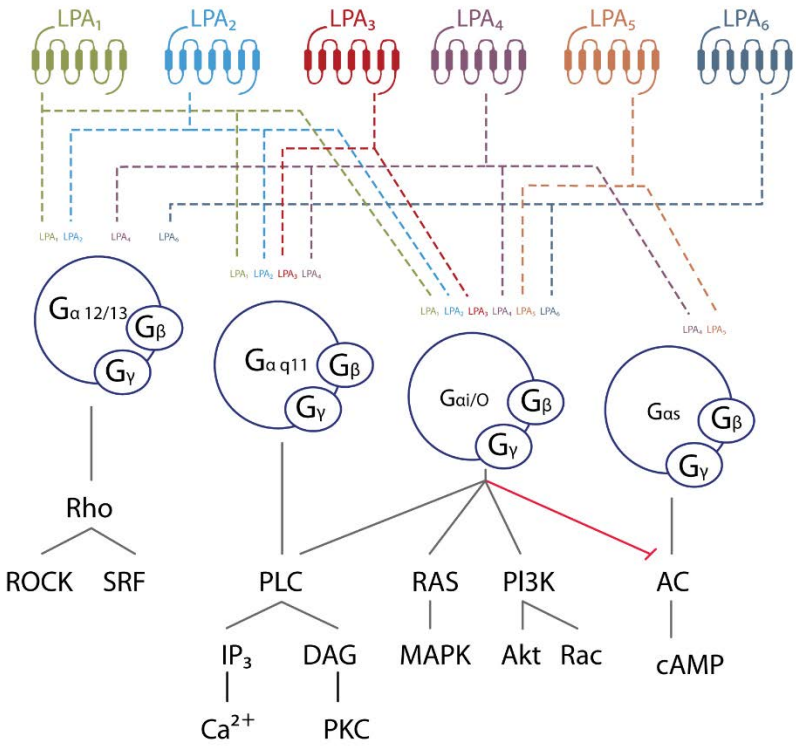


Figure 6. LPA receptors and signalling pathways.

4. Lysophosphatidic Acid in the Central Nervous System

LPA plays an important role as an intercellular signaling lipid that producing or regulating many physiological functions. In spite of evoking a variety of cellular responses in the majority of the tissues, little is known about the role of LPA in the nervous system, and most of its known functions arise from in vitro studies. Regarding the LPA receptors, the nervous system is one of the major loci for LPA receptor expression, and LPA exists in the brain at relatively high concentrations. LPA receptors are expressed in most cell types of the nervous system, including neural progenitors, primary neurons, astrocytes, microglia, oligodendrocytes, and Schwan cells (Chun, 2011).

Here, we summarize some of the cellular effects that LPA exert on both, neural and glial cells.

- **Neurons.** The effects of LPA signaling have been mainly evaluated *in vitro* using primary neurons and neural cell lines. These effects are largely related to morphological changes involving growth cone collapse and neurite retraction, especially in primary cultures of different neuron populations (sympathetic ganglion cells, dorsal root ganglion neurons –DRG-, retinal and cortical neurons), from both embryonic and adult mice (Saito, 1997; Fukushima et al., 2002; Bouquet

et al., 2007). LPA has also effects on inhibiting neural migration in cortical explants of embryonic mice (Fukushima et al., 2002). In addition, LPA has the ability to modulate neuronal activity *in vitro*, inducing release of neurotransmitters such as noradrenalin and dopamine (Nishikawa et al., 1989). On the other hand, *in vivo* studies showed that LPA elicits neuronal activity in adult rat spinal cord neurons and in DRG neurons (Elmes et al., 2004). LPA promotes neuronal death both by apoptosis and necrosis, associated with mitochondrial alterations and the generation of ROS (Holtsberg et al., 1998). Finally, LPA also regulates other neuronal function involving migration, cell death and survival, synapse formation, and synaptic transmission.

- **Astrocytes.** This type of glial cells is in charge of regulating many biological as well as pathological processes that are epitomized by astrogliosis. In fact, many *in vitro* effects of LPA signaling are related to astrogliosis, affecting proliferation, migration, morphological changes, and activation of related intracellular signaling (Choi and Chun, 2013). Another prominent function of astrocytes related to LPA signaling is the neural differentiation, since LPA-primed astrocytes secrete soluble factors that increase neuronal differentiation. Moreover, this differentiation might be induced by epidermal growth factor signaling pathway and the activation of the mitogen-activated protein (MAP) kinase (MAPK) cascade together with PKA activation in response to LPA (Spohr et al., 2011, 2014). In terms of morphology, 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 and Moolenaar, 1998). LPA has also been found to decrease both glutamate an glucose uptake and increase lipid peroxidation in astrocytes (Keller et al., 1996). Such effect may have consequences on neuron health, as it exacerbates neurotoxicity and reduces energy supply. Finally, LPA stimulate the expression of several cytokine genes, including IL-1β, IL-3 and IL-6, suggesting a pro-inflammatory role that would influence wound healing after CNS trauma. However, LPA stimulates the synthesis and secretion of NGF, suggesting also a neuroprotective role (Tabuchi et al., 2000).

- **Oligodendrocytes.** This type of glia plays a major role in myelination in the CNS. Their functions are also important for CNS development as well as repair after injuries, particularly remyelination that is composed of a series of events like

proliferation, migration, and differentiation of oligodendrocyte progenitor cells (Choi and Chun, 2013). LPA effect on oligodendrocytes seems to depend on the degree of maturation of these cells. For example, in oligodendrocyte progenitor cells (OPCs) and immature differentiating oligodendrocytes, LPA induces process retraction and inhibits oligodendrocyte maturation (Dawson et al., 2003). Moreover, a more recent study found that LPA may play a role in regulating the later stages of oligodendrocyte maturation, inducing an increase in the extension of oligodendrocyte's processes and an up-regulation of the myelin basic protein (MBP) (Nogaroli et al., 2009). However, in primary cultured mature oligodendrocytes, LPA did not show any influence in survival, maturation, cytoskeleton organization or myelination (Stankoff et al., 2002).

- **Microglia.** Microglia are categorized as a non-neural cell type, but are CNS resident cells. Upon activation, microglia respond to mediate neuroinflammatory processes. LPA signaling was reported to regulate proliferation, membrane ruffling and hyperpolarization, metabolic changes, migration, chemokinesis, and growth factor upregulation upon activation of the different LPA receptors (Choi and Chun, 2013). More specifically, there are some controversies regarding the LPA receptor expression on microglial cells, since there are several microglial cell types *in vitro*. 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. These events 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, so far there are no many studies addressing the role of LPA in CNS pathologies. As an exception, the involvement of the LPA in the development of the CNS has been described in detail. Indeed, at least 2 LPA receptors (LPA_1 and LPA_2) are expressed in the developing brain, contributing to neurogenesis. More specifically, the absence of LPA_1 signaling has been found to be directly related to defective cortical development in mice (Estivill-Torrús et al., 2008). Moreover, some years ago, a work

revealed that the administration of B3, an antibody that is able to bind to LPA and other lysophospholipids, prevented them from interacting with their receptors, promoting functional recovery after a spinal cord hemisection in mice (Goldshmit et al., 2012). Although we have tested this antibody after contusion injury, a more clinical relevant model of SCI, no beneficial effects were found in this pathology.

4.1 Role of Lysophosphatidic Acid in the spinal cord injury physiopathology

Little is known about the involvement of the LPA in the SCI physiopathology. We have recently shown that LPA levels are increased following a contusion injury in mice and that exogenous LPA exposure led to demyelination of the spinal cord tissue and an activation of the microglial cells surrounding the site of injection (Santos-Nogueira et al., 2015). As previously mentioned, demyelination and microgliosis are two important processes that take place during the secondary phase after the injury.

We have also demonstrated for the first time that LPA signaling via LPA_1 receptor contributes to the secondary damage after SCI, and that pharmacological blockade of LPA_1 results in enhanced locomotor recovery and myelin preservation after SCI, revealing the important contribution of LPA-LPA_1 axis in secondary damage (Santos-Nogueira et al., 2015). Interestingly, LPA-LPA_1 signaling has been recently shown to impede the sprouting of corticospinal axons after axotomy, and disruption of this axis enhanced axonal arborization and functional recovery after pyramidectomy (Fink et al., 2017). We have also unpublished data indicating that LPA-LPA_2 signaling also contribute to SCI since the lack of LPA_2 leads to greater locomotor skills and reduced myelin loss following contusion injury. However, LPA_3 does not seems to contribute to this pathology.

Over the last years, we have therefore collected several evidences that support the detrimental involvement of LPA to SCI. However, there are several key questions that need to be elucidated: (i) how LPA_1/LPA_2 signaling leads to demyelination; (ii) are the non-Edg family LPA receptors involved in secondary damage?; (iii) can we impede the detrimental actions of LPA following SCI by targeting autotaxin, the main enzyme that synthesizes this lipid in tissues? The present thesis will address these critical questions.

Objectives

Chapter I. Microglial LPA_1 and LPA_2 signalling mediates oligodendrocyte cell death

- To identify the glial cells that are associated with the demyelinating lesion induced by intraspinal injection of LPA.
- To study whether microglial cells become cytotoxic after LPA stimulation.
- To evaluate the contribution of microglial LPA_1 and LPA_2 activation to oligodendrocyte cell death.
- To elucidate the detrimental mediators produced by microglial cells upon LPA stimulation that cause oligodendrocyte cell death.

Chapter II. Role of non-endothelial differentiation gene family LPA receptors in secondary damage after spinal cord injury.

- To quantify the changes in mRNA levels of LPA_4 and LPA_5 in the spinal cord after contusion injury in adult mice.
- To evaluate the contribution of LPA_4 and LPA_5 to functional deficits after spinal cord contusion injury in adult mice.
- To assess whether LPA_4 and LPA_5 are important mediators of secondary damage after spinal cord contusion injury in adult.

Chapter III. Autotaxin inhibition does not enhance functional recovery after spinal cord injury.

- To quantify the changes in mRNA levels of autotaxin in the spinal cord after contusion injury.
- To assess whether the administration of autotaxin inhibitors enhances functional outcomes after spinal cord injury.
- To evaluate whether autotaxin inhibition reduces secondary tissue damage after spinal cord injury.

Chapter IV. Effects of combinatory targeting of LPA_1 and LPA_2 after spinal cord injury in mice

- To evaluate whether the suppression of both, LPA_1 and LPA_2 signalling, has additive therapeutic effects on neurological recovery after spinal cord injury.

Material and Methods

1. Animal Genotyping

For the present work, we used mice lacking LPA_2, LPA_4 and LPA_5 receptors, which were gently provided by Jerold Chun, from the Scripps Research Institute (La Jolla, CA, EEUU). The colonies were established at the animal facilities of the Universitat Autònoma de Barcelona. PCR analysis was used to detect the presence of the wild type and mutated alleles. Gentra Puregene Tissue Kit from Qiagen® was used for genomic DNA extraction from a piece of the mouse tail. Briefly, tail tissue was digested in 0.1 mg/mL proteinase K solution from the same kit, diluted in lysis buffer at 55°C overnight. After digestion, proteins were eliminated by precipitation using a protein precipitation buffer and centrifugation during 3 min at 16000 g. Supernatants were then mixed with 2-propanol (Panrea) to DNA precipitation. After a centrifugation of 1 min at 16000g, the pellets were washed with ethanol 70%. Finally, DNA pellets were completely dried at room temperature before DNA solubilisation by adding 50µl of DNA hydration solution at 65°C for 1 hour. Allele amplification was performed by PCR reaction, using the Taq DNA Polymerase kit (Invitrogen), and PCR products at a concentration of 50 ng/µL were analysed by standard electrophoresis in 2% agarose gels. Primer sequences, PCR conditions and product sizes are the following (Table 1):

	LPA_2	LPA_4	LPA_5
Forward primer	5´- AGTGTGCTGGT ATTGCTGACCA -3´	5'-GCAGATTTGGTG ACTCATTCTACTT-3'	5'-CAG AGT CTG TAT TGC CAC CAG-3'
Reverse primer	5´- CTCTCGGTAG CGGGGATGG -3´	5'-ATTTTGAAAGGTA GCGTACAAACAAA-3'	5'-GTC CAC GTT GAT GAG CAT CAG-3'
Mutation primer	5´-CAGCTGGGGCTC GACTAGAGGAT -3´	5'-GTAATGGGATAGG TTACGTTGGTGTA-3' (LacZ)	5'-GTG GTG CAG ATG AAC TTC AGG-3' (GFP)
PCR conditions	Initialization: 94°C 3 min Denaturation: 94°C 30 sec Annealing: 60,7°C 45 sec Elongation: 72°C 2 min Final elongation: 72°C 7 min 40 cycles	Initialization: 94°C 3 min Denaturation: 95°C 30 sec Annealing: 56°C 1 min Elongation: 72°C 1 min Final elongation: 72°C 5 min 35 cycles	Initialization: 94°C 5 min Denaturation: 94°C 30 sec Annealing: 60°C 45 sec Elongation: 72°C 1 min Final elongation: 72°C 7 min 35 Cycles
Product sizes	Wild-type band: 576 bp Knockout band: 328 bp	Wild-type band: 406 bp Knockout band: 559 bp	Wild-type band: 450 bp Knockout band : 220 bp

Table 1. Primer sequences, PCR conditions and product sizes used for genotyping wild type and mutated alleles from *lpa2*, *lpa4* and *lpa5* genes in mice colonies.

2. Surgical procedures

All animal procedures were approved by the Universitat Autònoma de Barcelona Animal Experimental Ethical Committee and followed the European Communities Council Directive, and the methods for each procedure were carried out in accordance with the approved guidelines.

2.1 Spinal cord contusion model

Contusion injury to the mouse spinal cord was performed using the Infinite Horizons Impactor device (Precision Scientific Instrumentation, Fairfax Station, VA). This instrument 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 the performance of graded magnitudes of injury. As a consequence of the impact, there is a displacement produced to the spinal cord, which is also registered. Before the contusion, the vertebral column is rigidly stabilized with the aid of two Adson forceps attached to two articulated support arms, and a contusive tip is placed in the middle of the spinal cord level. For this study, a moderate contusion injury is performed at the thoracic T11 level by applying a force of 60$\pm$5 kdynes and 400-600 μm in displacement, which produces a severe injury. The resulting injury is very similar to what is observed in humans, in terms of histological changes (i.e loss of grey 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 trunk. However, spontaneous recovery of some motor function is produced, permitting to detect changes due to potential treatments.

2.2 Behavioral scoring

After injury, spontaneous locomotor recovery of mice was assessed by using the standardized Basso Mouse Scale (BMS) (Basso et al., 2006). BMS is a widely used 9-point scale in which two blinded observers score the animal's motor performance (Table 2). 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. Once the animal is able to step frequently (5 points), a sub-score of 11-point scale is performed in parallel, in order to assess fine aspects of the locomotion (Table 3). BMS evaluation was performed prior to surgery (day 0) and at 1, 3, 5, 7, 10, 14, 21 and 28 days after injury (dpi).

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 2. Scores for the Basso Mouse Scale (BMS), for the evaluation of spontaneous locomotor recovery after spinal cord injury in mice. Maximum score of 9 points.

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

Table 3. Basso Mouse Scale (BMS) sub-scores, used to complement BMS scores. This scale evaluates more fine aspects of locomotion. Maximum score of 11 points.

2.3 Drug administration

To study the effect of autotaxin inhibition in the functional recovery and the secondary phase after SCI, we used two different ATX-selective inhibitors. **Compound X** and **PAT-048** (patent publication number WO 2012024620 A2), kindly provided by PharmAkea (San Diego, CA), were suspended in 0.5% methylcellulose (Sigma-Aldrich, St. Louis, MO, USA) and administered by oral gavage at a dose of 20 mg/kg for Compound X and 10 mg/kg dose for PAT-048. These inhibitors were given at one-hour post-injury and then daily for the first 14 dpi.

3. 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). 8 mm length of spinal cord containing the lesion site centered was hasvested, post-fixed for 1 hour in 4% paraformaldehyde in 0.1 M PB and cryoprotected with 30% sucrose in 0.1 M PB at 4°C supplemented with azide (1:100), for a minimum of 48 hours. Spinal cords were fast-frozen at -60°C in cryoembedding compound (Tissue-Tek OCT, Sakura) and cut on a cryostat (Leica). Six series of 15-µm-thick transversal sections were picked up on glass slides, so adjacent sections on the same slide were 90 µm apart.

3.1 Luxol Fast Blue (LFB) staining

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 37°C. Sections were then washed in 95% ethanol and distilled water before placing them into a solution of 0.5 mg/mL Li_2CO_3 in distilled water for 1 min at room temperature. After washing in distilled water, sections were dehydrated and mounted in DPX mounting media (Sigma).

3.2 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 4°C with primary antibodies diluted in BB. After several washes in PBST, sections were incubated for 2 hours at room temperature with the appropriate Alexa 594- or Alexa 488-conjugated secondary antibodies (Invitrogen) diluted in BB. Finally, sections were incubated with PBST containing DAPI (1 µg/mL; Sigma) and washed with

PBST, PBS and PB, dehydrated in 50%, 70%, 80% and 100% ethanol and coverslipped in DPX mounting medium.

3.3 Quantification of myelin sparing after SCI

Myelin sparing was calculated by delineating total LFB-stained areas, respectively, on each spinal cord section. Quantifications were done at the epicentre and in sections every 180 μm until 1020 μm at both, rostral and caudal sides of the epicentre.

3.4 Quantification of neuronal survival

Neuronal survival was assessed by manually counting NeuN+ cells in the ventral horn of spinal cord slices corresponding to the epicentre, and every 180 μm until 1020 μm at both, rostral and caudal sides of the epicentre.

4. Cell cultures

4.1 Oligodendrocyte primary cultures

Primary oligodendrocyte progenitor cells (OPCs) were isolated from mice cortices at 2-4 post-natal days (P2-4), as described previously (Saura et al., 2003). After removal of the meninges, cortical tissue was warmed to 37°C and minced using a P1000 pipette tip. Tissue was then dissociated by incubation in papain solution (Table 4) for 20 min at 37°C. Mixed glial culture media (MGCM; Table 4) 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) pre-coated with 10 μg/mL poly-D-lysine for 1h at 37°C, and cultured at 37°C in a humidified incubator with 5% CO_2 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 with 5 μg/mL insulin (Seralab). After 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 4). OPC maturation in oligodendrocytes was achieved after 5-6 days *in vitro*. For maintenance of the culture half of the medium was changed every 2-3 days.

Papain solution	**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)	DMEM medium (Gibco)
	10% inactivated Fetal Bovine Serum (FBS, Sigma)
	0,33% penicillin-streptomycin (PS, Sigma
	1% Glutamax 100x (Gibco)
Oligodendrocyte medium (OLM)	DMEM medium (Gibco)
	1% Glutamax 100x (Gibco)
	2% B27 (Gibco)
	0,5% FBS
	50 pg/mL recombinant mouse ciliary-neurotrophic factor (CNTF, BioTrend)
	1% OL supplement
OL supplement	DMEM medium (Gibco)
	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)

Table 4. Media recipes for oligodendrocyte enriched primary cultures.

4.2 Primary microglial cultures

Primary microglial cells were isolated from P4-P7 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 5), and cultured at 37°C in a humidified incubator with 5% CO_2 supplementation. Medium was replaced every 4-5 days and confluence 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 37°C. 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 enzyme 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.

Microglia Medium (MGM)	**DMEM medium (Gibco)**
	10% FBS
	5% P/S
Mild trypsin solution	DMEM medium (Gibco)
	25% trypsin-EDTA (0,25%) (Gibco)
PB-Buffer	PBS
	0.1% BSA

Table 5. Media recipes for microglial primary cultures.

4.3 Microglial conditioned medium generation

For the generation of microglial conditioned medium (MCM), postnatal microglial primary cultures were incubated for 24 hours in new medium (control), or with 1 µM LPA. Then, medium was replaced by oligodendrocyte specific culture medium, leading microglial cells to continue releasing factors induced by the treatment for 24 hours.

Afterwards, the MCM was centrifuged and frozen, and applied to oligodendrocytes to assess cell survival. Depending on the experiment, the MCM was supplemented with:

- 50 nM of BBG (Brilliant Blue G; Sigma-Aldrich), a P_2X_7 receptor antagonist.
- 30 µM of a CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; Alomone Labs, Jerusalem, Israel), an AMPA/Kainate receptor antagonist.

4.4 Oligodendrocyte treatment with LPA-stimulated microglial conditioned medium

After 5-6 days *in vitro*, mature oligodendrocyte cells were treated with MCM from LPA-activated microglia, with the different conditions mentioned above. The entire medium was removed and 500 µL/well of MCM were added to the cultures. After 24 hours in the incubator at 37°C and 5% CO_2, cells were fixed in 4% paraformaldehyde for 20 min.

4.5 Regular fluorescent immunocytochemistry

For immunocytochemistry, wells were fixed in 4% paraformaldehyde for 20 min, washed in 0.1 M PBS and incubated for 1 hour at RT 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. Afterwards, wells were incubated with PBST containing DAPI (1 µg/mL; Sigma) and washed with PBST, PBS and PB. Finally, culture coverslips were dehydrated in 50%, 70%, 80% and 100% ethanol and removed from the

24-well culture plates to mount over glass slides with DPX mounting media. Oligodendrocyte survival was determined by counting the number of nucleated MBP^+ cells.

4.6 Assessment of cytokine levels

MCM of non-stimulated and LPA-stimulated microglia was collected as described above. Cytokine protein levels in the MCM were then analyzed using the Milliplex MAP Mouse Cytokine/Chemokine magnetic bead panel (Millipore) on a Luminex (Millipore) as per manufacturers' protocol.

4.7 Assessment of nitrate levels

For assessment of nitric oxide metabolites in MCM, samples were measured by Nitrate/Nitrite Colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) as per manufacturers' protocol.

5. Real-Time PCR

To evaluate the expression level of mRNAs for LPA receptor subtypes (LPA_1, LPA_2, LPA_3, LPA_4 and LPA_5) in non-activated and activated microglia, quantitative real-time (RT)-PCR was performed. RNA from cell cultures was extracted using the RNeasy® Mini Kit (Quiagen), according to the manufacturer's protocol. Cells were collected using a Buffer RTL (supplemented with Beta-mercaptoethanol), and after processing them, 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. 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. The expression level of the target mRNA was normalized to the relative ratio of the expression of the GAPDH housekeeping gene (Livak and Schmittgen, 2001). Primer sequences used in RT-PCR reaction are detailed in Table 6.

In the case of the intact or lesioned mouse spinal cord, 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 manufacturer's protocol. RNA was treated with DNaseI (Qiagen) to eliminate genomic DNA contamination. 1 µg of obtained RNA and the following procedure was performed as in the case of the microglial cells.

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
LPA_6	ACTGCTGCTTTGACCCTATTG	AAGGTCTGTAGGTTGTGTTGG
GAPDH	TCAACAGCAACTCCCACTCTTCCA	ACCCTGTTGCTGTAGCCGTATTCA

Table 6. Primer sequences used in Real Time-PCR reaction for quantifying basal mRNA expression of LPA receptor subtypes in the spinal cord and the microglial cells.

REFERENCES

Basso DM, Fisher LC, Anderson AJ, Jakeman LBYNB, Tigue DMMC, Popovich PG, McTigue DM, Popovich PG (2006) Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. J Neurotrauma 23:635–659.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and. Methods 25:402–408.

Saura J, Tusell JM, Serratosa J (2003) High-yield isolation of murine microglia by mild trypsinization. Glia 44:183–189.

Chapter I. Microglial LPA_1 and LPA_2 signaling mediate oligodendrocyte cell death

Clara López-Serrano[1], Eva Santos-Nogueira[1], Jerold Chun[2], Rubèn López-Vales[1]

[1]Departament de Biologia Cellular, Fisiologia i Immunologia, Institut de Neurociències, Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Universitat Autònoma de Barcelona, Bellaterra, Spain; [2]Molecular and Cellular Neuroscience Department, Dorris Neuroscience Center, The Scripps Research Institute, La Jolla, USA.

ABSTRACT

Lysophosphatidic acid (LPA) is an extracellular lipid mediator involved in many physiological functions by signaling through six known G-protein-coupled receptors (LPA_1-LPA_6). In the central nervous system (CNS), LPA mediates a wide range of effects, including neural progenitor cell physiology, astrocyte and microglia activation, neuronal cell death, axonal retraction, as well as CNS contributions to pain, schizophrenia and hydrocephalus. We recently reported that LPA contributes to secondary tissue damage after spinal cord injury (SCI). Interestingly, selective blockade of LPA_1 after spinal cord contusion lesion reduced functional deficits and myelin loss. We also have unpublished data demonstrating that LPA_2 leads to similar effects in SCI. To gain insights into the detrimental action of LPA in the spinal cord, we performed more *in vivo* and cell culture work. We found that LPA-induced demyelination in the spinal cord is closely associated with areas of high microglial activation. We demonstrated that the conditioned medium of microglial cells stimulated with LPA exerts harmful action on oligodendrocyte survival, which is induced by LPA_1, and to greater extend by LPA_2 signaling. Moreover, we also found that the cytotoxic effects underlying by microglial cells upon LPA stimulation were mediated, in part, by the release of purines that induce oligodendrocyte cell death via P_2X_7. Overall, this study provides new mechanistic insights into how LPA contributes to SCI physiopathology.

Key words: lysophosphatidic acid, microglia, oligodendrocytes purines, demyelination.

Part of this chapter was published in Journal of Neurosciences: Santos-Nogueira, et. al., 2015 (See annex).

INTRODUCTION

Spinal cord injury (SCI) causes functional deficits produced by the loss of axons and neurons, as well as to the death of oligodendrocytes, and the limited ability of the CNS to regenerate axons (Rowland et al., 2008; David et al., 2012a). The pathophysiology of SCI involves two stages of tissue degeneration, known as primary and secondary injury. Primary injury results from the direct mechanical trauma to the spinal cord, which is followed by secondary injury, consisting of a wave of tissue degeneration that occurs over a period of several weeks, and is associated with inflammation and other mechanisms triggered by injury (David et al., 2012a; David et al., 2012b; Popovich, 2014). Secondary injury is thought to contribute importantly to the functional deficits seen after SCI, and may 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; 1-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; Yung et al., 2014). LPA exerts this wide variety of effects by binding to specific G protein-coupled receptors (GPCR), 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; Kihara et al., 2014). These receptors differ in their ability to alter downstream signaling pathways, including 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 (Yung et al., 2011), fetal hypoxic brain damage (Herr et al., 2011) 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). We have recently demonstrated that LPA contributes to demyelination and functional deficits after spinal cord contusion injury. Moreover, we have published (Santos-Nogueira et al., 2015) and unpublished data

demonstrating that LPA_1 and LPA_2 activation are involved in such pathological events *in vivo*. However, how LPA induces demyelination remains unknown.

In the present chapter, we aimed at investigating the mechanisms underlying LPA-mediated demyelination after SCI. We found, using *in vitro* studies, that activation of both, LPA_1 and LPA_2, in microglial cells are responsible of the demyelinating actions of LPA. In addition, we identified purines as the potential cytotoxic factor release by microglial cells upon LPA stimulation.

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 C57BL/6 mice were anesthetized with ketamine:xylazine (90:10 mg/kg, i.m.). After performing a laminectomy at the 11th thoracic vertebrae, the exposed spinal cord was injected with LPA. 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 (Avanti Polar Lipids, Alabaster, AL, USA) 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.

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. 8 mm length of spinal cord containing the injection 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 4°C, for a minimum of 48h. Spinal cords were fast-frozen at -60°C in cryoembedding compound (Tissue-Tek® OCT, Sakura) and cut on a cryostat (Leica). Ten series of 15 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 37°C. Then, sections were placed into a solution of 0.5 mg/mL Li_2CO_3 in distilled water for 1 min at RT, washed, dehydrated and mounted in DPX mounting media (Sigma). For assessing microgliosis and astrogliosis tissue sections rehydrated in PBS and blocked with 5% FBS in PBST (blocking buffer, BB) for 1h at RT. Sections were then incubated overnight at 4°C with rabbit anti-Iba1 (1:500, Wako) or rabbit anti-GFAP (1:500, Chemicon). After several washes in PBST, sections were incubated for 1h at RT with biotinilated-conjugated antibodies (1:500, Invitrogen) diluted in BB. Finally, sections were revealed using avidin-biotin complex technique (Thermo) and coverslipped in DPX.

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.

Cell cultures

Oligodendrocyte primary cultures

Primary oligodendrocyte progenitor cells (OPCs) were isolated from C57Bl/6 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 37°C. 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 1h at 37°C and cultured at 37°C in a humidified incubator with 5% CO_2 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). 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 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* (div) and for maintenance of the culture half of the medium was changed every 2-3 days.

Microglial cells

Primary microglial cells were isolated from LPA_1, LPA_2 null and their respective wildtype littermates as described previously (Saura et al., 2003). At P4 of age cortices were removed, and 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 37°C in a humidified incubator with 5% CO_2 supplementation. Medium was replaced every 4-5 days and confluency was achieved after

10-12 div. 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. The remaining adhered microglial cells were cultured for 24 hours before starting experiments.

For the generation of microglial conditioned medium (MCM), LPA_1, LPA_2 null WT microglial cells were incubated for 24 hours in new medium (control) or with 1 μM LPA (LPA). Then, this culture medium was replaced with new oligodendrocyte 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 to assess cell survival. The MCM was supplemented with 30 μM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) (Alomone Labs, Jerusalem, Israel) or 50 nM BBG (Brillian Blue G) (Sigma Aldrich) to block the AMPA/Kainate and P_2X_7 receptors in oligodendrocytes, respectively.

Assessment of oligodendrocyte cell death

To assess whether microglia LPA signaling leads to cell death, oligodendrocytes were exposed to MCM of LPA_1, LPA_2 and WT microglia for 24 hours. 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 1h at RT. Afterwards, cells were incubated with PBST containing DAPI (1 μg/mL; Sigma) and washed with PBST, PBS and PB. Cells were dehydrated in 50%, 70%, 80% and 100% ethanol and removed from the 24-well culture plates to mount over glass slides with DPX mounting media. Oligodendrocyte survival was determined by counting the number of nucleated MBP^+ cells.

Assessment of cytokine and nitrate levels

MCM of non-stimulated and LPA-stimulated microglia was collected as described above. Cytokine protein levels in the MCM were then analyzed using the Milliplex MAP Mouse Cytokine/Chemokine magnetic bead panel (Millipore) on a Luminex (Millipore) as per manufacturers' protocol. For assessment of nitric oxide metabolites in MCM, samples were measured by Nitrate/Nitrite Colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) as per manufacturers' protocol.

Isolation of microglia from CNS tissue

Briefly, spinal cord and brain from adult C57/Bl6 mice (8-10 weeks old) were removed and enzymatically digested with a collagenase B 0.2% (Roche Diagnostics GmbH) and trypsine-EDTA 0.2% at 37°C for 30 min, and then passed through a cell strainer of 40 µm (BD falcon). Cell suspension was centrifuged twice at 300g for 10 minutes at 4°C, and microglial cells were first isolated by magnetic sorting using a CD11b antibody (MiltenyiBiotec) and then stained with PerCP-Cy5.5-conjugated CD45 and PE-Cy7-conjugated CD11b antibodies for further purification on cell sorter (FACSARIA™ III, BD Bioscience). Microglial cells were assessed on a flow cytometer (FACSCalibur; BD Biosciences), and only showing population >90% purity was used for gene expression analysis.

RNA isolation, reverse transcription and real-time PCR

mRNA from cultured and *in vivo* sorted microglia was 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. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene: Primer sequences included the following:

- **LPA_1:**
    - Forward primer: 5'-TGTCCTGGCCTATGAGAAGTTCT-3'.
    - Reverse primer: 5'-TTGTCGCGGTAGGAGTAGATGA-3'.

- **LPA_2:**
    - Forward primer: 5'-CTCACTGGTCAATGCAGTGGTATAT-3'.
    - Reverse primer: 5'-GAAGGCGGCGGAAGGT-3'.

- **GAPDH:**
    - Forward primer: 5'-TCAACAGCAACTCCCACTCTTCCA-3'.
    - Reverse primer: 5'-ACCCTGTTGCTGTAGCCGTATTCA-3'.

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

Statistical analysis

All analyses were conducted through GraphPad Prism 6. 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 was 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

Microglial cells are associated to demyelinating lesions after intraspinal injection of LPA

We previously reported that deletion of LPA_1 (Santos-Nogueira et al., 2015) or LPA_2 (unpublished data) receptors resulted in an improvement of the functional recovery after SCI (Fig. 1A and 1B), and that increased LPA levels in the spinal cord leads to demyelination via LPA_1 and LPA_2 signaling. However, this is not due to a cytotoxic effect of LPA on oligodendrocytes, since cell culture experiments revealed that stimulation of this glial cells with LPA caused minimal effects on cell death (Santos-Nogueira et al., 2015).

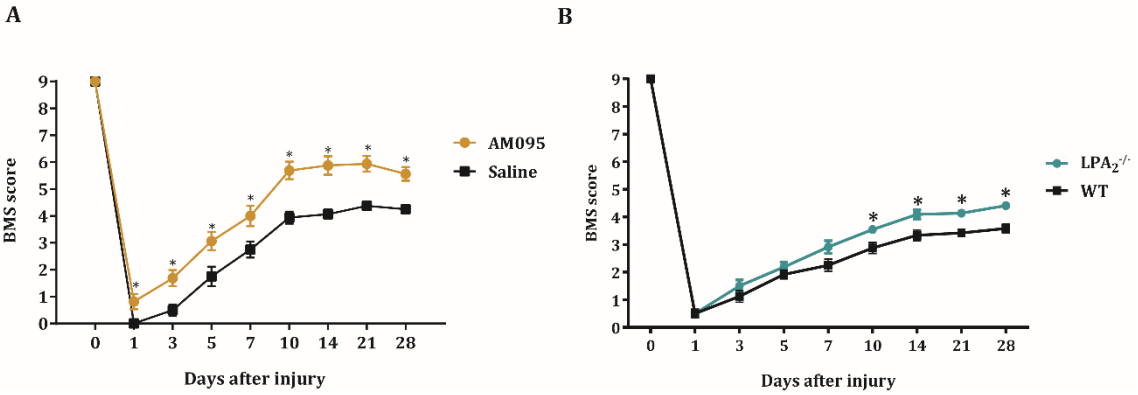


Figure 1. Effects of LPA_1 and LPA_2 gene deletion of functional outcomes after SCI. Graphs showing the locomotor recovery assessed using the BMS score of mice treated with a selective antagonist of LPA_1 receptor, AM095 (Santos-Nogueira et al., 2015) (A), as well as in LPA_2 deficient mice after SCI (unpublished data) (B). (*p<0.05). Error bars indicate SEM.

To gain insights into the mechanisms underlying the demyelinating effects triggered by intraspinal injection of LPA, we evaluated the glial cells that were associated with the demyelinated lesion (Fig. 2). Histological sections of LPA-injected spinal cords revealed the presence of regions with abundant microgliosis that were tightly associated with the demyelinating lesions. Nevertheless, the reactive astrocytes were scarce in these areas, suggesting that microglia rather than astrocytes could be responsible of triggering demyelination upon LPA stimulation (Santos-Nogueira et al., 2015).

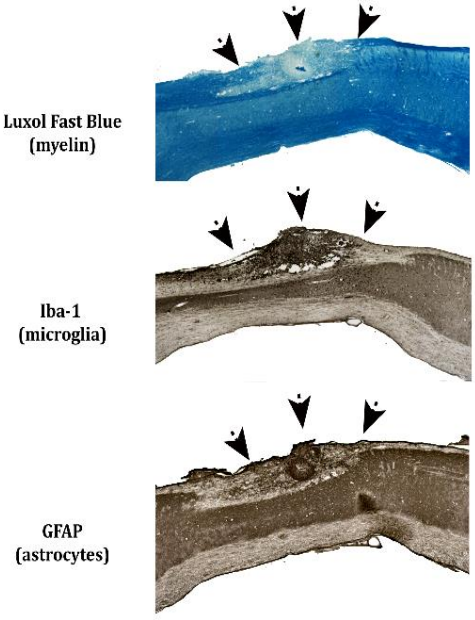


Figure 2. Representative spinal cord sections from mice that received intraspinal injection of LPA. The demyelinating lesion is associated with areas enriched in microglia and, astrocytes to lesser extent.

Microglial LPA$_1$ and LPA$_2$ activation leads to oligodendrocyte cell death

We then studied whether microglial cells expressed the receptors LPA$_1$ and LPA$_2$. Real time PCR (qPCR) analysis of microglial cell cultures revealed that microglia expressed both LPA receptors (Fig. 3A and 3B). Similarly, QPCR analysis of microglial cells sorted from the CNS of adult mice also revealed the presence of LPA$_1$ and LPA$_2$ *in vivo* (on-way ANOVA; *post-hoc* Bonferroni's test; n=4 per group). These experiments reveal that microglial cells express LPA$_1$ and LPA$_2$ receptors.

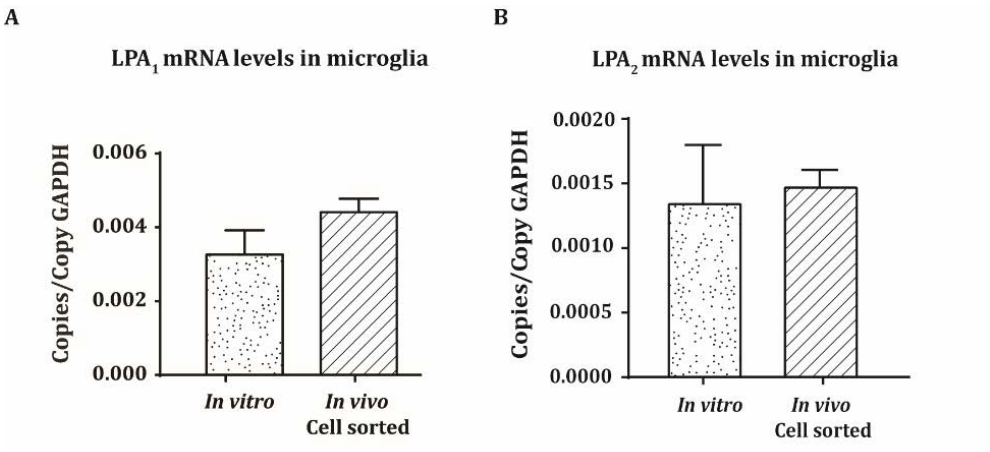


Figure 3. Quantification of LPA$_1$ and LPA$_2$ expression in cultured and cell sorted-microglia. Cultured microglial cells as well as microglia isolated from cell sorter show constitutive expression LPA$_1$ (A) and LPA$_2$ (B). Error bars indicate SEM.

Knowing that microglial cells express these two LPA receptors, we stimulated microglial cells with LPA and applied the conditioned medium to oligodendrocytes to elucidate whether microglia become cytotoxic upon LPA exposure. These experiments revealed that conditioned medium of primary microglial cells stimulated with LPA led to a marked reduction in oligodendrocyte survival (Fig. 4A) (one-way ANOVA; *post-hoc* Bonferroni's test $p<0.0001$; n=3 per group), suggesting that microglia become cytotoxic after LPA stimulation. Interestingly, oligodendrocyte cell death was reduced ~3 times when these cells were incubated with conditioned medium of LPA-stimulated microglial cells isolated from LPA_1 null mice (maLPA_1 mice) (Fig. 4B) (one-way ANOVA; *post-hoc* Bonferroni's test; $p<0.05$; n=3 per group). This data indicates that the activation of LPA_1 in microglial cells is responsible, in part, for the cytotoxic effects mediated by LPA-stimulated microglia. We also assessed whether microglia become cytotoxic upon LPA_2 activation. Interestingly, conditioned medium of LPA-stimulated microglia harvested from LPA_2 deficient mice lead to minimal, if any, toxicity in oligodendrocytes as compared to those cultured from WT littermate mice (Fig. 4C) (one-way ANOVA; *post-hoc* Bonferroni's test; $p<0.05$; n=4 per group). Therefore, these experiments revealed that both, LPA_1 and, to greater extend, LPA_2 signaling in microglial cells mediates the release of unknown factors that are harmful to oligodendrocytes.

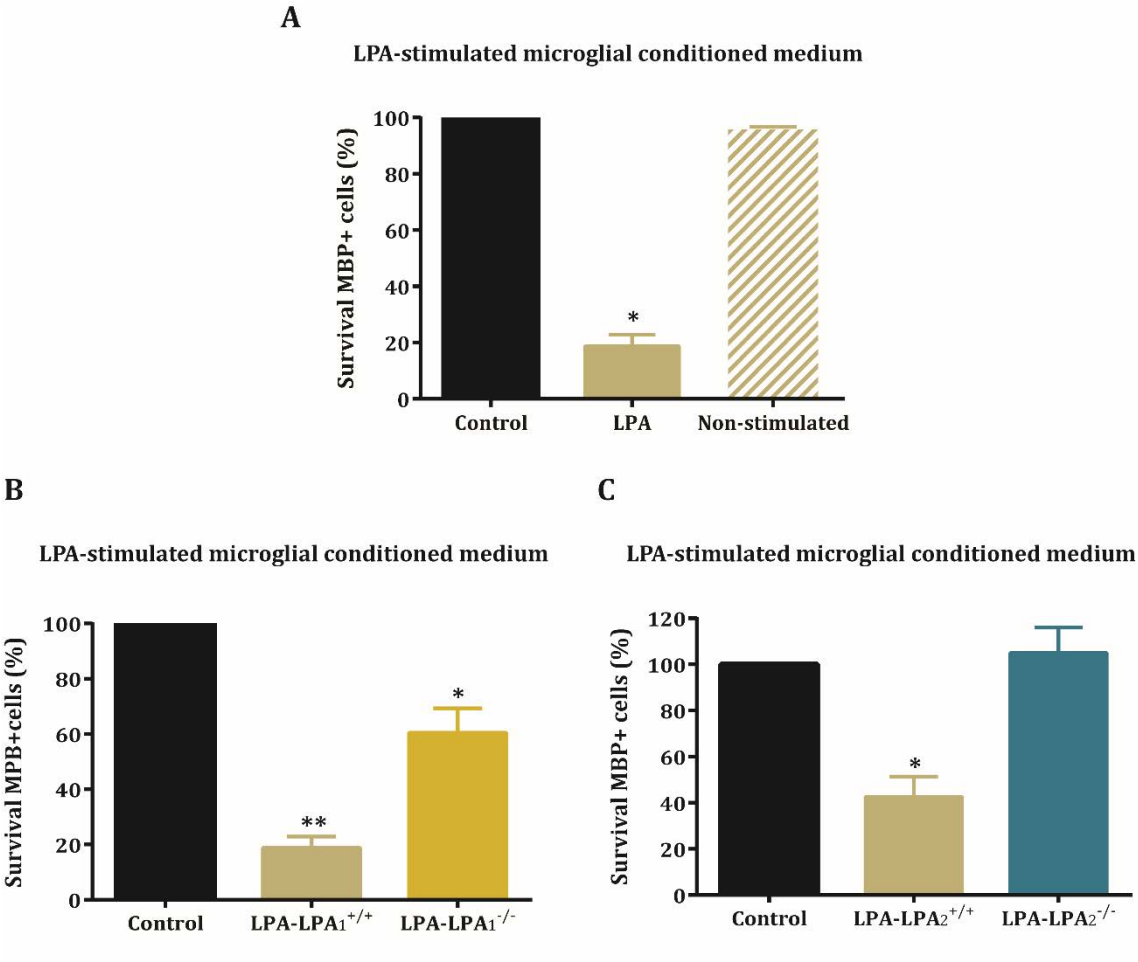


Figure 4. Oligodendrocyte survival after incubation with LPA-stimulated microglial conditioned medium. (A) Plot showing the quantification of MBP+ cells upon incubation with the conditioned medium from primary microglial cells stimulated or not with LPA. Representation of the MBP+ cell survival incubated with the conditioned medium from LPA_1 WT ($LPA_1^{+/+}$) and LPA_1-deficient ($LPA_1^{-/-}$) microglia (B) or from LPA_2 WT ($LPA_2^{+/+}$) or LPA_2-deficient ($LPA_2^{-/-}$) microglia (-c). The control column corresponds to oligodendrocyte cultures incubated with non-LPA stimulated microglial conditioned medium. Error bars indicate SEM. (*p<0.05 vs. Control; **p>0.01 vs. Control).

Elucidating the detrimental mediators produced by microglia upon LPA activation

We next conducted cell culture experiments to identify the cytotoxic factors released by microglia after LPA exposure. Since microglial cells are one of the main sources of cytokines, which can cause cell death (Jessen and Mirsky, 2008; Hartung et al., 2014), we examined the protein levels of 20 cytokines in the supernatants of LPA-stimulated microglia. However, Luminex assay revealed that none of the cytokines studied was significant enhanced in the conditioned medium of LPA-stimulated microglia, suggesting that cytokines are unlikely to be the detrimental factors released by microglial cells upon LPA stimulation (T test; n=4 per group for cytokine evaluation; n=3 for nitrate measurement). (Fig. 5A, B and C).

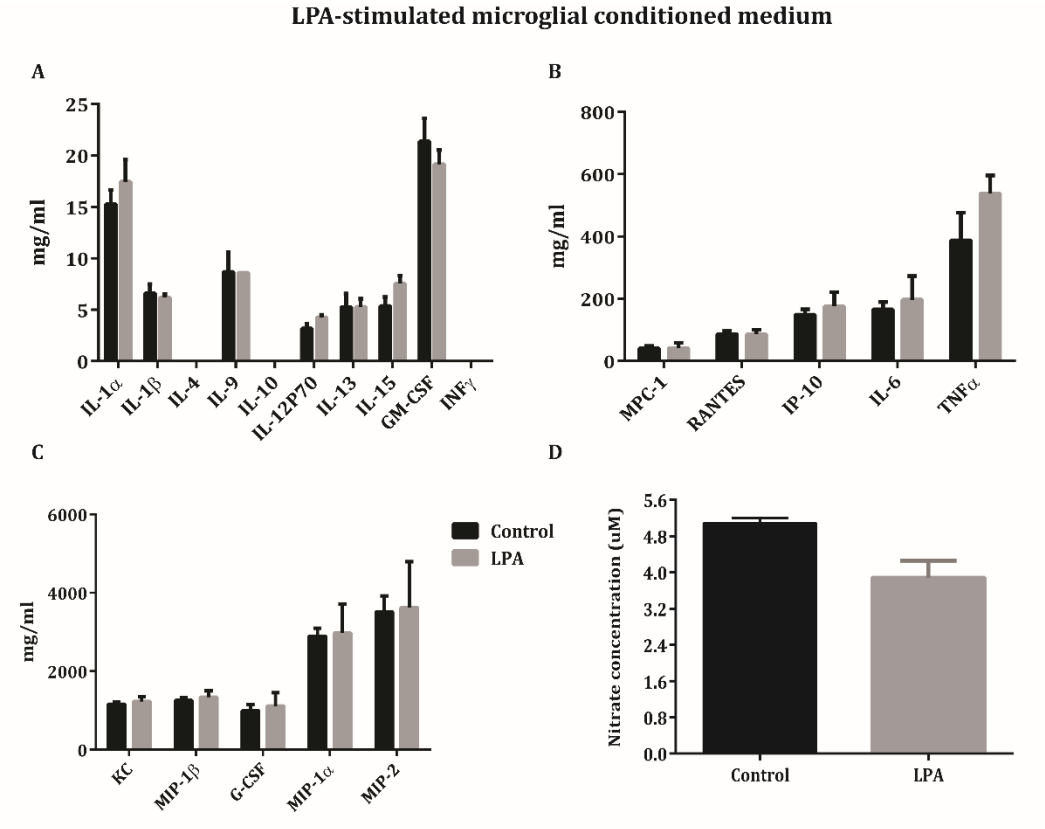


Figure 5. Cytokine and nitrate levels in the conditioned medium from LPA-stimulated microglia. Measurements of different concentrations of cytokines (A, B, C) and nitrates (D) in the supernatant of the conditioned medium from LPA-stimulated microglia. Error bars indicate SEM.

Previous studies demonstrated that activated microglial cells produce reactive oxygen species (ROS), which may induce cell death (Valencia and Moran, 2004; Circu and Aw, 2010). We therefore assessed whether ROS levels are augmented in the supernatants from LPA-stimulated microglia conditioned medium, since oligodendrocytes are very susceptible to oxidative stress (Merrill et al., 1993). However, we found that LPA stimulation did not increase ROS formation (Fig. 5D).

We also assessed whether glutamate, which is released by activated microglial cells and mediates cytotoxicity to oligodendrocytes, was one of the harmful mediators present in the LPA-stimulated microglial conditioned medium. With this aim, we exposed oligodendrocytes to conditioned medium of LPA-stimulated microglia in the presence or not of CNQX, an AMPA/kainate receptor antagonist. However, CNQX failed to reduce oligodendrocyte cell death, suggesting that glutamate is unlikely to be involved in the harmful effects of the LPA-stimulated microglial conditioned medium (one-way ANOVA; *post-hoc* Bonferroni's test; $p<0.05$; n=7). (Fig. 6A).

A previous work reveals that LPA induces the release of ATP in microglial cells (Fujita et al., 2008). Interestingly, ATP exerts potent excitotoxic effects in oligodendrocytes by acting through P_2X_7 receptor (Matute et al., 2007). Thus, we investigated whether ATP was a detrimental factor that induces oligodendrocyte cell death when microglia is stimulated with LPA. To this aim, we exposed oligodendrocytes to conditioned medium of LPA-stimulated microglia together with BBG, a selective P_2X_7 antagonist. These experiments revealed that the cytotoxic effects of LPA-conditioned medium to oligodendrocytes was completely abrogated death upon application of the P_2X_7 antagonist (one-way ANOVA; *post-hoc* Bonferroni's test; $p<0.05$; n=4). (Fig 6B). These results provided clear evidence that LPA stimulation of microglia induces the release of ATP to the extracellular milieu that triggers oligodendrocyte cell through P_2X_7 receptor activation.

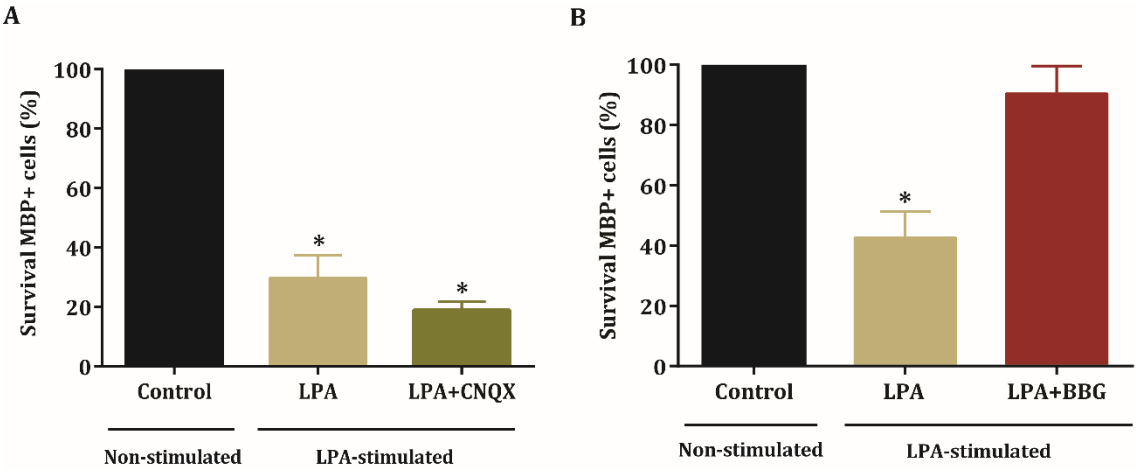


Figure 6. Oligodendrocyte survival after incubation with LPA-stimulated microglial conditioned medium and CNQX or BBG. Quantification of MBP+ cells incubated with LPA-stimulated microglial conditioned medium showed around 30% of oligodendrocyte cell death, which was not rescued in presence of the AMPA/Kainate receptor antagonist CNQX (A). Quantification of MBP+ cells incubated with LPA-stimulated microglial conditioned medium showed around 40% of oligodendrocyte cell death, which was completely rescued in presence of the P_2X_7 receptor antagonist BBG (B). Error bars indicate SEM. (*$p<0.05$ vs. Control).

DISCUSSION

Lysophospholipids (LPs), which include LPA and sphingosine 1-phosphate (S1P), have emerged as key modulators of inflammation, including in nervous system disorders (Chun and Brinkmann, 2011; David et al., 2012c; Choi and Chun, 2013). The contribution of LPs to CNS pathology includes experiments identifying lysolecithin (lysophosphatidylcholine (LPC)) as a potent demyelinating and inflammation-inducing agent in the CNS (Ousman and David, 2000, 2001). S1P has been demonstrated to play a key role in multiple sclerosis (Brinkmann et al., 2010) through the actions of fingolimod (FTY720) (Chun and Brinkmann, 2011), a non-selective S1P receptor modulator, that was the first worldwide oral treatment for relapsing forms of multiple sclerosis (Brinkmann et al., 2010).

LPA is a lipid signaling molecule, capable of 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 0.1μM in plasma to over 10μM in serum. LPA is synthesized *in vivo* from membrane phospholipids by multiple pathways (Aoki et al., 2002; Choi et al., 2010; Choi and Chun, 2013). Exogenous LPA can initiate a large range of neural sequelae within the nervous system that includes effects on most cell types and activities in disease models. Cell types include: developing neural cells (Dubin et al., 1999; Kingsbury et al., 2003; Dubin et al., 2010), astrocytes (Shano et al., 2008; Spohr et al., 2008), microglia (Moller et al., 2001; Ma et al., 2013), oligodendrocytes and Schwann cells (Weiner et al., 1998; Weiner et al., 2001), as well as neurons (Fukushima et al., 2002; Trimbuch et al., 2009; Lin et al., 2012). Disease models include: pain (Inoue et al., 2004; Lin et al., 2012; Ma et al., 2013), hypoxia and ischemia (Herr et al., 2011; Halder et al., 2013), demyelination (Inoue et al., 2004; Inoue et al., 2008a; Inoue et al., 2008b; Nagai et al., 2010) and behavioral disorders (Contos et al., 2000; Contos et al., 2002; Santin et al., 2009; Castilla-Ortega et al., 2012; Pedraza et al., 2014). These physiological responses linked to LPA activation are triggered by a variety of downstream pathways, including Ca^{2+} mobilization, adenylyl cyclase inhibition, activation of Rho, mitogen-activated, protein kinase, phospholipase C, and Akt (Yung et al., 2014; Sheng et al., 2015; Yung et al., 2015).

We previously identified LPA as a new trigger of secondary damage in SCI (Santos-Nogueira et al., 2015). The mechanism involves LPA_1, that is the mostly highly expressed LPA receptor in the spinal cord, and LPA_2, since pharmacological inhibition of LPA_1 using AM095, a selective LPA_1 antagonist, (Santos-Nogueira et al., 2015) or gene deletion of LPA_2

(unpublished data) ameliorates myelin and functional loss after SCI. These effects appear to involve microglia/macrophages, since these glial cells were found in close association with the demyelinating lesion induced by intraspinal injection of LPA into the intact spinal cord. We also noticed mild astrogliosis in the spinal cord after LPA injection, however, the effects of the LPA were more pronounced in microglia/macrophages, suggesting that they are more susceptible to activation by LPA.

LPA_1 and LPA_2 are found on oligodendrocytes (Weiner et al., 1998) and their 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. Similarly, 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). Previous data from the group indicate that LPA exerts very mild toxicity in oligodendrocytes at doses ≥ 1μM, but not in oligodendrocyte precursor cells. However, this effect is not mediated via LPA_1, or LPA_2, since the pharmacological blockade of LPA_1 (Santos-Nogueira et al., 2015) or gene deletion of LPA_2 (unpublished data) failed to rescue oligodendrocyte cell death, at least in cell culture. Hence, LPA-mediated demyelination is likely to be mediated by the activation of LPA_1 and LPA_2 in other cell types, rather than to a direct activation of these two receptors in oligodendrocytes, consistent with our data showing that intraspinal injection of LPA leads to activation of microglia/macrophages, which express LPA_1 and LPA_2 *in vivo* and *in vitro,* as shown here. Conditioned medium of microglia stimulated with LPA mediates marked oligodendrocyte cell death, indicating that microglial cells become cytotoxic upon LPA stimulation. Interestingly, gene deletion of LPA_1 in LPA-stimulated microglial cells appears to increase oligodendrocyte cell survival. This result suggests that microglia LPA_1 is involved in the development of the demyelinating injury triggered by LPA injection into the spinal cord. However, LPA_1 deletion did not completely suppress the harmful effects induced by LPA in microglia, suggesting that others microglial LPA receptors may contribute to oligodendrocyte cell death. Indeed, we found that the conditioned medium of LPA-stimulated microglia lacking LPA_2 has very mild, if any, toxic effects in oligodendrocytes, suggesting microglial LPA_2 signaling also triggers toxic actions in microglia, even to greater extent than LPA_1.

These results indicate that microglial cells release unknown cytotoxic factors upon LPA stimulation via LPA_1 and LPA_2 activation. There are multiple mediators released by microglial cells that cause oligodendrocyte cell death. Among them, cytokines and nitric

oxide produces by microglia are known to be strong inducers of oligodendrocyte cell death (Jessen and Mirsky, 2008; Hartung et al., 2014). However, we did not detect any significant increase in cytokines or nitrate levels in the conditioned medium of microglial cells stimulated with LPA, suggesting that these are not the deleterious mediators induced by LPA. Other works have shown that the glutamate released by microglial cells induces oligodendrocyte cell death, mainly through AMPA/Kainate receptor mediated signaling (Garcia-Barcina and Matute, 1998; McDonald et al., 1998). Therefore, we evaluated indirectly the effects of glutamate released by LPA-activated microglia on oligodendrocyte cell death by using CNQX, a selective AMPA/Kainate antagonist. CNQX treatment did not rescue oligodendrocyte cells from the cytotoxic effects of the LPA-conditioned medium, suggesting that glutamate released by microglia is not the harmful mediator that causes oligodendrocyte cell death. More recent studies have shown that microglial cells release ATP upon LPA stimulation (Fujita et al., 2008). Interestingly, oligodendrocyte cells express the purinergic P_2X_7, and stimulation of oligodendrocytes with ATP induces cell death via P_2X_7 activation (Matute et al., 2007; Peng et al., 2009). Indeed, inhibition of the purinergic receptor P_2X_7 confers protection against experimental autoimmune encephalomyelitis (EAE) (Matute et al., 2007) and SCI (Peng et al., 2009). Here, we showed that treatment with BBG, a potent P_2X_7 antagonist, almost completely rescued oligodendrocyte cell death induced by the conditioned medium of LPA-activated microglia. These findings suggest that activation of LPA receptors in microglial cells stimulate the release of purines to the extracellular milieu, which in turn, activate P_2X_7 in oligodendrocytes, provoking cell death and consequently, demyelination.

Overall, the results of the present chapter provide clear and novel evidence that LPA_1 and LPA_2 signaling in microglia seems to be the responsible of the demyelinating effects of LPA via purine. 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, but probably to CNS conditions where myelin loss is a key contributor to the disease.

REFERENCES

Aoki J, Taira A, Takanezawa Y, Kishi Y, Hama K, Kishimoto T, Mizuno K, Saku K, Taguchi R, Arai H (2002) Serum lysophosphatidic acid is produced through diverse phospholipase pathways. J Biol Chem 277:48737-48744.

Bot M, Bot I, Lopez-Vales R, van de Lest CH, Saulnier-Blache JS, Helms JB, David S, van Berkel TJ, Biessen EA (2010) Atherosclerotic lesion progression changes lysophosphatidic acid homeostasis to favor its accumulation. The American journal of pathology 176:3073-3084.

Brinkmann V, Billich A, Baumruker T, Heining P, Schmouder R, Francis G, Aradhye S, Burtin P (2010) Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. Nat Rev Drug Discov 9:883-897.

Castilla-Ortega E, Pedraza C, Chun J, de Fonseca FR, Estivill-Torrus G, Santin LJ (2012) Hippocampal c-Fos activation in normal and LPA(1)-null mice after two object recognition tasks with different memory demands. Behav Brain Res 232:400-405.

Choi JW, Chun J (2013) Lysophospholipids and their receptors in the central nervous system. Biochim Biophys Acta 1831:20-32.

Choi JW, Herr DR, Noguchi K, Yung YC, Lee CW, Mutoh T, Lin ME, Teo ST, Park KE, Mosley AN, Chun J (2010) LPA receptors: subtypes and biological actions. Annu Rev Pharmacol Toxicol 50:157-186.

Chun J, Brinkmann V (2011) A mechanistically novel, first oral therapy for multiple sclerosis: the development of fingolimod (FTY720, Gilenya). Discov Med 12:213-228.

Circu ML, Aw TY (2010) Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med 48:749-762.

Contos JJ, Fukushima N, Weiner JA, Kaushal D, Chun J (2000) Requirement for the lpA1 lysophosphatidic acid receptor gene in normal suckling behavior. Proc Natl Acad Sci U S A 97:13384-13389.

Contos JJ, Ishii I, Fukushima N, Kingsbury MA, Ye X, Kawamura S, Brown JH, Chun J (2002) Characterization of lpa(2) (Edg4) and lpa(1)/lpa(2) (Edg2/Edg4) lysophosphatidic acid receptor knockout mice: signaling deficits without obvious phenotypic abnormality attributable to lpa(2). Mol Cell Biol 22:6921-6929.

David S, Lopez-Vales R, Wee Yong V (2012a) Harmful and beneficial effects of inflammation after spinal cord injury: potential therapeutic implications. Handb Clin Neurol 109:485-502.

David S, Zarruk JG, Ghasemlou N (2012b) Inflammatory pathways in spinal cord injury. Int Rev Neurobiol 106:127-152.

David S, Greenhalgh AD, Lopez-Vales R (2012c) Role of phospholipase A2s and lipid mediators in secondary damage after spinal cord injury. Cell Tissue Res 349:249-267.

Dubin AE, Herr DR, Chun J (2010) Diversity of lysophosphatidic acid receptor-mediated intracellular calcium signaling in early cortical neurogenesis. J Neurosci 30:7300-7309.

Dubin AE, Bahnson T, Weiner JA, Fukushima N, Chun J (1999) Lysophosphatidic acid stimulates neurotransmitter-like conductance changes that precede GABA and L-glutamate in early, presumptive cortical neuroblasts. J Neurosci 19:1371-1381.

Frisca F, Sabbadini RA, Goldshmit Y, Pebay A (2012) Biological effects of lysophosphatidic acid in the nervous system. Int Rev Cell Mol Biol 296:273-322.

Fujita R, Ma Y, Ueda H (2008) Lysophosphatidic acid-induced membrane ruffling and brain-derived neurotrophic factor gene expression are mediated by ATP release in primary microglia. J Neurochem 107:152-160.

Fukushima N, Weiner JA, Kaushal D, Contos JJ, Rehen SK, Kingsbury MA, Kim KY, Chun J (2002) Lysophosphatidic acid influences the morphology and motility of young, postmitotic cortical neurons. Mol Cell Neurosci 20:271-282.

Garcia-Barcina JM, Matute C (1998) AMPA-selective glutamate receptor subunits in glial cells of the adult bovine white matter. Brain Res Mol Brain Res 53:270-276.

Garcia-Diaz B, Riquelme R, Varela-Nieto I, Jimenez AJ, de Diego I, Gomez-Conde AL, Matas-Rico E, Aguirre JA, Chun J, Pedraza C, Santin LJ, Fernandez O, Rodriguez de Fonseca F, Estivill-Torrus G (2014) Loss of lysophosphatidic acid receptor LPA alters oligodendrocyte differentiation and myelination in the mouse cerebral cortex. Brain Struct Funct.

Halder SK, Yano R, Chun J, Ueda H (2013) Involvement of LPA1 receptor signaling in cerebral ischemia-induced neuropathic pain. Neuroscience 235:10-15.

Hartung HP, Aktas O, Menge T, Kieseier BC (2014) Immune regulation of multiple sclerosis. Handb Clin Neurol 122:3-14.

Herr KJ, Herr DR, Lee CW, Noguchi K, Chun J (2011) Stereotyped fetal brain disorganization is induced by hypoxia and requires lysophosphatidic acid receptor 1 (LPA1) signaling. Proc Natl Acad Sci U S A 108:15444-15449.

Inoue M, Ma L, Aoki J, Chun J, Ueda H (2008a) Autotaxin, a synthetic enzyme of lysophosphatidic acid (LPA), mediates the induction of nerve-injured neuropathic pain. Mol Pain 4:6.

Inoue M, Rashid MH, Fujita R, Contos JJ, Chun J, Ueda H (2004) Initiation of neuropathic pain requires lysophosphatidic acid receptor signaling. Nat Med 10:712-718.

Inoue M, Xie W, Matsushita Y, Chun J, Aoki J, Ueda H (2008b) Lysophosphatidylcholine induces neuropathic pain through an action of autotaxin to generate lysophosphatidic acid. Neuroscience 152:296-298.

Jessen KR, Mirsky R (2008) Negative regulation of myelination: relevance for development, injury, and demyelinating disease. Glia 56:1552-1565.

Kihara Y, Maceyka M, Spiegel S, Chun J (2014) Lysophospholipid receptor nomenclature review: IUPHAR Review 8. Br J Pharmacol 171:3575-3594.

Kingsbury MA, Rehen SK, Contos JJ, Higgins CM, Chun J (2003) Non-proliferative effects of lysophosphatidic acid enhance cortical growth and folding. Nat Neurosci 6:1292-1299.

Lin ME, Rivera RR, Chun J (2012) Targeted Deletion of LPA5 Identifies Novel Roles for Lysophosphatidic Acid Signaling in Development of Neuropathic Pain. J Biol Chem 287:17608-17617.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Ma L, Nagai J, Chun J, Ueda H (2013) An LPA species (18:1 LPA) plays key roles in the self-amplification of spinal LPA production in the peripheral neuropathic pain model. Mol Pain 9:29.

Matute C, Torre I, Perez-Cerda F, Perez-Samartin A, Alberdi E, Etxebarria E, Arranz AM, Ravid R, Rodriguez-Antiguedad A, Sanchez-Gomez M, Domercq M (2007) P2X(7) receptor blockade prevents ATP excitotoxicity in oligodendrocytes and ameliorates experimental autoimmune encephalomyelitis. J Neurosci 27:9525-9533.

McDonald JW, Althomsons SP, Hyrc KL, Choi DW, Goldberg MP (1998) Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. Nat Med 4:291-297.

Merrill JE, Ignarro LJ, Sherman MP, Melinek J, Lane TE (1993) Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. J Immunol 151:2132-2141.

Moller T, Contos JJ, Musante DB, Chun J, Ransom BR (2001) Expression and function of lysophosphatidic acid receptors in cultured rodent microglial cells. J Biol Chem 276:25946-25952.

Nagai J, Uchida H, Matsushita Y, Yano R, Ueda M, Niwa M, Aoki J, Chun J, Ueda H (2010) Autotaxin and lysophosphatidic acid1 receptor-mediated demyelination of dorsal root fibers by sciatic nerve injury and intrathecal lysophosphatidylcholine. Mol Pain 6:78.

O'Meara RW, Ryan SD, Colognato H, Kothary R (2011) Derivation of enriched oligodendrocyte cultures and oligodendrocyte/neuron myelinating co-cultures from post-natal murine tissues. J Vis Exp.

Ousman SS, David S (2000) Lysophosphatidylcholine induces rapid recruitment and activation of macrophages in the adult mouse spinal cord. Glia 30:92-104.

Ousman SS, David S (2001) MIP-1alpha, MCP-1, GM-CSF, and TNF-alpha control the immune cell response that mediates rapid phagocytosis of myelin from the adult mouse spinal cord. J Neurosci 21:4649-4656.

Pedraza C, Sanchez-Lopez J, Castilla-Ortega E, Rosell-Valle C, Zambrana-Infantes E, Garcia-Fernandez M, Rodriguez de Fonseca F, Chun J, Santin LJ, Estivill-Torrus G (2014) Fear extinction and acute stress reactivity reveal a role of LPA(1) receptor in regulating emotional-like behaviors. Brain Struct Funct 219:1659-1672.

Peng W, Cotrina ML, Han X, Yu H, Bekar L, Blum L, Takano T, Tian GF, Goldman SA, Nedergaard M (2009) Systemic administration of an antagonist of the ATP-sensitive receptor P2X7 improves recovery after spinal cord injury. Proc Natl Acad Sci U S A 106:12489-12493.

Popovich PG (2014) Neuroimmunology of traumatic spinal cord injury: a brief history and overview. Exp Neurol 258:1-4.

Rowland JW, Hawryluk GW, Kwon B, Fehlings MG (2008) Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon. Neurosurg Focus 25:E2.

Santin LJ, Bilbao A, Pedraza C, Matas-Rico E, Lopez-Barroso D, Castilla-Ortega E, Sanchez-Lopez J, Riquelme R, Varela-Nieto I, de la Villa P, Suardiaz M, Chun J, De Fonseca FR, Estivill-Torrus G (2009) Behavioral phenotype of maLPA1-null mice: increased anxiety-like behavior and spatial memory deficits. Genes Brain Behav 8:772-784.

Santos-Nogueira E, Lopez-Serrano C, Hernandez J, Lago N, Astudillo AM, Balsinde J, Estivill-Torrus G, de Fonseca FR, Chun J, Lopez-Vales R (2015) Activation of Lysophosphatidic Acid Receptor Type 1 Contributes to Pathophysiology of Spinal Cord Injury. J Neurosci 35:10224-10235.

Saura J, Tusell JM, Serratosa J (2003) High-yield isolation of murine microglia by mild trypsinization. Glia 44:183-189.

Shano S, Moriyama R, Chun J, Fukushima N (2008) Lysophosphatidic acid stimulates astrocyte proliferation through LPA1. Neurochem Int 52:216-220.

Sheng X, Yung YC, Chen A, Chun J (2015) Lysophosphatidic acid signalling in development. Development 142:1390-1395.

Spohr TC, Choi JW, Gardell SE, Herr DR, Rehen SK, Gomes FC, Chun J (2008) Lysophosphatidic acid receptor-dependent secondary effects via astrocytes promote neuronal differentiation. J Biol Chem 283:7470-7479.

Trimbuch T et al. (2009) Synaptic PRG-1 modulates excitatory transmission via lipid phosphate-mediated signaling. Cell 138:1222-1235.

Valencia A, Moran J (2004) Reactive oxygen species induce different cell death mechanisms in cultured neurons. Free Radic Biol Med 36:1112-1125.

Weiner JA, Hecht JH, Chun J (1998) Lysophosphatidic acid receptor gene vzg-1/lpA1/edg-2 is expressed by mature oligodendrocytes during myelination in the postnatal murine brain. J Comp Neurol 398:587-598.

Weiner JA, Fukushima N, Contos JJ, Scherer SS, Chun J (2001) Regulation of Schwann cell morphology and adhesion by receptor-mediated lysophosphatidic acid signaling. J Neurosci 21:7069-7078.

Yung YC, Stoddard NC, Chun J (2014) LPA receptor signaling: pharmacology, physiology, and pathophysiology. J Lipid Res 55:1192-1214.

Yung YC, Stoddard NC, Mirendil H, Chun J (2015) Lysophosphatidic Acid signaling in the nervous system. Neuron 85:669-682.

Yung YC, Mutoh T, Lin ME, Noguchi K, Rivera RR, Choi JW, Kingsbury MA, Chun J (2011) Lysophosphatidic acid signaling may initiate fetal hydrocephalus. Sci Transl Med 3:99ra87.

Chapter II. Role of the non-endothelial differentiation gene family LPA receptors in the secondary damage after spinal cord injury

Clara López-Serrano[1], Eva Santos-Nogueira[1], Jerold Chun[2], Rubèn López-Vales[1]

[1]Departament de Biologia Cellular, Fisiologia i Immunologia, Institut de Neurociències, Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Universitat Autònoma de Barcelona, Bellaterra, Spain; [2]Molecular and Cellular Neuroscience Department, Dorris Neuroscience Center, The Scripps Research Institute, La Jolla, USA.

ABSTRACT

Lysophosphatidic acid (LPA) is a bioactive lipid that acts as an intracellular messenger regulating different physiological functions by binding to six G protein-coupled receptors (GPCRs), known as LPA_{1-6}. Our laboratory has characterized the role of the different endothelial gene differentiation (Edg) family LPA receptors (LPA_{1-3}) in the physiopathology of spinal cord injury (SCI) in mice. However, it is currently unknown the involvement of the non-Edg family LPA receptors after CNS trauma. The aim of the present study was to assess whether LPA_4 and LPA_5, two out of the three LPA receptors that belong to the non-Edg family, are involved in triggering secondary tissue damage and functional deficits after spinal cord contusion in mice. Our results reveal that the LPA_4 mRNA does not change in the spinal cord parenchyma after injury, and that the gene deletion of this receptor worsened, although not significantly, locomotors skills after contusion. Contrary to LPA_4, the transcripts of LPA_5 were markedly up-regulated in the contused spinal cord. However, the lack of LPA_5 did not conferred protection against functional deficits or myelin and neuronal loss after SCI. Therefore, these findings demonstrate that both, LPA_4 and LPA_5 receptors do not have any key contribution to secondary damage and functional impairments after SCI.

Key words: lysophosphatidic acid, non-Edg LPA family receptors, spinal cord injury.

INTRODUCTION

Spinal cord injury (SCI) causes functional deficits due to the loss of axons, neurons and oligodendrocytes after trauma. Injury to the spinal cord results in primary damage, which is a direct consequence of the trauma, followed by a secondary phase of tissue damage that occurs over a period of several weeks (Schwab and Bartholdi, 1996). Out of the multiple mechanisms that trigger the secondary phase, the inflammatory response that takes place after the SCI is considered one of the main contributors (David et al., 2012). Endogenous microglial and astroglial cells, as well as peripheral monocytes and neutrophils that enter into the spinal cord after lesion, are the main cells involved in such physiological response after SCI. Although they have a crucial role in the clearance of dead cells and debris to recover homeostasis, they also release several factors such as cytokines, free radicals, proteases, eicosanoids, etc., that causes damage to neurons, astroglia and myelin (Donnelly and Popovich, 2008).

A molecule that has been recently described to have a key involvement in regulating the inflammatory response is the lysophosphatidic Acid (LPA) (Noguchi et al., 2009; Choi et al., 2010). LPA signals through six known G protein-coupled receptors (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 LPA receptors 4, 5 and 6 (LPA_{4-6}), which belong to the non-Edg family LPA receptors and are more closely related to the P2Y purinergic receptor family (Non-Edg family) (Choi et al., 2010). LPA receptors are involved in the pathogenesis of some CNS disorders and therefore may have therapeutic relevance to treat such conditions in human CNS, such as multiple sclerosis, congenital hydrocephalus, ischemic stroke, neurotrauma, neuropsychiatric and developmental disorders, seizures, hearing loss, etc. (Choi and Chun, 2013). Due to the variety of LPA receptors, LPA might have either helpful or harmful effects in the CNS depending on the receptors it signals through, and thus, there is a need to elucidate the role of the different LPA receptors in neurodegeneration.

We have recently reported that LPA levels are increased in the spinal cord parenchyma after a spinal cord contusion, leading to demyelination in the spinal cord through the activation of the microglial LPA_1 (Santos-Nogueira et al., 2015). Unpublished data from the laboratory together with the results shown in the previous chapter also reveal that LPA_2 receptor also mediates deleterious effects in the injured CNS. Moreover, we have also unpublished data revealing that LPA_3 receptor does not contribute to the

physiopathology after the SCI in mice. However, whether non-Edg family LPA receptors are involved in the secondary damage after a SCI need to be addressed.

LPA_4 and LPA_5 receptors are genetically distinct from the Edg-family LPA receptors. LPA_4, also known as GPR23 or P2Y9 receptor, shares significant homology to P2Y purinergic receptors, even though it does not respond to any nucleotides or nucleosides tested (Noguchi et al., 2003). LPA_4 receptor signals through the $G_{\alpha 12/13}$ and Rho/Rho-kinase pathways mediating neurite retraction, increases intracellular cAMP through the activation of the $G_{\alpha s}$ protein signalling, and induces Ca^{2+} mobilization upon Gαq activation (Lee et al., 2007). This receptor has a suppressive effect on cell motility and therefore, LPA_4 deficiency enhances cell migration and motility in fibroblasts and colon cancer cells in response to LPA (Lee et al., 2008). However, the role of LPA_4 in pathologies affecting the CNS has not been explored yet.

On the other hand, LPA_5, also known as GPR92, is a G protein-coupled receptor that signals through $G\alpha_{12/13}$ and G_q protein activation, increasing Ca^{2+} and cAMP levels and inositol phosphate production (Choi et al., 2010). The biological functions of LPA_5 are still not well known. *In vitro* studies have shown that LPA_5 is part of the microglial sensome, probably taking part of the microglial polarization to M1 phenotype after an inflammatory stimulus (Plastira et al., 2016). Related to CNS pathologies, LPA_5-deficient mice have been observed to have protective effects after partial sciatic nerve ligation, suggesting the involvement of the LPA-LPA_5 signalling in the development of neuropathic pain (Lin et al., 2012).

In the present work, we aim to elucidate whether either LPA_4 or LPA_5 receptors play any role in the SCI physiopathology. We provide clear evidence that these 2 non-Edg family LPA receptors are not involved in secondary damage after SCI.

MATERIAL AND METHODS

LPA_4 and LPA_5 deficient mice

LPA_4 and LPA_5 deficient mice were kindly given by Professor Jerold Chun, from the Scripps Research Institute and were bred in the animal facility at Universitat Autònoma de Barcelona.

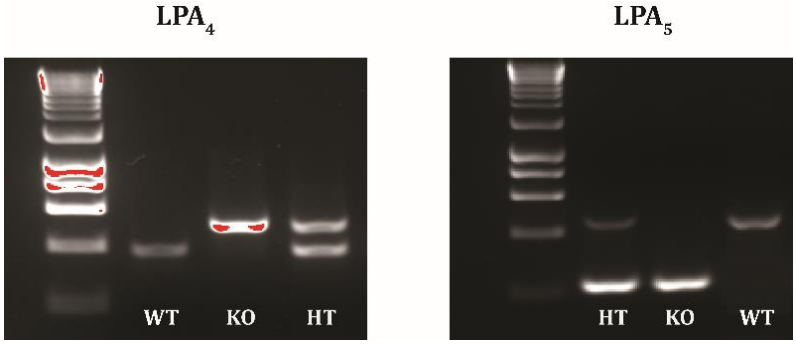
PCR analysis was used to detect the presence of *Lpar4* and *Lpar5* natural or mutated alleles. For tissue sampling, the tip of the tail was cut at the time of weaning the mice. Genomic DNA extraction was carried out by using the ArchivePure DNA Purification System (5PRIME), as described by the manufacturer. Allele amplification was performed by PCR reaction, using the Taq DNA Polymerase kit (Invitrogen), and PCR products were analysed by standard electrophoresis in 2% agarose gels with a ChemiDoc XRS+ device (BIO RAD). Primers sequences were the following:

- **LPA_4:**
    - Forward primer: 5'-GCAGATTTGGTGACTCATTCTACTT-3'.
    - Reverse primer: 5'-ATTTTGAAAGGTAGCGTACAAACAAA-3'.
    - Mutation primer (LacZ): 5'-GTAATGGGATAGGTTACGTTGGTGTA-3'.

  Product sizes are 406 bp (base pair) for wild-type and 559 bp for knockout band.

- **LPA_5:**
    - Forward primer: 5'-CAG AGT CTG TAT TGC CAC CAG-3'.
    - Reverse primer: 5'-GTC CAC GTT GAT GAG CAT CAG-3'.
    - Mutation primer (GFP): 5'-GTG GTG CAG ATG AAC TTC AGG-3'.

  Product sizes are 450 bp for wild-type band and 220 bp for knockout band.



Spinal cord injury

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_4 and LPA_5 deficient mice in a C57BL/6J and wildtype littermate (WT) mice were anesthetized with ketamine:xylazine (90:10mg/kg, i.m.). After performing a laminectomy at the 11^{th} thoracic vertebrae, the exposed spinal cord was contused by applying a force of 60±5 kilodynes and 400-600μm in displacement with a Horizons Impactor device (Precision Scientific Instrumentation, Fairfax Station, VA).

RNA isolation, reverse transcription and real-time PCR

To evaluate the mRNA expression levels of LPA_4 and LPA_5 receptors in the spinal cord, quantitative real-time (RT)-PCR (qPCR) was performed. Mice with intact or lesioned spinal cords were perfused with sterile saline and 5 mm length of spinal cord containing the lesion site in the centre was harvested. 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. 1μg of obtained RNA was primed with random hexamers (Promega) and reverse transcribed using Omniscript RT kit (Qiagen). RNase inhibitor (Roche) was added (1U/μl final concentration) to avoid RNA degradation. qPCR analysis was performed using a MyiQ Single-Color Real-Time PCR Detection System (BIO RAD). qPCR reactions were performed using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The amount of cDNA was calculated based on the Cycle Threshold cycle (CT) value, and was standardized by the amount of housekeeping gene using the $2^{-\Delta\Delta C_T}$ method. The expression level of the target mRNA was normalized to the relative ratio of the expression of the GAPDH housekeeping gene (Livak and Schmittgen, 2001). Primer sequences used in RT-PCR reaction are the following:

- **LPA_4:**
    - Forward primer: 5'- GATGGAGTCGCTGTTTAAGACTGA-3'.
    - Reverse primer: 5'-TGTTTGATCACTAACTTCCTCTTGGATA-3'.
- **LPA_5:**
    - Forward primer: 5'- CCGTACATGTTCATCTGGAAGAT-3'.
    - Reverse primer: 5'- CAGACTAATTTCTCTTCCCACCT-3'.

- **GAPDH:**
    - Forward primer: 5'- TCAACAGCAACTCCCACTCTTCCA-3'.
    - Reverse primer: 5'- ACCCTGTTGCTGTAGCCGTATTCA-3'.

Behavioural scoring

The spontaneous locomotor recovery of the injured animals was assessed prior to surgery and 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), which was specifically developed for locomotor testing after contusion injuries in mice. In the BMS, zero points represent total absence of movement of the hindlimbs, whereas nine points represent the total recovery of the movement of the hindlimbs. An eleven-point sub-score scale was used for a more detailed punctuation, considering tail direction, trunk stability, etc. (Basso et al., 2006).

Histological assessment

At 28 dpi, mice were deeply anaesthetized using Dolethal (pentobarbital sodium; Vétoquinol E.V.S.A.) and perfused with 4% paraformaldehyde in 0.1M phosphate buffer (PB). The epicentre of the lesion and 8 mm length ventral and dorsal to it was post-fixed for 1 hour in 4% paraformaldehyde in 0.1M PB and cryoprotected with 30% sucrose with azide (1:100), in 0.1M PB at 4°C. Spinal cords were fast-frozen at -60°C in cryoembedding compound (Tissue-Tek OCT, Sakura) and cut on a cryostat (Leica) in 15-µm-thick transversal sections.

For assessment of myelin sparing, tissue sections were dyed with Luxol Fast Blue (LFB). Briefly, tissue sections were hydrated in 0.1M PBS and gradually dehydrated in 50%, 70%, 80% and 95% ethanol. Sections were placed in a 1mg/mL LFB solution in 95% ethanol and 0.05% acetic acid overnight at 37°C. Sections were then washed in 95% ethanol and distilled water before placing them into a solution of 0.5mg/mL of lithium carbonate in distilled water for 1 min at room temperature. After washing in distilled water, sections were dehydrated in ethanol and mounted in DPX mounting media (Sigma).

For neuronal sparing, tissue sections were immunostained against NeuN, which is a neuronal nuclear antigen cell commonly used as a biomarker for neurons. 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 4°C 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, gradually dehydrated in ethanol and mounted in DPX mounting media (Sigma).

Statistical analysis

All analyses were conducted through GraphPad Prism 6. LPA_4 and LPA_5 expression in the microglia and spinal cord by RT-PCR was analysed using one-way ANOVA with *post-hoc* Bonferroni's test. BMS score and histological parameters were analysed using two-way repeated measure (RM)-ANOVA with *post-hoc* Bonferroni's test. Results are expressed as mean ± SEM. Differences were considered significant at $p<0.05$.

RESULTS

mRNA expression levels of LPA$_4$ and LPA$_5$ receptors after SCI

We first assessed the changes in mRNA levels of the non-Edg family LPA receptors, LPA$_4$ and LPA$_5$, in the spinal cord parenchyma after a contusion injury by using RT-PCR assay. Our data revealed different expression patterns for LPA$_4$ and LPA$_5$ after SCI. In the case of the LPA$_4$, it tended to increase its transcripts, although not significantly, at day 1 and 3 post-injury (one-way ANOVA; *post-hoc* Bonferroni's test; $p > 0.05$; n=3 per group) (Fig. 1A). Contrarily, LPA$_5$ mRNA levels were markedly increased after injury from 7 to 21 days post-injury, peaking at day 7 (one-way ANOVA; *post-hoc* Bonferroni's test; $p < 0.05$; n=3 per time point (Fig. 1B).

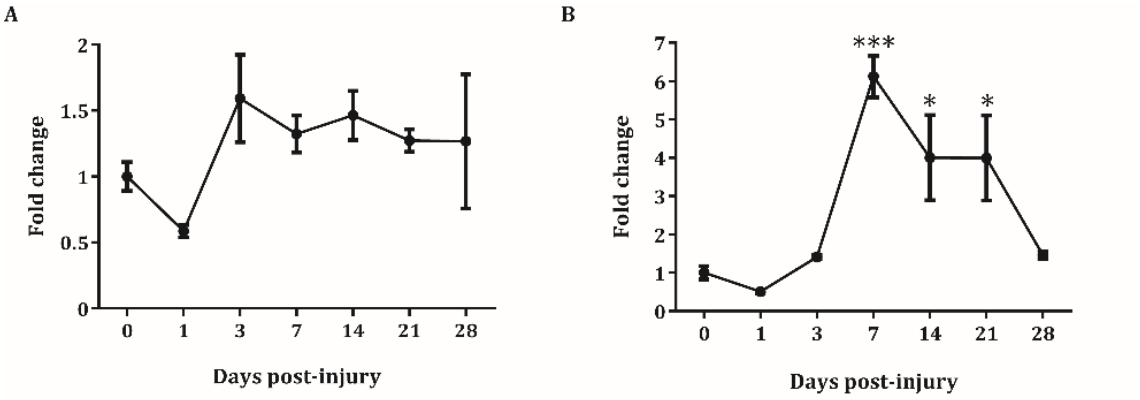


Figure 1. LPA$_4$ mRNA expression does not change in the spinal cord injury, whereas LPA$_5$ mRNA expression is highly up-regulated. Real Time-PCR quantification of LPA$_4$ (A) and LPA$_5$ (B) mRNA levels from spinal cord harvested at different time points after contusion injury. Error bars indicate SEM. (*$p < 0.05$, ***$p < 0.0001$).

LPA$_4$ or LPA$_5$ are not major contributors to locomotor deficits after SCI

To study the potential role of these two non-Edg family LPA receptor in SCI, we induced moderate contusion injuries in LPA$_4$ and LPA$_5$ deficient or WT littermate mice. We observed that the genetical deletion of LPA$_4$ tended to worsen behavioural outcomes after spinal cord contusion (Fig. 2A). Post-hoc analysis revealed that the lack of LPA$_4$ resulted in significant locomotor impairments at day 14 post-injury (two-way RM-ANOVA; *post-hoc* Bonferroni's test; $p < 0.05$; n= 14 per group) but not at any other time point (Fig. 2A). The absence of the LPA$_5$, however, did not result in any significant difference on motor skills after SCI (two-way RM-ANOVA; *post-hoc* Bonferroni's test; n= 20 for WT and n=18 for LPA$_5$ deficient mice) (Fig. 2B).

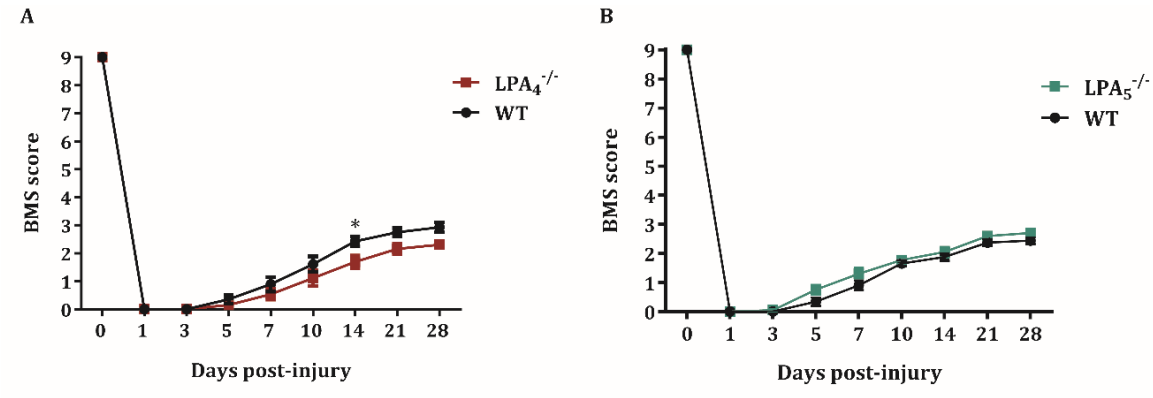


Figure 2. Effects of LPA_4 and LPA_5 gene deletion on functional outcomes after SCI. Graph showing the locomotor recovery after spinal cord contusion injury in LPA_4 (A) or LPA_5 (B) deficient mice and their respective WT littermates was assessed using the 9-point Basso Mouse Scale. *p<0.05; Error bars indicate SEM.

The lack of LPA_4 and LPA_5 does not enhance myelin preservation after SCI

Even though LPA_4 or LPA_5 did not show to have a major contribution to motor impairments after SCI, we examined whether LPA_4 or LPA_5 signalling leads to tissue damage. Histological sections of the injured spinal cord from LPA_4 and LPA_5 deficient and WT mice were processed with a Luxol Fast Blue staining to evaluate myelin preservation. We observed that lack of LPA_4 (Fig. 3A) or LPA_5 (Fig. 3B) did not show significant differences in the myelin preservation, compared to their respective wild-type littermates (two-way RM-ANOVA; *post-hoc* Bonferroni's test; LPA_4: n=11 for WT and n=10 for null mice; LPA_5: n= 20 for WT and n=18 for null mice).

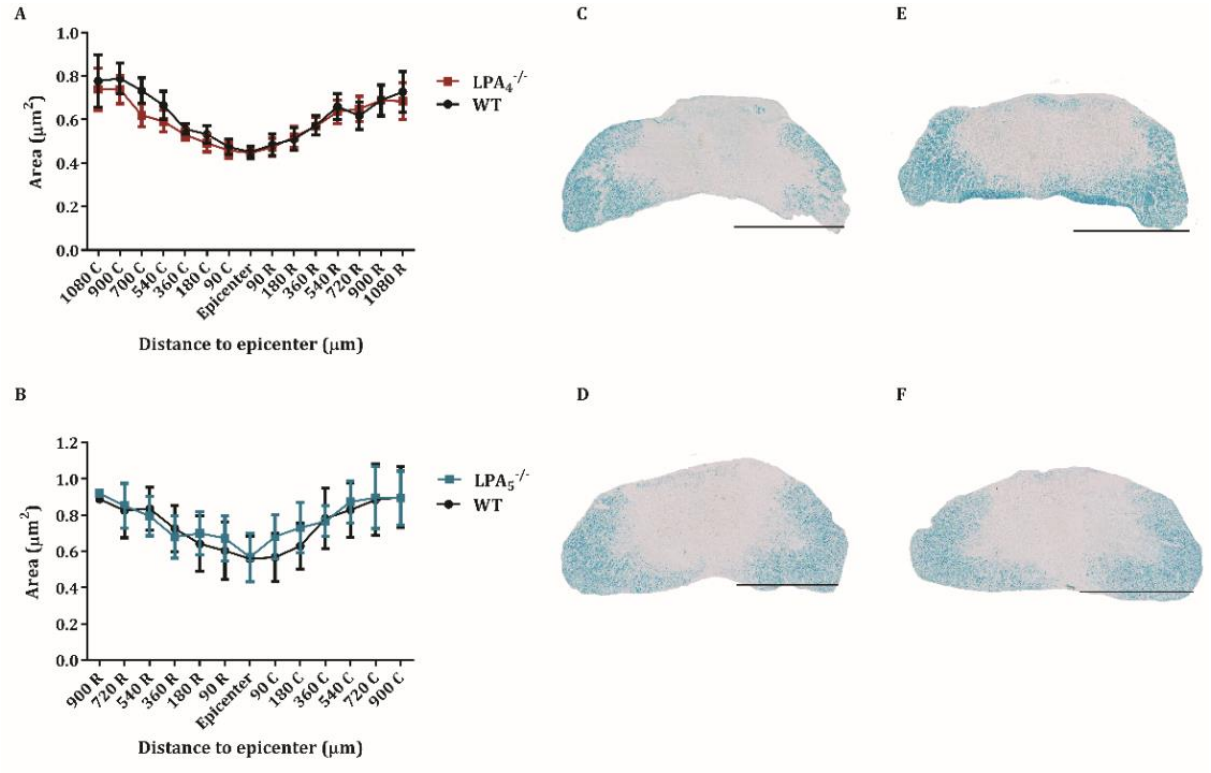


Figure 3. The lack of LPA_4 or LPA_5 does not enhance myelin sparing after SCI. Plots showing the quantification of the Luxol Fast Blue staining at different distances rostral and caudal to the injury epicentre in both deficient mice (A, B). Representative micrographs show myelin sparing at the injury epicentre in LPA_4 (C) and LPA_5 (D) deficient mice, and their respective WT littermates (E, F). Error bars indicate SEM. Scale bar=500μm.

The lack of LPA_4 and LPA_5 does not enhance neuronal survival after SCI

Histological sections were also immunostained with a NeuN antibody to evaluate the neuronal survival in the ventral horn of the spinal cord. Like myelin preservation, quantification of NeuN positive cells in the ventral horns showed very similar neuronal counts in the LPA_4 (Fig. 4A) and LPA_5 (Fig. 4B) deficient mice, respect to their wild-types (two-way RM-ANOVA; *post-hoc* Bonferroni's test; LPA_4: n=11 for WT and n=10 for null mice; LPA_5: n= 20 for WT and n=18 for null mice).

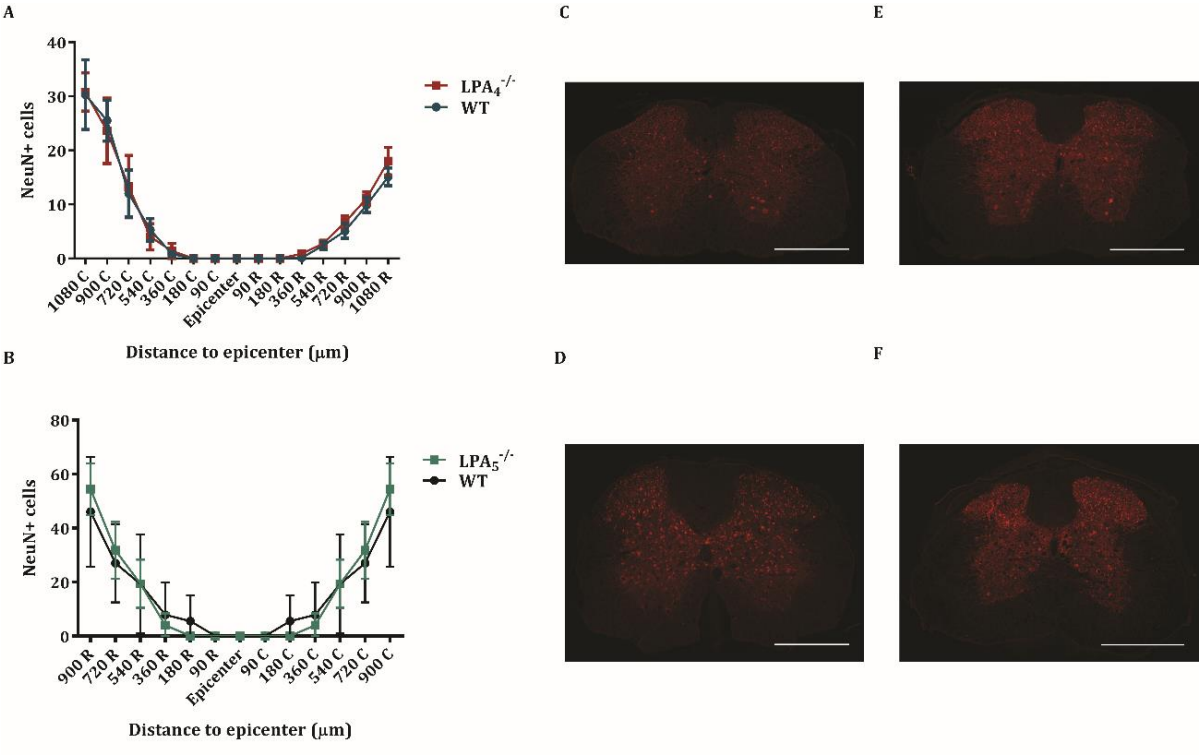


Figure 4. The lack of LPA_4 or LPA_5 does not enhance neural preservation after SCI. Graph showing the quantification of NeuN+ cells in the ventral horn of the injured spinal cords at different distances rostral and caudal to the injury epicentre in both LPA_4 and LPA_5 deficient mice (A, B). Representative micrographs show neuronal sparing at rostral levels in LPA_4 (C) and LPA_5 (D) deficient mice, and their respective WT littermates (E, F). Error bars indicate SEM. Scale bar=500μm.

DISCUSSION

We recently reported that LPA contributes to secondary tissue damage after SCI. Interestingly, selective blockade of LPA_1 after spinal cord contusion lesion reduced functional deficits and myelin loss, linking LPA_1 signalling to demyelination, which was, in part, mediated by microglial cells (Santos-Nogueira et al., 2015). Moreover, in Chapter I in the present thesis together with unpublished data from the laboratory, we provide clear evidence on the deleterious contribution of LPA_2 receptor, whose selective blockade improved functional recovery of the animals, and reduced myelin and neuronal loss after SCI.

In this chapter, we studied whether LPA_4 and LPA_5 exert harmful, beneficial or neutral effects after a spinal cord contusion. As mentioned before, LPA_{4-6} have been classified in the non-Edg family (Chun, 2011), since they show a dissimilar predicted amino acid sequence from the other LPA receptor genes (LPA_{1-3}). Originally, it was thought that the cellular effects of LPA were mediated by the activation of the cell-surface GPCRs that belong to the Edg family. This family share 50-57% amino acid identity in humans and for years it was considered the only LPA receptor family (Ishii et al., 2009). However, right after the identification of these LPA receptors, several studies reported the existence of new receptors that are activated after LPA stimulation. For instance, in a study using fibroblasts from LPA_1/LPA_2 double-deficient mouse embryos, some LPA-induced responses, such as adenylyl cyclase inhibition and stress fiber formation, were severely reduced. Moreover, the LPA_3 mRNA in this double-deficient fibroblast was not detected (Contos et al., 2002). Another study reported that LPA-induced human platelet aggregation showed ligand specificities, which were not carried out by the Edg family-receptor mediated responses, suggesting the presence of other LPA receptors (Gueguen et al., 1999; Hooks et al., 2001; Tokumura et al., 2002).

In 2003, during the "de-orphaning" project of G protein-coupled receptors, it was found that the $p2y_9$/GPR23 receptor responded specifically to LPA, inducing adenylyl cyclase stimulation and intracellular Ca^{2+} mobilization (Noguchi et al., 2003). LPA_4 only shares 20-24% amino acid identities with the Edg family, being phylogenetically distant from it (Noguchi et al., 2003). Among all the biological functions, LPA-LPA_4 axis activation induces Ca^{2+} mobilization via both G_q- and $G_{i/o}$-mediated pertussis toxin-sensitive pathway, and cAMP accumulation through $G_{\alpha s}$ activation (Lee et al., 2007). Moreover, LPA_4 stimulation induces $G_{12/13}$-mediated Rho activation, producing neurite retraction and stress fiber formation *in vitro*, a function shared with the other LPA receptors, except LPA_3 (Ishii

et al., 2000). Lee et al. also showed that the absence of LPA_4 increases cell motility and migration upon LPA stimulation, suggesting that this receptor acts as a barrier that prevents the migratory response to LPA, again through Rho activation *in vitro* (Lee et al., 2008). Although adult *Lpa4*$^{-/-}$ mice do not display evident abnormalities, there are some issues related to its absence, like a decreased prenatal survival or affections in the circulatory system development (Noguchi et al., 2009). LPA_4 is located in chromosome X, and is present in multiple murine tissues including heart, skin, thymus, platelets, bone marrow, and embryonic brain. Moreover, it is highly expressed in female organs, such as ovary, uterus and placenta. Within the central nervous system, LPA_4 mRNA expression has been found in different neural cell lines (Lee et al., 2008), undifferentiated human neural stem/progenitor cells (Dottori et al., 2008), rat embryonic hippocampal neurons (Fujiwara et al., 2003), immortalized hippocampal progenitor cells (Jin Rhee et al., 2006) and mouse primary astrocytes (Spohr et al., 2011), suggesting its role in neurodevelopmental processes. Nevertheless, little is known about the role of LPA_4 in human pathologies, especially in those affecting the central nervous system.

In the present chapter, we showed that LPA_4 mRNA expression is not up-regulated in the spinal cord parenchyma after a spinal cord contusion. We also observed that LPA_4 deficient mice undergo a slight worsening of the functional recovery after the lesion compared to their wild-type littermates. However, this was not accompanied with a greater myelin and/or neuronal loss. We do not discard that LPA_4 activation could modulate neuronal plasticity after injury, since its mRNA have been described in neuronal cells (Fujiwara et al., 2003). This might explain the slight, but consistent, functional deficits observed in the LPA_4 knockout mice after SCI, but further experiments need to be done to demonstrate this possibility.

In 2006, the orphan receptor LPA_5,or GPR92, was identified as a fifth LPA receptor (Lee et al., 2006). This receptor shares ~35% amino acid identity with the LPA_4/GPR23 receptor (Ishii et al., 2009). It also shares some functions with other LPA GPCRs, like the concentration-dependent neurite retraction mediated by $G_{12/13}$ and Rho; receptor internalization; increased LPA binding; increased cAMP accumulation; intracellular Ca^{2+} mobilization, which is mediated by G_q; and evoked electrophysiological currents (Ishii et al., 2009). LPA_5 is part of the microglial sensome, recently reported to be involved in microglial polarization (Plastira et al., 2016). LPA_5 is also expressed in many murine tissues, with high expression in spleen, heart, platelets and gastrointestinal lymphocytes (Noguchi et al., 2009). Moreover, LPA_5 is highly expressed in the peripheral nervous system, specifically in dorsal root ganglia, suggesting its contribution to neuropathic pain. Indeed, in previous

studies, LPA_1 receptor was shown to be essential for the initiation of neuropathic pain in the partial sciatic nerve ligation mouse model (PSNL) (Inoue et al., 2004). However, the distribution of LPA_5 is found in a subset of dorsal root ganglion neurons and within neurons of the spinal cord dorsal horn, which is distinct from LPA_1 (Lin et al., 2012). By using LPA_5 deficient mice, Chun's group reported that LPA_5 activation caused increased cAMP levels and up-regulation of phosho-CREB and hyperalgesia, which has been strongly associated with neuropathic pain. Interestingly, LPA_5 causes neuropathic pain in a different way to LPA_1. For instance, LPA_5 mice did not show reduced astrogliosis or $Ca_{\alpha 2\delta 1}$ levels after PNSL, and did not prevent demyelination after PSNL, which suggests that this receptor is not involved in the demyelination process induced by nerve injury (Lin et al., 2012). Here, we found that LPA_5 receptor showed an up-regulation of in the mRNA levels from seven to twenty-one days after SCI, peaking at day 7. The expression of these receptor correlates with the dynamics of microglia counts in the spinal cord after injury (David et al., 2012). Indeed, previous studies using RNA sequencing revealed that this receptor is selective expressed in microglia and not in macrophages (Butovsky et al., 2014). Indeed, a very recent study has showed that microglia cells stimulated with LPA adopt a cytotoxic/pro-inflammatory phenotype, and that this effect is mainly lost by using a LPA_5 antagonist (Plastira et al., 2016). These evidences indicate that LPA_5 could be potentially involved in inflammation and secondary damage after SCI. Surprisingly, our data using LPA_5 deficient reveals that the lack of this receptors does not have any impact on functional recovery and secondary tissue damage after SCI. Due to time constraints, we did not study whether microglial cell counts or phenotype is affected in the LPA_5 null following SCI, but these experiments will be done in the future. Moreover, we do not discard that these mice could be protected against neuropathic pain, as it has been previously described after nerve injury (Lin et al., 2012)(Lin et al., 2012). However, this needs to be elucidated after SCI.

A part from LPA_4 and LPA_5, there is another member of the non-Edg family LPA receptors known as LPA_6 or P2Y5 (Yanagida et al., 2009). There is currently limited information on the role of this receptor due to the lack of LPA_6 null mice. However, LPA leads to neurite retraction in B103-LPA6 cells in RH7777-LPA6 cells (Yanagida et al., 2009), which may suggest an important contribution of this receptor to the failure of axonal regeneration in the CNS. Indeed, our previous work reveals that LPA_6 is the LPA receptor most expressed in the spinal cord parenchyma after LPA_1, which makes LPA_6 an attractive target to study its role after CNS trauma.

Overall, the results of the present chapter suggest that, unlike LPA_1 and LPA_2, the non-Edg family LPA receptors, LPA_4 and LPA_5, does not have a major contribution to SCI physiopathology. Therefore, this data, together with our previous results from the laboratory, suggest that there is only a need to selectively block LPA_1 and LPA_2 to minimize functional deficits after SCI.

REFERENCES

Basso DM, Fisher LC, Anderson AJ, Jakeman LBYNB, Tigue DMMC, Popovich PG, McTigue DM, Popovich PG (2006) Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. J Neurotrauma 23:635–659.

Butovsky O, Jedrychowski MP, Moore CS, Cialic R, Lanser AJ, Gabriely G, Koeglsperger T, Dake B, Wu PM, Doykan CE, Fanek Z, Liu L, Chen Z, Rothstein JD, Ransohoff RM, Gygi SP, Antel JP, Weiner HL (2014) Identification of a unique TGF-β-dependent molecular and functional signature in microglia. Nat Neurosci 17:131–143.

Choi JW, Chun J (2013) Lysophospholipids and their receptors in the central nervous system. Biochim Biophys Acta 1831:20–32

Choi JW, Herr DR, Noguchi K, Yung YC, Lee C-W, Mutoh T, Lin M-E, Teo ST, Park KE, Mosley AN, Chun J (2010) LPA receptors: subtypes and biological actions. Annu Rev Pharmacol Toxicol 50:157–186.

Chun J (2011) Lysophospholipid receptors.

Contos JJA, Ishii I, Fukushima N, Kingsbury MA, Ye X, Kawamura S, Brown JH, Chun J, Heller Brown J, Chun J (2002) Lysophosphatidic Acid Receptor Knockout Mice : Signaling Deficits without Obvious Phenotypic Abnormality Attributable to lpa 2. Mol Cell Biol 22:6921–6929.

David S, Greenhalgh AD, López-Vales R (2012) Role of phospholipase A 2s and lipid mediators in secondary damage after spinal cord injury. Cell Tissue Res 349:249–267.

Donnelly DJ, Popovich PG (2008) Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Exp Neurol 209:378–388.

Dottori M, Leung J, Turnley A, Pebay A (2008) Lysophosphatidic acid inhibits neuronal differentiation of neural stem/progenitor cells derived from human embryonic stem cells. Stem Cells:1146–1154.

Fujiwara Y, Sebök A, Meakin S, Kobayashi T, Murakami-Murofushi K, Tigyi G (2003) Cyclic phosphatidic acid elicits neurotrophin-like actions in embryonic hippocampal neurons. J Neurochem 87:1272–1283.

Gueguen G, Gaigé B, Grévy J-M, Rogalle P, Bellan J, Wilson M, Klaébé A, Pont F, Simon M-F, Chap H (1999) Structure−Activity Analysis of the Effects of Lysophosphatidic Acid on Platelet Aggregation. Biochemistry 38:8440–8450.

Hooks SB, Santos WL, Im DS, Heise CE, Macdonald TL, Lynch KR (2001) Lysophosphatidic Acid-induced Mitogenesis Is Regulated by Lipid Phosphate Phosphatases and Is Edg-receptor Independent. J Biol Chem 276:4611–4621.

Inoue M, Rashid H, Fujita R, Contos JJA, Chun J, Ueda H (2004) Initiation of neuropathic pain requires lysophosphatidic acid receptor signaling. 10.

Ishii I, Contos JJ, Fukushima N, Chun J (2000) Functional comparisons of the lysophosphatidic acid receptors, LP(A1)/VZG-1/EDG-2, LP(A2)/EDG-4, and LP(A3)/EDG-7 in neuronal cell lines using a retrovirus expression system. MolPharmacol 58:895–902.

Ishii S, Noguchi K, Yanagida K (2009) Non-Edg family lysophosphatidic acid (LPA) receptors. Prostaglandins Other Lipid Mediat 89:57–65.

Jin Rhee H, Nam J-S, Sun Y, Kim M-J, Choi H-K, Han D-H, Kim N-H, Huh S-O (2006) Lysophosphatidic acid stimulates cAMP accumulation and cAMP response element-binding protein phosphorylation in immortalized hippocampal progenitor cells. Neuroreport 17.

Lee CW, Rivera R, Dubin AE, Chun J (2007) LPA4/GPR23 is a lysophosphatidic acid (LPA) receptor utilizing Gs-, Gq/Gi-mediated calcium signaling and G12/13-mediated Rho activation. J Biol Chem 282:4310–4317.

Lee CW, Rivera R, Gardell S, Dubin AE, Chun J (2006) GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5. J Biol Chem 281:23589–23597.

Lee Z, Cheng C-T, Zhang H, Subler MA, Wu J, Mukherjee A, Windle JJ, Chen C-K, Fang X (2008) Role of LPA4/P2Y9/GFP23 in negative regulation of cell motility. Mol Biol Cell 19:5435–5445.

Lin M-E, Rivera RR, Chun J (2012) Targeted Deletion of LPA5 Identifies Novel Roles for Lysophosphatidic Acid Signaling in Development of Neuropathic Pain. J Biol Chem 287:17608–17617.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and. Methods 25:402–408.

Noguchi K, Herr D, Mutoh T, Chun J (2009) Lysophosphatidic acid (LPA) and its receptors. Curr Opin Pharmacol 9:15–23.

Noguchi K, Ishii S, Shimizu T (2003) Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. J Biol Chem 278:25600–25606.

Plastira I, Bernhart E, Goeritzer M, Reicher H, Kumble VB, Kogelnik N, Wintersperger A, Hammer A, Schlager S, Jandl K, Heinemann A, Kratky D, Malle E, Sattler W (2016) 1-Oleyl-lysophosphatidic acid (LPA) promotes polarization of BV-2 and primary murine microglia towards an M1-like phenotype. J Neuroinflammation 13:205.

Santos-Nogueira E, López-Serrano C, Hernández J, Lago N, Astudillo AM, Balsinde J, Estivill-Torrús G, de Fonseca FR, Chun J, López-Vales R (2015) Activation of Lysophosphatidic Acid Receptor Type 1 Contributes to Pathophysiology of Spinal Cord Injury. J Neurosci 35:10224–10235.

Spohr TCLDSE, Dezonne RS, Rehen SK, Gomes FCA (2011) Astrocytes treated by lysophosphatidic acid induce axonal outgrowth of cortical progenitors through extracellular matrix protein and epidermal growth factor signaling pathway. J Neurochem 119:113–123.

Tokumura A, Sinomiya J, Kishimoto S, Tanaka T, Kogure K, Sugiura T, Satouchi K, Waku K, Fukuzawa K (2002) Human platelets respond differentially to lysophosphatidic acids having a highly unsaturated fatty acyl group and alkyl ether-linked lysophosphatidic acids. Biochem J 365:617–628.

Yanagida K, Masago K, Nakanishi H, Kihara Y, Hamano F, Tajima Y, Taguchi R, Shimizu T, Ishii S (2009) Identification and characterization of a novel lysophosphatidic acid receptor, p2y5/LPA6. J Biol Chem 284:17731–17741.

Chapter III. Autotaxin inhibition does not enhance functional recovery after spinal cord injury

Clara López-Serrano[1], Eva Santos-Nogueira[1], Jerold Chun[2], Rubèn López-Vales[1]

[1]Departament de Biologia Cellular, Fisiologia i Immunologia, Institut de Neurociències, Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Universitat Autònoma de Barcelona, Bellaterra, Spain; [2]Molecular and Cellular Neuroscience Department, Dorris Neuroscience Center, The Scripps Research Institute, La Jolla, USA.

ABSTRACT

Lysophosphatidic acid (LPA) is a simple and bioactive phospholipid with an important role as an intracellular messenger, acting through six G protein-coupled receptors (GPCRs), LPA_{1-6}, to regulate several physiological functions. One of the enzymes related to the different LPA synthesis pathways is autotaxin (ATX), a secreted enzyme that generates LPA from different lysophospholipids (LPLs) through its lysophospholipase D (lysoPLD) activity. We have recently shown that LPA levels increase after a spinal cord injury (SCI), which causes functional impairment and demyelination through the activation of LPA_1 and LPA_2 receptors. In the present work, we studied whether the deleterious effects of LPA in the contused spinal cord could be prevented by targeting ATX. Our results showed that administration of two different selective and potent ATX inhibitors does not have any impact on functional and histological outcomes after spinal cord trauma, which suggests that ATX does not have importance in LPA synthesis in the spinal cord parenchyma after injury.

Key words: Lysophosphatidic acid, spinal cord injury, autotaxin, PAT-048, Compound X.

INTRODUCTION

Traumatic spinal cord injury (SCI) causes permanent functional deficits due to the loss of axons and neurons, as well as to death of oligodendrocytes and the limited ability of the central nervous system (CNS) to regenerate axons (Rossignol et al., 2007; Rowland et al., 2008). Two ways of tissue degeneration after SCI have been described: a primary phase, which is immediate after the trauma, and a secondary injury, where multiple mechanisms are involved, including an inflammatory response (Carlson et al., 1998). During this inflammatory response, endogenous glial cells (microglia and astrocytes) and peripheral immune cells release several factors, such as cytokines, free radicals, proteases, eicosanoids, etc., that cause damage to neurons, glia, axons and myelin (Donnelly and Popovich, 2008; David et al., 2012). Among these factors, we have recently identified a lipid mediator that contributes to the secondary damage after SCI: lysophosphatidic acid (LPA) (Santos-Nogueira et al., 2015).

LPA is a bioactive lipid that exhibits a wide range of biological functions. We have recently shown that LPA has important contribution to myelin loss and functional impairments after SCI (Santos-Nogueira et al., 2015). The deleterious effects of LPA in the SCI can be prevented by using drugs that target LPA receptors. However, current drugs developed to inhibit LPA receptors activity have very low stability and show poor efficacy in blocking the receptor, as shown with LPA_2 (Chun, 2011), one the two LPA receptors that greater contribute to demyelination, as shown in chapter I.

Another different approach to minimize secondary injury events after SCI is to prevent the production of LPA by targeting the enzymes involved in the synthesis of this lysophospholipid (LPL). LPA is generated from membrane phospholipids through several different enzymatic pathways, being two the major synthetic routes. The first one involves hydrolysis of phosphatidic acids (PAs) by the action of phospholipases A_1 and A_2 (PLA_1 and PLA_2), and is thought to happen mainly in the inside or on the membrane of the cell, since the substrate PAs are located on cell membranes (Aoki et al., 2008). The second most important pathway involves the cleavage of LPLs, such as lyosphosphatidilcoline (LPC) and lysophosphatidilserine (LPS), by the enzyme autotaxin (ATX), which has lysophospholipase D (lysoPLD) properties (Lin et al., 2010). There are at least two additional pathways that can produce LPA: the acylation of glycerol 3-phosphate by glycerolphosphate acyltransferase (GPAT) and the phosphorylation of monoacylglycerol by monoacylgylcerol kinase (MAG-kinase). However, the LPA resulting from these synthetic pathways appears to serve as precursor for glycerolipid synthesis, rather than a source of extracellular

signalling molecules (Pagès et al., 2001). A previous study shows that PLA_2 enzymes have divergent actions in SCI, and thus, inhibitors targeting PLA_2 activity should be avoided (López-Vales et al., 2011). However, inhibition of ATX could be an alternative approach to prevent the synthesis of LPA in the injured spinal cord, and consequently, to minimize neurological deficits. In the present chapter, we therefore sought to study the therapeutic effects of two ATX inhibitors 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) C57BL/6J (Charles Rivers) 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 by applying a force of 60±5 kdynes kilodynes and 400-600μm in displacement with a Horizons Impactor device (Precision Scientific Instrumentation, Fairfax Station, VA).

RNA isolation, reverse transcription and real-time PCR

To evaluate the mRNA expression levels of ATX in the spinal cord, quantitative real-time polymerase chain reaction (qPCR) was performed. Mice with intact or injured lesioned spinal cords were perfused with sterile Saline solution and 5 mm length of spinal cord was harvested. 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. 1μg of obtained RNA was primed with random hexamers (Promega) and reverse transcribed using Omniscript RT kit (Qiagen). RNase inhibitor (Roche) was added (1U/μL final concentration) to avoid RNA degradation. qPCR analysis was performed using a MyiQ Single-Color Real-Time PCR Detection System (BIO-RAD). qPCR reactions were performed using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The amount of cDNA was calculated based on the Cycle Threshold cycle (CT) value, and was standardized by the amount of housekeeping gene using the $2^{-\Delta\Delta C_T}$ method. The expression level of the target mRNA was normalized to the relative ratio of the expression of the GAPDH housekeeping gene (Livak and Schmittgen, 2001). Primer sequences used in RT-PCR reaction are the following:

- **Autotaxin:**
    - Forward primer: 5'- TGGATGGATTCCGTGCATCGTACA-3'.
    - Reverse primer: 5'- ACCAGTGGCCAGCGTATACAGATT-3'.
- **GAPDH:**
    - Forward primer: 5'- TCAACAGCAACTCCCACTCTTCCA-3'.
    - Reverse primer: 5'- ACCCTGTTGCTGTAGCCGTATTCA-3'.

Autotaxin inhibitor administration

To study the effect of autotaxin inhibition in the functional recovery and the secondary phase after SCI, we used two different ATX-selective inhibitors. **Compound X** and **PAT-048** (patent publication number WO 2012024620 A2), kindly provided by PharmAkea (San Diego, CA), were suspended in 0.5% methylcellulose (Sigma-Aldrich, St. Louis, MO, USA) and administered by oral gavage at a dose of 20 mg/kg for Compound X and 10 mg/kg dose for PAT-048. These inhibitors were given at one-hour post-injury and then daily for the first 14 days post-injury (dpi).

Behavioural scoring

The spontaneous locomotor recovery of the injured animals was assessed prior to surgery and at 1, 3, 5, 7, 10, 14, 21 and 28 dpi in an open-field test using the nine-point Basso Mouse Scale (BMS), which was specifically developed for locomotor testing after contusion injuries in mice. In the BMS, zero points represent total absence of movement of the hindlimbs, whereas nine points represent the total recovery of the movement of the hindlimbs. An eleven-point sub-score scale was used for a more detailed punctuation, considering tail direction, trunk stability, etc. (Basso et al., 2006).

Histological assessment

At 28 dpi, mice were deeply anaesthetized using Dolethal (pentobarbital sodium; Vétoquinol E.V.S.A.) and perfused with 4% paraformaldehyde in 0.1M phosphate buffer (PB). The epicentre of the lesion and 8 mm length ventral and dorsal to it was post-fixed for 1 hour in 4% paraformaldehyde in 0.1M PB and cryoprotected with 30% sucrose with azide (1:100), in 0.1M PB at 4°C. Spinal cords were fast-frozen at -60°C in cryoembedding compound (Tissue-Tek OCT, Sakura) and cut on a cryostat (Leica) in 15µm-thick transversal sections.

For assessment of myelin sparing, tissue sections were dyed with Luxol Fast Blue (LFB). Briefly, tissue sections were hydrated in 0.1M PBS and gradually dehydrated in 50%, 70%, 80% and 95% ethanol. Sections were placed in a 1mg/mL LFB solution in 95% ethanol and 0.05% acetic acid overnight at 37°C. Sections were then washed in 95% ethanol and distilled water before placing them into a solution of 0.5mg/mL of lithium carbonate in distilled water for 1 min at room temperature. After washing in distilled water, sections were dehydrated in ethanol and mounted in DPX mounting media (Sigma).

For neuronal sparing, tissue sections were immunostained against NeuN, which is a neuronal nuclear antigen commonly used as a biomarker for neurons. 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 4°C with mouse anti-NeuN biotin conjugated (1:200, 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, gradually dehydrated in ethanol and mounted in DPX mounting media (Sigma).

Statistical analysis

All analyses were conducted through GraphPad Prism 6. Autotaxin expression in spinal cord tissue by RT-PCR was analysed using one-way ANOVA with Bonferroni's *post-hoc* test. BMS score and histological parameters were analysed using two-way repeated measure (RM)-ANOVA with Bonferroni's *post-hoc* test. Results are expressed as mean ± SEM. Differences were considered significant at $p<0.05$.

RESULTS

Autotaxin mRNA expression levels decrease after SCI

To assess the changes in mRNA levels of ATX in the spinal cord parenchyma of the injured mice, we used qPCR assay. We observed that ATX levels were markedly decreased in the spinal cord parenchyma already at day 1 post-injury (one-way ANOVA; *post-hoc* Bonferroni's test: $p<0.01$; n=3 per group) and were maintained significantly decreased until day 3 (one-way ANOVA; *post-hoc* Bonferroni's test; $p<0.05$; n=3 per group) (Fig. 1). From this time point until day 28 post-contusion, transcripts for ATX tended to remain at lower levels, although it did not reach statistically significance.

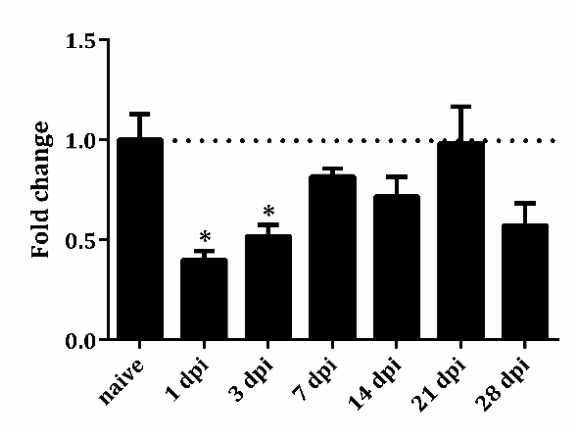


Figure 1. Autotaxin mRNA expression levels are significantly decreased in the spinal cord from 1 to 3 days post-injury. Real Time-PCR quantification of autotaxin mRNA levels from spinal cords harvested at different time points after contusion injury. Error bars indicate SEM (*$p<0.05$).

Effects of autotaxin to neurological outcomes after SCI

To assess the role of ATX in the functional recovery after SCI, we induced spinal cord contusion injury to C57BL/6J mice and performed orogastric administration of the two different ATX inhibitors, PAT-048 and Compound X. A third group of mice received methylcellulose (vehicle) and were used as an experimental control. We observed that the administration of ATX inhibitors PAT-048 (Fig. 2A) or Compound X (Fig. 2B) did not have any effects in the functional recovery after SCI (Two-way RM ANOVA; *post-hoc* Bonferroni's test; $p>0.05$; n=10 for PAT-048; n=11 for Compound X; n=9 for vehicle).

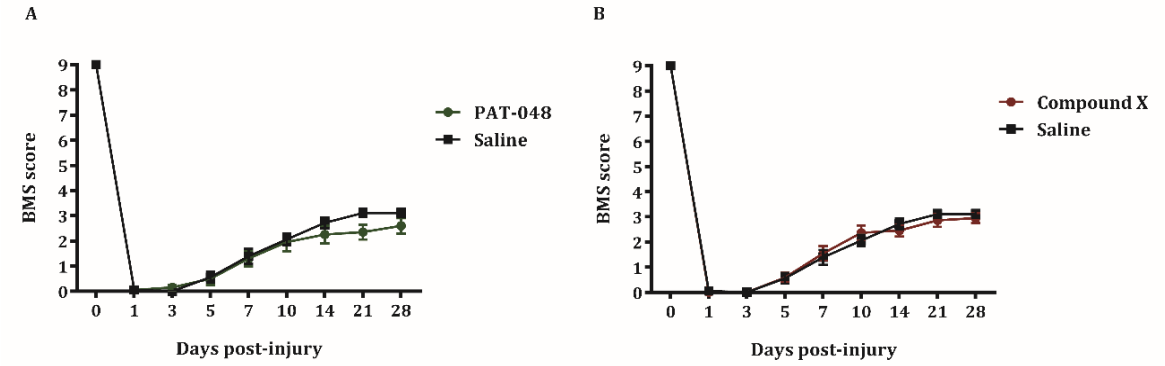


Figure 2. The inhibition of autotaxin activity does not promote functional recovery after spinal cord injury. Locomotor recovery after spinal cord contusion injury in mice treated with autotaxin inhibitors PAT-048 (A) and Compound X (B) was assessed using the 9-point Basso Mouse Scale. There were no differences in the different groups compared to the vehicle. Error bars indicate SEM.

Autotaxin inhibitors do not enhance myelin preservation after SCI

To study whether ATX contributes to myelin loss after SCI, we evaluated the effects of the two ATX inhibitors on myelin preservation by using a Luxol Fast Blue staining. We observed that the administration of either PAT-048 (Fig. 3A) or Compound X (Fig. 3B) did not enhance myelin preservation at the injury site or adjacent sites after SCI, as compared to the vehicle-treated mice (two-way RM-ANOVA; *post-hoc* Bonferroni's test; n=6 for Compound X; n=7 for PAT-048; n=7 for vehicle).

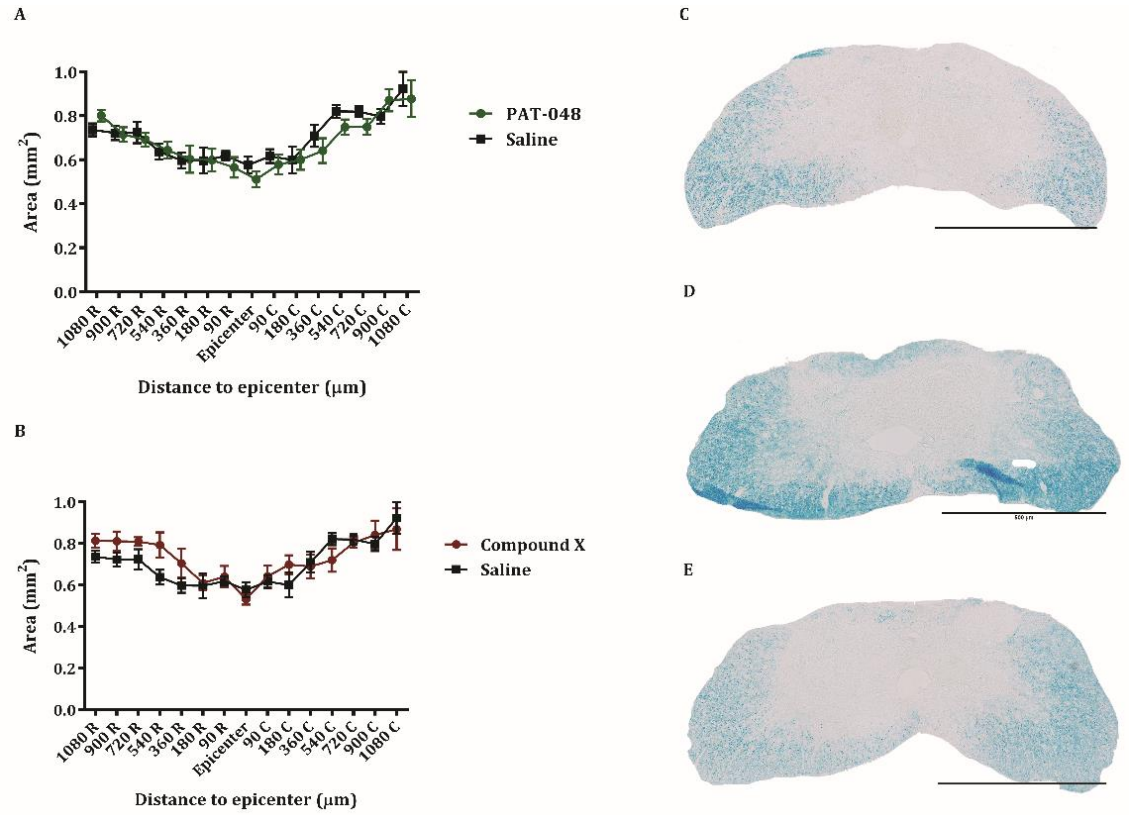


Figure 3. Autotaxin inhibition does not contribute to myelin preservation after spinal cord injury. Quantification of the Luxol Fast Blue staining at different distances rostral and caudal to the injury epicentre in both treatments, PAT-048 (A) and Compound X (B), compared to the vehicle. Representative micrographs show myelin sparing at the injury epicentre of spinal cords treated with PAT-048 (C), Compound X (D), or vehicle (E). Error bars indicate SEM. Scale bar=500μm.

Autotaxin inhibition does not enhance neural survival after SCI

We then evaluated whether the ATX inhibitors used in the present study enhanced neural survival in the ventral horn of the spinal cord by doing immunostaining against NeuN. Similar to myelin preservation, quantification of the NeuN-positive cells in the ventral horns showed similar neuronal counts in mice treated with PAT-048 (Fig. 4A), Compound X (Fig. 4B), and vehicle (Fig. 4C) (two-way RM-ANOVA; *post-hoc* Bonferroni's test; n=4 for Compound X; n=4 for PAT-048; n=6 for vehicle), suggesting that these inhibitors did not prevent neuronal death.

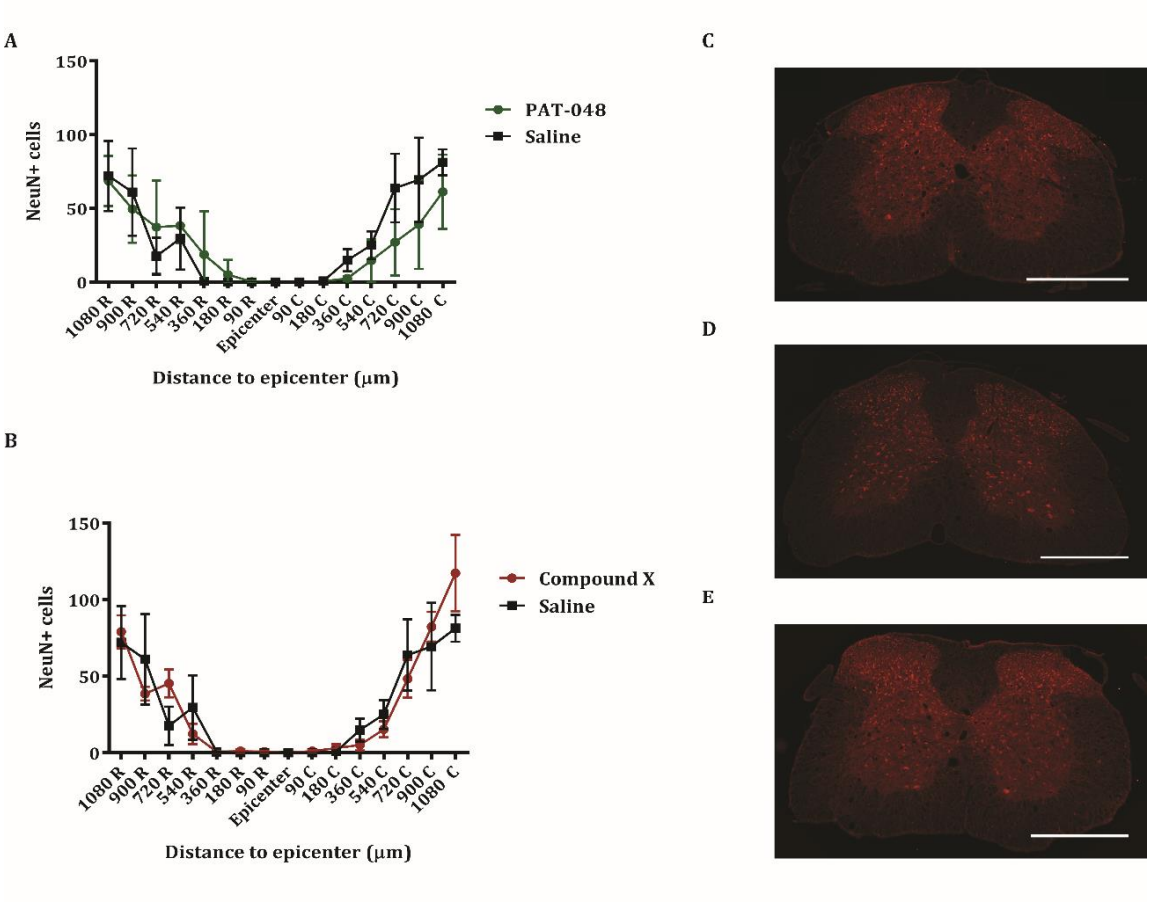


Figure 4. Autotaxin inhibition does not contribute to neural preservation after spinal cord injury. Quantification of cells positive for NeuN antibody in the ventral horn of the injured spinal cords at different distances rostral and caudal to the injury epicentre in spinal cords treated with PAT-048 (A) and Compound X (B). Representative micrographs show neuronal sparing at rostral levels of spinal cords treated with PAT-048 (C), Compound X (D), or vehicle (E). Error bars indicate SEM. Scale bar=500μm.

DISCUSSION

Recent studies have shown that LPA and LPA-producing enzymes are involved in many physiological and pathological functions. Specifically, the ATX-LPA signalling pathway has been implicated in some pathological conditions, such as cancer, fibrosis, inflammation, and pain (Okudaira et al., 2010). For instance, high activity of the ATX enzyme has been observed in the cerebrospinal fluid of multiple sclerosis patients, implying its relation with this pathology (Zahednasab et al., 2014). Moreover, it has been shown that ATX heterozygous mice had about 50% recovery of nerve injury-induced neuropathic pain, compared to wild-type mice (Inoue et al., 2008). However, in other pathologies, such as lung injury, ATX inhibition with the potent inhibitor PAT-048 did not decrease pulmonary LPA production nor fibrosis, even though ATX activity has been proven to be increased after the injury (Black et al., 2016).

ATX, also known as ENPP2, is a 125-kDa secreted enzyme that belongs to the seven-membered family of ectonucleotide pyrophosphatases/phosphodiesterases (ENPPs), which have the ability to catalyse the hydrolysis of pyrophosphate or phosphodiester bonds in nucleotides (Stefan et al., 2005). Among the ENPPs family, ATX is unique, since it has lysoPLD activity, transforming LPC into the phospholipid LPA. ATX expression seems necessary to embryonic development of vascular and neuronal components. In adult life, is widely expressed, with the highest mRNA levels in brain, placenta, ovary, and intestine (van Meeteren et al., 2006). Within the adult mammalian nervous system, ATX is present in the cerebrospinal fluid (Sato et al., 2005) and in the adult brain, including the white matter, choroid plexus and leptomeningeal cells (Savaskan et al., 2007).

The crystalized structure of ATX has two somatomedin B (SMB)-like binding domains at the amino terminus, followed by a central catalytic domain which binds two zinc ions and contains an active site threonine and an N-glycan, all of which are critical for the lysoPLD activity. On the other end, the C terminus contains the nuclease-like domain, which is catalytically inert, but, together with the SMB-like domains, sandwich the catalytic domain of ATX, stabilizing it. This catalytic site consists of a hydrophobic lipid-binding pocket and a second hydrophobic channel through which the LPC substrates enter and the LPA product is delivered (Johnson et al., 2015).

In the present work, we aimed to elucidate the therapeutic effects of two ATX inhibitors after SCI. We previously showed that LPA levels are increased in contused spinal cords, with a peak of expression at day three post-injury, and remaining significantly high up to day 14 (Santos-Nogueira et al., 2015). Surprisingly, we observed that ATX mRNA levels

after a spinal cord contusion were significantly down-regulated in the spinal cord parenchyma for the first three days, which it did not align to the notably increased LPA production.

The lower expression of ATX at the mRNA level we found in the injured spinal cord does not necessarily imply that this enzyme is not involved in the LPA synthesis observed after contusion injury. Indeed, ATX is highly expressed in platelets, which cannot induce transcript due to the lack of cellular nuclei (Machlus et al., 2014). Indeed, platelet aggregation take place in the spinal cord parenchyma after contusion injury due to the high haemorrhagic events that occurs as a consequence of the trauma (Ersoz et al., 1999) and thus, the activity of ATX can be high despite the drop in mRNA levels. For this reason, we decided to block the activity of ATX to assess whether this enzyme has a key contribution to LPA synthesis, and consequently, to secondary tissue damage and functional deficits. We used two ATX inhibitors after SCI: PAT-048 and the Compound X. PAT-048 is a very potent and selective ATX inhibitor that was patented in 2012 (Hutchinson et al., 2012) and has been previously used to study the role of ATX in lung injury (Black et al., 2016), and dermal fibrosis (Castelino et al., 2016). The Compound X, whose identity has not been published yet due to intellectual property issues, is also a very potent and selective ATX inhibitor. After administration of the two compounds over the first fifteen days after the lesion, we did not observe any difference in functional recovery, as compared to vehicle-treated mice. Furthermore, histological analysis revealed that these two ATX inhibitors did not result in enhancement of myelin or neuronal preservation, suggesting that these inhibitors do not have therapeutic effects in SCI.

Our data does not necessarily imply that ATX does not contribute to LPA synthesis, since the lack of effect of the inhibitors used can be explained, in part, to the low penetrance of these drugs into the central nervous system (CNS). It has to be highlighted, however, that there is a prominent disruption of the blood-brain barrier (BBB) and blood vessel breakdown in the spinal cord following injury, which leads to increase in transendothelial vesicular transport of proteins and extravasation of plasma (Schlosshauer, 1993; Mautes et al., 2000). It is therefore expected that exogenous molecules, such as drugs and other substances, could also diffuse to the site of lesion, even though they are not-permeable under normal physiological conditions. Due to time constrains, we were unable to analyse whether the inhibitors used here were able to reach the contused CNS, and if so, whether they reduce LPA levels. These experiments have key importance to answer our main question, and thus, will be done in the future.

As mentioned above, there are other LPA synthesis pathways that might be involved in the LPA production after SCI. For instance, PLA_2 superfamily is directly involved in the LPA synthesis, hydrolysing phosphatidic acid (PA) and obtaining LPA (Choi et al., 2010). Indeed, this is the main route of LPA synthesis in most tissues. Previous studies from López-Vales have shown that members of PLA_2 superfamily, such as calcium-dependent PLA_2 GIVA (c PLA_2 GIVA), calcium-independent PLA_2 GVIA (iPLA_2 GVIA), and secreted PLA_2 GIIA (sPLA_2 GIIA) are up-regulated after SCI. Moreover, by using selective inhibitors and null mice, they observed that these PLA_2 have distinct, and even opposite effects after a spinal cord contusion (López-Vales et al., 2011. Indeed, a pan PLA_2 inhibitor resulted in detrimental effects after SCI, and thus, therapies used to treat SCI should avoid to target this family member (López-Vales et al., 2011). This is due because PLA_2 are involved in the synthesis of a great variety of lipid mediators, many of them being key mediators that induce the resolution phase of inflammation and with great therapeutic potential (David et al., 2012).

Taking together, these results highlight the importance of targeting the different LPA receptors involved in SCI to avoid the deleterious effects of LPA, rather than the enzymes involved in its synthesis. We previously showed that mice treated with a pharmacological LPA_1-selective antagonist conferred protection against functional loss and demyelination (Santos-Nogueira et al., 2015). Moreover, in Chapter I of the present thesis, we show that similar to LPA_1, LPA_2 might have even a greater effect in promoting myelin loss. Therefore, selective inhibition of these two LPA receptors is likely the best therapeutic approach to ameliorate the detrimental actions of LPA production in SCI.

REFERENCES

Aoki J, Inoue A, Okudaira S (2008) Two pathways for lysophosphatidic acid production. Biochim Biophys Acta 1781:513–518.

Basso DM, Fisher LC, Anderson AJ, Jakeman LBYNB, Tigue DMMC, Popovich PG, McTigue DM, Popovich PG (2006) Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. J Neurotrauma 23:635–659.

Black KE, Berdyshev E, Bain G, Castelino F V., Shea BS, Probst CK, Fontaine BA, Bronova I, Goulet L, Lagares D, Ahluwalia N, Knipe RS, Natarajan V, Tager AM (2016) Autotaxin activity increases locally following lung injury, but is not required for pulmonary lysophosphatidic acid production or fibrosis. FASEB J 30:2435–2450.

Carlson SL, Parrish ME, Springer JE, Doty K, Dossett L (1998) Acute Inflammatory Response in Spinal Cord Following Impact Injury. Exp Neurol 151:77–88.

Castelino F V., Bain G, Pace VA, Black KE, George L, Probst CK, Goulet L, Lafyatis R, Tager AM (2016) An Autotaxin/Lysophosphatidic Acid/Interleukin-6 Amplification Loop Drives Scleroderma Fibrosis. Arthritis Rheumatol 68:2964–2974.

Choi JW, Herr DR, Noguchi K, Yung YC, Lee C-W, Mutoh T, Lin M-E, Teo ST, Park KE, Mosley AN, Chun J (2010) LPA receptors: subtypes and biological actions. Annu Rev Pharmacol Toxicol 50:157–186.

Chun J (2011) Lysophospholipid receptors.

David S, Greenhalgh AD, López-Vales R (2012) Role of phospholipase A 2s and lipid mediators in secondary damage after spinal cord injury. Cell Tissue Res 349:249–267.

Donnelly DJ, Popovich PG (2008) Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Exp Neurol 209:378–388.

Ersoz G, Ficicilar H, Pasin M, Yorgancioglu R, Yavuzer S (1999) Platelet aggregation in traumatic spinal cord injury. Spinal Cord 37:644–647.

Hutchinson JH, Parr TA, Roppe JR, Stock NS, Volkots D (2012) PAT-048 patent: WO 2012024620 A2.

Inoue M, Ma L, Aoki J, Chun J, Ueda H (2008) Autotaxin, a synthetic enzyme of lysophosphatidic acid (LPA), mediates the induction of nerve-injured neuropathic pain. Mol Pain 4:6.

Johnson DH, Shrier BM, Neal JSO, Knutzen JA (2015) Structural Basis for Inhibition of Human Autotaxin by Four Potent Compounds with Distinct Modes of Binding. Mol Pharmacol 88:982–992.

Lin ME, Herr DR, Chun J (2010) Lysophosphatidic acid (LPA) receptors: Signaling properties and disease relevance. Prostaglandins Other Lipid Mediat 91:130–138.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and. Methods 25:402–408.

López-Vales R, Ghasemlou N, Redensek A, Kerr BJ, Barbayianni E, Antonopoulou G, Baskakis C, Rathore KI, Constantinou-Kokotou V, Stephens D, Shimizu T, Dennis E a, Kokotos G, David S (2011) Phospholipase A2 superfamily members play divergent roles after spinal cord injury. FASEB J 25:4240–4252.

Machlus KR, Thon JN, Italiano JE (2014) Interpreting the developmental dance of the megakaryocyte: a review of the cellular and molecular processes mediating platelet formation. Br J Haematol 165:227–236.

Mautes AEM, Weinzierl MR, Donovan F, Noble LJ (2000) Vascular Events After Spinal Cord Injury: Contribution to Secondary Pathogenesis. Phys Ther 80:673 LP-687.

Noguchi K, Herr D, Mutoh T, Chun J (2009) Lysophosphatidic acid (LPA) and its receptors. Curr Opin Pharmacol 9:15–23.

Okudaira S, Yukiura H, Aoki J (2010) Biological roles of lysophosphatidic acid signaling through its production by autotaxin. Biochimie 92:698–706.

Pagès C, Simon M-F, Valet P, Saulnier-Blache JS (2001) Lysophosphatidic acid synthesis and release. Prostaglandins Other Lipid Mediat 64:1–10.

Rossignol S, Schwab M, Schwartz M, Fehlings MG (2007) Spinal Cord Injury: Time to Move? J Neurosci 27:11782 LP-11792.

Rowland JW, Hawryluk GWJ, Kwon B, Fehlings MG (2008) Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon. Neurosurg Focus 25:E2.

Santos-Nogueira E, López-Serrano C, Hernández J, Lago N, Astudillo AM, Balsinde J, Estivill-Torrús G, de Fonseca FR, Chun J, López-Vales R (2015) Activation of Lysophosphatidic Acid Receptor Type 1 Contributes to Pathophysiology of Spinal Cord Injury. J Neurosci 35:10224–10235.

Sato K, Malchinkhuu E, Muraki T, Ishikawa K, Hayashi K, Tosaka M, Mochiduki A, Inoue K, Tomura H, Mogi C, Nochi H, Tamoto K, Okajima F (2005) Identification of autotaxin as a neurite retraction-inducing factor of PC12 cells in cerebrospinal fluid and its possible sources. J Neurochem 92:904–914.

Savaskan NE, Rocha L, Kotter MR, Baer A, Lubec G, Van Meeteren LA, Kishi Y, Aoki J, Moolenaar WH, Nitsch R, Bräuer AU (2007) Autotaxin (NPP-2) in the brain: Cell type-specific expression and regulation during development and after neurotrauma. Cell Mol Life Sci 64:230–243.

Schlosshauer B (1993) The blood-brain barrier: Morphology, molecules, and neurothelin. BioEssays 15:341–346.

Stefan C, Jansen S, Bollen M (2005) NPP-type ectophosphodiesterases: unity in diversity. Trends Biochem Sci 30:542–550.

van Meeteren LA, Ruurs P, Stortelers C, Bouwman P, van Rooijen MA, Pradère JP, Pettit TR, Wakelam MJO, Saulnier-Blache JS, Mummery CL, Moolenaar WH, Jonkers J (2006) Autotaxin, a secreted lysophospholipase D, is essential for blood vessel formation during development. Mol Cell Biol 26:5015–5022.

Zahednasab H, Balood M, Harirchian MH, Mesbah-Namin SA, Rahimian N, Siroos B (2014) Increased autotaxin activity in multiple sclerosis. J Neuroimmunol 273:120–123.

Chapter IV. Effects of combinatory targeting of LPA_1 and LPA_2 after spinal cord injury in mice

Clara López-Serrano[1], Jerold Chun[2], Rubèn López-Vales[1]

[1]Departament de Biologia Cellular, Fisiologia i Immunologia, Institut de Neurociències, Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Universitat Autònoma de Barcelona, Bellaterra, Spain; [2]Molecular and Cellular Neuroscience Department, Dorris Neuroscience Center, The Scripps Research Institute, La Jolla, USA.

ABSTRACT

Secondary tissue damage that occurs after spinal cord injury (SCI) contributes significantly to permanent functional disabilities. Although regeneration of damaged axons and replacement of lost neurons are important goals to repair the injured spinal cord, the secondary damage to axons, neurons, myelin and glial cells that follows the initial trauma is likely to be more easily amenable to treatment. Therefore, preventing or minimizing such secondary damage after SCI is expected to substantially reduce functional deficits. We have recently identified lysophosphatidic acid (LPA) as a new trigger of secondary damage after SCI. Our previous studies, together with the works shown in the present thesis, characterized the functional role of five out of the six LPA receptors currently described. Among them, we demonstrated that only LPA_1 and LPA_2 are key contributors to secondary damage after SCI, and that targeting their activity conferred protection against functional deficits and myelin loss. Here, we addressed whether targeting both LPA_1 and LPA_2 after SCI had additive therapeutic effects in this pathology. Our data reveal that animals lacking LPA_1 or LPA_2 activity underwent greater functional recovery after a severe spinal cord contusion injury. Strikingly, we also show that the lack of both, LPA_1 and LPA_2, did not result in additive beneficial effects after SCI. Overall, this study reveals the lack of additive effects of the combinatory targeting of LPA_1 and LPA_2, in SCI, suggesting that these two LPA receptors may share similar actions in this pathology.

Keywords: lysophosphatidic acid, LPA_1, LPA_2, functional recovery, spinal cord injury.

RESULTS

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; David et al., 2012a). There are various mechanisms that contribute to secondary injury, not all of which are fully defined. However, a large number of studies suggest that the inflammatory response occurring after spinal cord injury (SCI) is one of the main contributors to secondary degeneration (David et al., 2012a; David et al., 2012b; Popovich, 2014). In this line, we have previously demonstrated that microglia mediates secondary damage when activated with lysophosphatidic acid (LPA) (Santos-Nogueira et al., 2015).

LPA is a lysophospholipid (LPL) that was originally described as an intermediate in *de novo* lipid synthesis. However, it has now emerged as an intra- and extracellular phospholipid messenger with a wide variety of biological activities. LPA regulates important physiological functions, but it has also been involved in the course of various human diseases such as atherosclerosis, cancer and pulmonary fibrosis, among others (Inoue et al., 2004; Halder et al., 2013; Ma et al., 2013; Yung et al., 2014; Yung et al., 2015). Our laboratory has demonstrated that LPA is also an important contributor to SCI physiopathology (Santos-Nogueira et al., 2015). Interestingly, we have also unpublished data that, together with that described in the present thesis, highlight the importance of microglial LPA_1 and LPA_2 in mediating the harmful effects of this LPL after SCI. In this line, the lack of either LPA_1 or LPA_2 signaling after spinal cord contusion in mice leads to functional improvement, opening a novel avenue for the treatment of this pathology.

In the present chapter, we aimed at studying whether suppressing both, LPA_1 and LPA_2 activity, after SCI confers additive beneficial effects against neurological deficits. To develop this goal, we performed a pilot study in which LPA_2 knockout mice and wild type littermates were administrated with AM095, a selective and potent LPA_1 antagonist. Since we expected that this combined approach would had additive therapeutic effects, we induced a very severe SCI, in which neurological recovery is markedly impaired.

Analysis of locomotor performance on an open field evaluated using the Basso Mouse Scale (BMS) revealed that pharmacological blockade of LPA_1 resulted in greater locomotor skills at the end of the follow up, even though the SCI we induced was very severe (Fig. 1). Despite the beneficial effects of AM095 treatment, statistically significant differences were not found, which is likely due to the low number of animals used (n=5 for WT vehicle; n=4 for WT AM095). Gene deletion of LPA_2 also led to locomotor recovery after

SCI (Fig. 1). Although functional outcome of LPA_2 knockout mice was very similar that those observed in wildtype littermates treated with AM095 at the end of the follow up, greater locomotor recovery was more evident in mice lacking LPA_2 over the first 3 weeks after injury. Despite the low number of mice used in the present experiment (n=6 for $LPA2^{-/-}$ vehicle), BMS scores reached statistical significance at 7 and 21 days post-injury (RM-two-way ANOVA; *post-hoc* Bonferroni's test) in LPA_2 knockout mice, as compared to their WT littermate controls. Contrary to our hypothesis, pharmacological inhibition of LPA_1 in the LPA_2 knockout mice did not have any additive effects in motor skills (Fig. 1) suggesting the lack of therapeutic effects of the combinatory targeting. As mentioned before, this is a pilot using a relatively low number of mice (n=6 $LPA2^{-/-}$ AM095). However, since the lack of both LPA_1 and LPA_2 activity did not show any trend to improve neurological outcomes after SCI, as compared to LPA_2 knockout mice, it reinforces the idea that targeting these two LPA receptors does not have any additive beneficial effect.

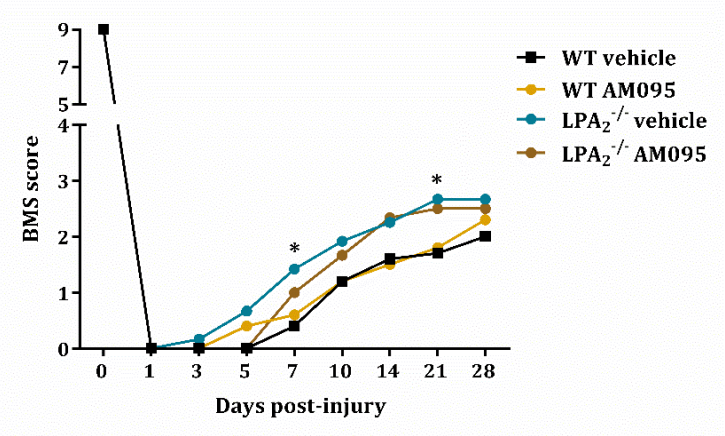


Figure 1. Assessment of locomotor recovery after SCI. Graph showing BMS score of mice lacking LPA_1, LPA_2 activity, or both. Note that pharmacological inhibition of LPA_1 or gene deletion of LPA_2 leads to neurological recovery at the end of the follow up. However, the lack of both, LPA_1 and LPA_2, did not promote additive therapeutic actions. Error bars indicate SEM. (*p<0.05).

In the present chapter, we therefore replicate that LPA_1 antagonism or gene deletion of LPA_2 improves functional outcomes in a severe model of SCI. Our data also suggest that targeting LPA_2 activity is likely to have greater therapeutic actions than blocking LPA_1 signaling. This agrees with our *in vitro* studies, in which we show that the toxic effects of microglia stimulated with LPA to oligodendrocytes are better abrogated when LPA_2, rather than LPA_1 signaling, is target. Interestingly, blockade of both, LPA_1 and LPA_2, does not result in beneficial additive effects after SCI, which may indicate that these two LPA receptors may share similar intracellular pathway. Since there is currently a lack of potent and selective LPA_2 antagonists, our data suggest that development of such compounds could open a new avenue for the treatment of acute SCI in human, for which there is no actual treatment.

METHODS

Surgical procedure

All surgical procedures were approved by the Universitat Autònoma de Barcelona Animal Care Committee and followed the European Communities Council Directive 2010/63/EU, and the methods for each procedure were carried out in accordance with the approved guidelines.

Adult (8-10 weeks old) female LPA_2 null and wildtype littermate mice (Charles River) were anesthetized with intraperitoneal injection of ketamine (90 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.). After performing a laminectomy at the 11th thoracic vertebrae, the exposed spinal cord was contused using the Infinite Horizon Impactor device (Precision Scientific Instrumentation). Injuries were made using a force of 60 kdynes and tissue displacement ranging between 500 and 700 μm. 1 hour after SCI, AM095 or saline was administered orally at a dose of 30 mg/kg, and then given every 12 hours for one week as previously described (Santos-Nogueira et al., 2015).

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.

Statistical analysis

All analyses were conducted through GraphPad Prism 6.0. Functional follow-up for BMS score was analyzed using repeated measures two-way ANOVA with Bonferroni's correction for multiple comparisons. Results are expressed as mean ± SEM. Differences were considered significant at $p<0.05$.

REFERENCES

Basso DM, Fisher LC, Anderson AJ, Jakeman LB, McTigue DM, Popovich PG (2006) Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. J Neurotrauma 23:635-659.

David S, Lopez-Vales R, Wee Yong V (2012a) Harmful and beneficial effects of inflammation after spinal cord injury: potential therapeutic implications. Handb Clin Neurol 109:485-502.

David S, Zarruk JG, Ghasemlou N (2012b) Inflammatory pathways in spinal cord injury. Int Rev Neurobiol 106:127-152.

Halder SK, Yano R, Chun J, Ueda H (2013) Involvement of LPA1 receptor signaling in cerebral ischemia-induced neuropathic pain. Neuroscience 235:10-15.

Inoue M, Rashid MH, Fujita R, Contos JJ, Chun J, Ueda H (2004) Initiation of neuropathic pain requires lysophosphatidic acid receptor signaling. Nat Med 10:712-718.

Ma L, Nagai J, Chun J, Ueda H (2013) An LPA species (18:1 LPA) plays key roles in the self-amplification of spinal LPA production in the peripheral neuropathic pain model. Mol Pain 9:29.

Popovich PG (2014) Neuroimmunology of traumatic spinal cord injury: a brief history and overview. Exp Neurol 258:1-4.

Santos-Nogueira E, Lopez-Serrano C, Hernandez J, Lago N, Astudillo AM, Balsinde J, Estivill-Torrus G, de Fonseca FR, Chun J, Lopez-Vales R (2015) Activation of Lysophosphatidic Acid Receptor Type 1 Contributes to Pathophysiology of Spinal Cord Injury. J Neurosci 35:10224-10235.

Schwab ME, Bartholdi D (1996) Degeneration and regeneration of axons in the lesioned spinal cord. Physiol Rev 76:319-370.

Yung YC, Stoddard NC, Chun J (2014) LPA receptor signaling: pharmacology, physiology, and pathophysiology. J Lipid Res 55:1192-1214.

Yung YC, Stoddard NC, Mirendil H, Chun J (2015) Lysophosphatidic Acid signaling in the nervous system. Neuron 85:669-682.

Discussion

Inflammation is an ubiquitous consequence of CNS trauma, differing the course of this response between brain and spinal cord (Donnelly and Popovich, 2008b). In regards to the secondary phase after SCI, the inflammatory response is initiated in the spinal cord parenchyma during the first hours after injury, persisting active for several months or even years, and contributing to tissue damage and functional loss (Fleming et al., 2006). Among all the molecules that contribute to the inflammatory response, lysophospholipids (LPLs) have been described as important lipidic mediators, regulating important aspects in this process (David et al., 2012).

LPLs are membrane-derived signalling molecules produced by phopholipases (PL) as a result of homeostatic lipid metabolism or as a response to stimulus-induced cellular activation (Sevastou et al., 2013). LPLs are glycerophospholipids in which one acyl chain is lacking, leaving only one hydroxyl group of the glycerol backbone acylated (D'Arrigo and Servi, 2010). LPLs are classified in two groups: lysoglycerophospholipids, with lysophosphatidic acid (LPA) and lysophosphatidilcoline (LPC) as the most representative lipid mediators; and lysosphingolipids, being sphingosine 1-phosphate (S1P) the most representative molecule in this case (Sevastou et al., 2013).

Among the different LPLs, LPC can induce rapid breakdown and removal of myelin from the adult mammalian tissue. Intraspinal injections of LPC in a spinal cord have shown that it is a potent demyelinating agent (Hall, 1972). Moreover, LPC has a crucial role in the inflammatory processes in the CNS, since it provokes a robust recruitment and activation of macrophages/microglia in the spinal cord (Ousman and David, 2000), together with an influx of T cells (Ghasemlou et al., 2007). Furthermore, LPC has been shown to promote and stabilize a strong M1 proinflammatory phenotype in macrophages (Qin et al., 2014), which is, in part, responsible of triggering different cytotoxic processes in the CNS (Kigerl et al., 2009; Kroner et al., 2014).

These findings have made LPC as one of the most extensively used agents to study demyelination responses in the central and peripheral nervous system (Hall, 1972; Blakemore et al., 1977; Ousman and David, 2000). In fact, from these evidences, LPLs have become of great importance in the course of the neuroinflammatory response, suggesting that one potential therapeutic intervention to avoid the detrimental actions of LPLs in neural pathologies is to focus on the enzymes involved in their synthesis.

As mentioned above, LPLs are synthetized by the action of PL family enzymes, being the different forms of phospholipase A_2s (PLA_2) the most important players. These enzymes catalyse the cleavage of fatty acids from the *sn*-2 position of phospholipids (Sun et al., 2004). For example, plasma lipoprotein-based PLA_2 hydrolyses oxidized phosphatidylcholine (PC), producing the majority of plasma LPC and oxidized non-esterified free fatty acid, such as arachidonic acid, which can also be metabolized into proinflammatory eicosanoids (Qin et al., 2014). Like LPC, eicosanoids, as prostaglandins, leukotrienes and thromboxanes, are potent inflammatory mediators, since they increase vascular permeability and induce chemotaxis of immune cells (Dennis et al., 1992; Funk, 2001). There are different types of PLA_2 enzymes, including secretory ($sPLA_2$) and cytosolic forms. The latest are divided into Ca^{2+}-dependent ($cPLA_2$) and Ca^{2+}-independent PLA_2s ($iPLA_2$) (Murakami et al., 1997). In the mammalian system, more than 19 different PLA_2s have been shown to participate in physiological events related to cell injury, inflammation, and apoptosis within the CNS (Sun et al., 2004).

PLA_2s are expressed mainly in neurons and oligodendrocytes after SCI, being its activity and protein levels increased after the lesion, inducing neuronal death and oligodendrocyte demyelination (Liu et al., 2006; López-Vales et al., 2011). Later investigation has shown that different PLA_2 subtypes play distinct roles in SCI. Some of them mediate protection after injury, such as $cPLA_2$, whereas others, like $sPLA_2$ and $iPLA_2$ have detrimental actions (López-Vales et al., 2011). These contrary effects are likely due to the multiple metabolites that PLA_2 actions generate. PLA_2s synthetize fatty acids and LPL that give rise to over two dozen of bioactive lipid mediators, which are classified into three classes. The first one includes metabolites of arachidonic acid, which are referred to as eicosanoids, as previously mentioned. The second one comprehends LPLs such as LPC, platelet-activating factor, S1P, endocannabinoids, and LPA, which is the form we focus on in this thesis. Finally, a third class of bioactive lipids includes the new group of anti-inflammatory pro-resolution mediators derived from omega-3 polyunsaturated fatty acids and omega-6 fatty acid released from plasma membrane glycerophospholipids by PLA_2s. These lipid mediators bind to specific receptors, regulating inflammatory responses (David et al., 2012). Since PLA_2s produce both pro-inflammatory mediators, and several lipids with high importance in regulating and resolving inflammation, inhibition of PLA_2 enzymes may interfere with the synthesis of molecules that exert harmful and beneficial effects in the SCI physiopathology, and thus, lead to weak therapeutic effects, and even to deleterious actions. Therefore, it is important to block selectively those lipid mediators generated by PLA_2s that mediate noxious actions in CNS conditions without impeding the production of the metabolites that may exert helpful effects.

In this thesis, we focused our work on LPA, which is a PLA_2-derived bioactive phospholipid, generated from PA, or from different LPLs by the hydrolysis of the head group by the secreted enzyme autotaxin (ATX) (Okudaira et al., 2010). LPA exerts its actions through six G-protein coupled receptors (GPCRs), LPA_{1-6}. These receptors are classified into two different families according their genetic similarities: LPA_{1-3} belong to the endothelial gene differentiation (Edg) family LPA receptor, whereas LPA_{4-6} belong to the non-Edg family LPA receptor (Chun, 2011). These receptors are expressed in several tissues by different cell types in the CNS. Indeed, we have previously demonstrated that all of them are constitutively expressed in the spinal cord parenchyma (Santos-Nogueira et al., 2015).

It has been shown that S1P receptor, which shares high homology to the Edg family LPA receptors (Van Brocklyn et al., 2000), plays an important role in demyelination (Chun and Hartung, 2010). In fact, fingolimod -known as FTY720 in the scientific literature and commercialized as Gilenya™ (Novartis AG, Basel)- is the first oral treatment for multiple sclerosis, being the first compound targeting LPL receptors that has become a human medicine to date (Brinkmann et al., 2010). Regarding LPA, there is currently emerging data demonstrating its involvement in the course of several CNS pathologies. For instance, a previous work revealed that the administration of the B3 antibody, that binds to LPA and other LPLs, preventing them from interacting with their receptors, enhances functional recovery in a spinal cord hemisection model in mice (Goldshmit et al., 2012). Despite the hemisection model is not clinically relevant, this work showed the potential deleterious actions of LPA in the SCI. However, previous work made in our laboratory administering B3 antibody after spinal cord contusion did not result in improvement of functional outcomes (data not shown). Our data suggested that an alternative and more potent method to inhibit LPA actions in the CNS is to target selective the LPA receptors. However, since there is a wide variety of LPA receptors, it is of great interest to elucidate which ones exert harmful or helpful effects in SCI physiopathology to develop a selective therapeutic target for those LPA receptors that exert detrimental action.

We have previously reported that LPA is a new factor involved in demyelinating events in the CNS. Indeed, LPA levels raised very rapidly in the spinal cord parenchyma after injury, remaining at high levels for at least two weeks (Santos-Nogueira et al., 2015). The increased 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 day 1 to day 14 post-injury, the last point evaluated, LPA synthesis is probably produced by neural and infiltrating cells in the spinal cord through PLD and PLA_2 activity, since these

enzymes are up-regulated after SCI (David et al., 2012), whereas ATX is downregulated, as we show in the present work.

Increased LPA levels after SCI may therefore act on the distinct cells, generating different physiological responses depending on the LPA receptors it signals through. We have previously proven by injecting LPA in the spinal cord that a substantial increase of LPA levels in the spinal cord tissue leads to demyelination, providing clear evidence that LPA production in the spinal cord parenchyma after SCI induces a demyelination process (Santos-Nogueira et al., 2015).

Myelin loss is mainly due to oligodendrocyte cell death, and these cells are particularly susceptible to death after SCI, undergoing necrosis and apoptosis acutely after injury, but also apoptosis at chronic stages of the disease at distant regions from the injury epicentre (Profyris et al., 2004). Demyelination that happens after SCI significantly contributes to functional impairments, since myelin is important to facilitate axonal conduction, it provides trophic support to axons (Nave, 2010), and is also crucial for axonal durability (Maxwell, 1996). Therefore, it is of high importance to elucidate the molecular mechanisms that cause myelin loss after injury, which has emerged as a key research field. Therapeutic interventions focused on reducing oligodendrocyte death or enhancing remyelination after SCI may lead to improvement in neurological outcomes, which make them of a great value to individuals living with SCI. In that line, some pharmacological approaches have been reported. For instance, rolipram, an inhibitor of phosphodiesterase 4, an enzyme that hydrolyses cAMP and is expressed in oligodendrocytes, has been found to significantly attenuate oligodendrocyte death after SCI (Whitaker et al., 2008). PTEN also promoted neural cell survival and oligodendrocyte-mediated myelination of axons (Walker and Xu, 2014). Contrarily, 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 same way, we showed for the first time that LPA is also a potent demyelinating factor (Santos-Nogueira et al., 2015). However, LPA-mediated demyelination is unlikely to be due to a cytotoxic effect of this LPL on oligodendrocytes, since we previously showed that LPA exerts mild cytotoxic effects in oligodendrocytes (Santos-Nogueira et al., 2015). In the present thesis, we revealed that LPA-triggered demyelination is mediated by the activation of LPA receptors in glial cells, rather to a direct effect of LPA in oligodendrocytes, results that were included in our first publication on the effects of LPA in SCI (Santos-Nogueira et al., 2015).

Inflammation is one of the main contributors to oligodendrocyte cell death after SCI. Indeed, intraspinal injection of LPA into the intact spinal cords leads to the development of

an inflammatory response, activating mainly microglia in the site of injection and in adjacent areas, and astrocytes in a lesser extent. In fact, stimulation of the microglial LPA receptors what induces the demyelinating injury. This is based on our findings, in which we observed that (i) after instraspinal injection of LPA in the intact spinal cord, microglia activation is associated with the areas of demyelination; and (ii) conditioned medium from microglia stimulated with LPA led to a remarkable induction of oligodendrocyte cell death *in vitro*.

In the present thesis, we demonstrate that microglial cells express LPA_1 and LPA_2 either *in vitro* and *in vivo* conditions. Here, we show that microglial LPA_1 is involved in oligodendrocyte cell death, since the lack of this receptor attenuated the cytotoxic effects of microglia upon LPA stimulation. Indeed, these findings were further confirmed by using a LPA_1 selective antagonist (Santos-Nogueira et al., 2015). We also demonstrate that microglial LPA_2 activation leads to even greater cytotoxic effects that LPA_1, since gene deletion of LPA_2 in microglia resulted in almost complete abolition of the cytotoxic effects that exerts these glial cells to oligodendrocytes after LPA stimulation *in vitro*. In line with these results, previous studies in our laboratory also showed that mice lacking LPA_1 (Santos-Nogueira et al., 2015) and LPA_2 activity (unpublished data) resulted in significant reduction of myelin loss after a spinal cord contusion in mice. These findings therefore suggest that activation of both microglial LPA_1 and LPA_2 is likely responsible of the demyelinating effects of LPA in the spinal cord parenchyma. However, further experiments using LPA_1 and LPA_2 knockout mice will be needed to completely demonstrate our findings.

LPA_1 and LPA_2 receptors can couple to the same or distinct G proteins, depending in the cell type (Noguchi et al., 2009). The G proteins activated by LPA_1 and LPA_2 signalling in microglia are currently unknown. It is possible that they could couple to different G proteins on microglia, and thus, activate divergent intracellular pathways to mediate cytotoxicity. If so, the blockade of both, LPA_1 and LPA_2, could have additive effects in abrogating the cytotoxic actions of microglia activation by LPA. To address this possibility, we combined the pharmacological blockade of LPA_1, by using a selective antagonist (AM095), with LPA_2 deficient mice in a severe SCI. However, LPA_1 antagonism did not enhance locomotor skills in the LPA_2 null mice after the contusion injury, which might indicate the both LPA_1 and LPA_2 signalling share common intracellular pathways. Indeed, our *in vitro* studies showed that gene deletion of LPA_2 blockade completely reverts the oligodendrocyte cell death mediated by microglial cells upon LPA stimulation, suggesting that microglial LPA_2 is the most powerful LPA receptor in mediating cytotoxicity of microglia upon LPA activation.

There is a wide variety of harmful mediators released by microglial cells that can cause oligodendrocyte death. For example, different studies have revealed that cytokines produced by microglial cells, like TNF-α or IL-1β, are potent promoters of oligodendrocyte cell death (D'Souza et al., 1995; Takahashi et al., 2003). Taking this into account, we measured the protein levels of 25 cytokines in the conditioned medium of LPA-activated microglia. We observed a mild production of some cytokines in conditioned medium of microglia after LPA stimulation, however, these levels were not significantly reduced as compared to that without stimulation. These data would suggest that the cytotoxic effects induced by LPA stimulated microglia are independent on cytokine production. Other, studies have correlated the oligodendrocyte cell death that occurs after a CNS insult to the nitric oxide produced by microglia (Merrill et al., 1993). Nevertheless, we did not observe significant differences in nitrate levels in the conditioned medium of LPA stimulated microglia. Moreover, other works have associated the glutamate released by microglial cells to oligodendrocyte cell death, induced by both, non-receptor mediated (Oka et al., 1993) and to a greater extend, to receptor mediated effects (García-Barcina and Matute, 1998; McDonald et al., 1998; Yoshioka et al., 2002). Activation of AMPA/Kainate receptor in oligodendrocytes causes oligodendrocyte cell death by different ways, such as hypoxic-ischemic death (Tekkök and Goldberg, 2001) or caspase-dependent and -independent mechanisms (Sánchez-Gómez et al., 2003). Therefore, we evaluated indirectly the effects of glutamate released by LPA-activated microglia on oligodendrocyte cell death by using CNQX, an AMPA/Kainate antagonist. CNQX treatment did not rescue oligodendrocyte cells from the cytotoxic effects of the LPA-conditioned medium, suggesting that glutamate released by microglia is not responsible for oligodendrocyte cytotoxicity. More recent studies have shown that microglial cells release ATP upon LPA stimulation (Fujita et al., 2008). Interestingly, oligodendrocyte cells express the purinergic P_2X_7, and stimulation of oligodendrocytes with ATP induces cell death via P_2X_7 activation (Matute et al., 2007). Indeed, inhibition of the purinergic receptor P_2X_7 confers protection against experimental autoimmune encephalomyelitis (EAE) and SCI (Matute et al., 2007; Peng et al., 2009). In this thesis, we showed that treatment with BBG, a potent P_2X_7 antagonist, almost completely rescue oligodendrocyte cell death induced by the conditioned medium of LPA-activated microglia. These findings suggest that activation of LPA receptors in microglial cells stimulates the release of purines to the extracellular milieu, which in turns activate P_2X_7 in oligodendrocytes, provoking cell death and consequently, demyelination.

In the present thesis, we also evaluated the functional role of LPA_4 and LPA_5, two out of the three members of the non-Edg LPA family receptors after SCI. Since these receptors are phylogenetically distant from the Edg family LPA receptor, they may play distinct, or

even neuroprotective effects upon LPA activation. LPA_4 couples to both Gs and Gq proteins, whereas LPA_5 couples to Gq and $G_{12/13}$ proteins, sharing some intracellular signalling events. However, while the role of LPA_4 in pathologies affecting the CNS is poorly understood, LPA_5 is known to be involved in some processes, such as the contribution to the development of neuropathic pain (Lin et al., 2012), or mediating microglial polarization to a pro-inflammatory phenotype *in vitro* (Plastira et al., 2016). Here, we observed that, even though LPA_4 is constitutively expressed in the spinal cord, its mRNA levels are not up-regulated after contusion. LPA_4 deficient mice, however, underwent slightly worse functional recovery after the lesion. This suggests that LPA_4 activation may translate LPA stimulation into some minor beneficial action during the inflammatory response of SCI, which needs to be elucidated. Contrarily, we found that LPA_5 mRNA expression levels are up-regulated almost five times in the spinal cord after contusion injury. However, mice deficient for LPA_5 showed neither enhancement in motor skills, nor greater myelin and neural sparing after SCI. Thus, these findings provide clear evidence that LPA_4 and LPA_5 do not have a central contribution to the secondary damage after SCI. Hence, the characterization of LPA_4 and LPA_5 receptors in the SCI physiopathology gives rise to some questions about the role of the third Non-Edg family LPA receptor, LPA_6, in this pathology. Since we do not have the LPA_6 deficient mice colony in our laboratory yet, we could not carry out these experiments, which will be assessed in the laboratory in the future. This will be crucial to elucidate whether LPA_6 also contributes to secondary damage after SCI.

As mentioned above, LPA is synthetized by the action of PLs through different pathways. In plasma, LPA is produced from LPC by a LysoPLD enzyme, also known as ATX, which is responsible for almost all the LPA present in plasma (Tsuda et al., 2006). ATX is over-expressed in many inflammatory conditions, such as glioblastoma, arthritis, or multiple sclerosis (Kehlen et al., 2001; Hammack et al., 2004; Kishi et al., 2006). Indeed, ATX inhibition by the potent selective inhibitor PF-8380 provided >95% reduction of plasma LPA during inflammation, suggesting that the ATX-LPA axis is the main source of LPA in inflammatory conditions (Gierse et al., 2010). Since we have previously discarded to target PLA_2 enzymes as a therapeutic approach to avoid the LPA production during SCI, in the present thesis, we aimed to evaluate the contribution of the ATX-LPA axis to the SCI physiopathology, which has not been described yet. Surprisingly, we found that after a spinal cord contusion, the ATX mRNA levels showed a significant down-regulation in the spinal cord parenchyma, which did not align to the notably increased LPA production after SCI. However, we went on to pharmacologically inhibit the ATX activity by using PAT-048 and Compound X, two potent and selective ATX inhibitors. As expected, PAT-048 or

Compound X administration did not result in either beneficial nor detrimental effects in the functional recovery of the injured mice. Since after SCI there is a prominent disruption of the blood-brain barrier (BBB), allowing plasma derived cells and other molecules to diffuse to the epicentre of the lesion, both PAT-048 and Compound X are likely to reach the injured spinal cord. However, in order to verify the presence, and consequent activity, of both inhibitors in blood and spinal cord parenchyma of the lesion site, we are currently measuring the levels of PAT-048 and Compound X l in both tissues, as well as, the changes in the LPA levels in plasma and spinal cord samples triggered by both inhibitors. Due to time constraints, we have not been able to show this data in this thesis, but we will have the results shortly. However, our functional and histological findings, together with qPCR analysis, suggest that ATX-LPA axis is not the main enzyme involved in LPA production after SCI, and reinforce the idea that PLA_2 enzymes are the main player in the production of this LPL in SCI.

Overall, the present thesis suggests that the demyelinating actions of LPA after SCI are mediated by activation of LPA_1 and LPA_2 in microglial cells, which in turn, induces the release of purines to the extracellular milieu, causing oligodendrocyte cell death by activation of P_2X_7 receptor. We also provide clear evidence that, in contrast to LPA_1 and LPA_2, the non-Edg LPA family receptors, LPA_4 and LPA_5, do not play any major contribution to secondary damage after SCI. We also show consistent data suggesting that the production of LPA in the contused spinal cord parenchyma is not via ATX, but to action of PLA_2 enzymes. Since the use of PLA_2 inhibitors to impede the production of LPA cannot be used to treat SCI, since pan-PLA_2 inhibitors worsen SCI pathology (López-Vales et al., 2011), there is a need to selectively block the activation of LPA receptors involved in secondary damage, LPA_1 and LPA_2, although the role of LPA_6 needs still to be elucidated. Our data also indicates that the lack of both, LPA_1 and LPA_2 activity, does not exert synergic beneficial effects on functional outcomes after SCI, which suggests that the use of selective inhibitors for LPA_1 or, preferably for LPA_2, may lead to the development of a new pharmacological approach to treat this pathology.

Conclusions

Chapter I:

- Microglial activation is tightly associated with areas of demyelination after intraspinal injection of LPA.
- Microglial cells become cytotoxic to oligodendrocytes after LPA stimulation.
- Microglia express LPA_1 and LPA_2 in the adult CNS, but also in cell culture conditions.
- LPA_1 and LPA_2 signalling in microglia triggers oligodendrocyte cell death.
- LPA induces the release of purines in microglial cells release purines that trigger oligodendrocyte cells death via P_2X_7 activation.

Chapter II:

- LPA_4 is constitutively expressed in the spinal cord and its RNA levels do not increase after contusion injury.
- Gene deletion of LPA_4 results in slight worsening of functional outcomes after spinal cord injury.
- The lack of LPA_4 has no effect on secondary tissue damage after spinal cord contusion.
- LPA_5 is constitutively expressed in the spinal cord parenchyma and its transcripts are markedly up-regulated after contusion injury.
- LPA_5 signalling does not contribute to functional and histological outcomes after spinal cord injury.

Chapter III:

- Autotaxin mRNA levels are down-regulated in the spinal cord after contusion injury.
- Pharmacological inhibition of autotaxin has no effect on functional recovery or secondary tissue damage after spinal cord injury.

Chapter IV:

- Combinatory targeting of LPA_1 and LPA_2 does not result in additive therapeutic actions after spinal cord injury.

References

Ackery A, Tator C, Krassioukov A (2004) A Global Perspective on Spinal Cord Injury Epidemiology. 21:1355–1370.

Alvarez-Palomo A, McLenachan S, Chen F, Da Cruz L, Dilley R, Requena J, Lucas M, Lucas A, Drukker M, Edel M (2015) Prospects for clinical use of reprogrammed cells for autologous treatment of macular degeneration. Fibrogenes Tissue Repair 8.

Aoki J, Inoue A, Okudaira S (2008) Two pathways for lysophosphatidic acid production. Biochim Biophys Acta 1781:513–518.

Araque A, Navarrete M (2010) Glial cells in neuronal network function. Philos Trans R Soc B Biol Sci 365:2375–2381.

Armand J (1982) The Origin, Course and Terminations of Corticospinal Fibers in Various Mammals. Prog Brain Res 57:329–360.

Baptiste DC, Fehlings MG (2006) Pharmacological Approaches To Repair the Injured Spinal Cord. J Neurotrauma 23:318–334.

Basu S, Hellberg A, Ulus AT, Westman J, Karacagil S (2001) Biomarkers of free radical injury during spinal cord ischemia. FEBS Lett 508:36–38.

Blakemore WF, Eames RA, Smith KJ, Mcdonald WI (1977) Remyelination in the spinal cord of the cat following intraspinal injections of lysolecithin. J Neurol Sci 33:31–43.

Bouquet C, Ravaille-Veron M, Propst F, Nothias F (2007) MAP1B coordinates microtubule and actin filament remodeling in adult mouse Schwann cell tips and DRG neuron growth cones. Mol Cell Neurosci 36:235–247.

Bregman BS, Kunkel-Bagden E, Schnell L, Dai HN, Gao D, Schwab ME (1995) Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. Nature 378:498–501.

Brinkmann V, Billich A, Baumruker T, Heining P, Schmouder R, Francis G, Aradhye S, Burtin P (2010) Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. Nat Rev Drug Discov 9:883–897.

Burns AS, Connell CO (2012) The challenge of spinal cord injury care in the developing world. J Spinal Cord Med 35:3–8.

Carlson SL, Parrish ME, Springer JE, Doty K, Dossett L (1998) Acute Inflammatory Response in Spinal Cord Following Impact Injury. Exp Neurol 151:77–88.

Casha S, Zygun D, McGowan MD, Bains I, Yong VW, John Hurlbert R (2012) Results of a phase II placebo-controlled randomized trial of minocycline in acute spinal cord injury. Brain 135:1224 LP-1236.

Chiu WT, Lin HC, Lam C, Chu SF, Chiang YH, Tsai SH (2010) Review paper: epidemiology of traumatic spinal cord injury: comparisons between developed and developing countries. Asia-Pacific J Public Heal 22:9–18.

Choi JW, Chun J (2013) Lysophospholipids and their receptors in the central nervous system. Biochim Biophys Acta 1831:20–32.

Choi JW, Herr DR, Noguchi K, Yung YC, Lee C-W, Mutoh T, Lin M-E, Teo ST, Park KE, Mosley AN, Chun J (2010) LPA receptors: subtypes and biological actions. Annu Rev Pharmacol Toxicol 50:157–186.

Choi JW, Lee CW, Chun J (2008) Biological roles of lysophospholipid receptors revealed by genetic null mice: An update. Biochim Biophys Acta - Mol Cell Biol Lipids 1781:531–539.

Chu X-P, Xiong Z-G (2013) Acid-Sensing Ion Channels in Pathological Conditions. In: Sodium Calcium Exchange: A Growing Spectrum of Pathophysiological Implications: Proceedings of the 6th International Conference

on Sodium Calcium Exchange (Annunziato L, ed), pp 419–431. Boston, MA: Springer US. Available at: http://dx.doi.org/10.1007/978-1-4614-4756-6_36.

Chun J (2011) Lysophospholipid receptors.

Chun J, Hartung H (2010) Mechanism of Action of Oral Fingolimod (FTY720) in Multiple Sclerosis. Clin Neuropharmacol 33:91–101.

Creasey G, Wilberger J, Maynard FM, Bracken MB, Creasey G, Ditunno JF, Donovan WH, Ducker B, Garber SL, Marino RJ, Stover SL, Tator CH, Waters RL, Wilberger JE (1997) International Standards for Neurological and Functional Classification of Spinal Cord Injury. Spinal Cord 35:266–274.

D'Arrigo P, Servi S (2010) Synthesis of lysophospholipids. Molecules 15:1354–1377.

D'Souza S, Alinauskas K, McCrea E, Goodyer C, Antel JP (1995) Differential susceptibility of human CNS-derived cell populations to TNF-dependent and independent immune-mediated injury. J Neurosci 15:7293 LP-7300.

David S, Greenhalgh AD, López-Vales R (2012) Role of phospholipase A 2s and lipid mediators in secondary damage after spinal cord injury. Cell Tissue Res 349:249–267.

David S, Kroner A (2011) Repertoire of microglial and macrophage responses after spinal cord injury. Nat Rev Neurosci 12:388–399.

Dawson J, Hotchin N, Lax S, Rumsby M (2003) Lysophosphatidic acid induces process retraction in CG-4 line oligodendrocytes and oligodendrocyte precursor cells but not in differentiated oligodendrocytes. J Neurochem 87:947–957.

Dennis EA, Rhee SG, Billah MM, HAnnun YA (1992) Role of phospholipases in generating lipid second messengers in signal transduction. FASEB J 5:2068–2077.

Doble A (1999) The Role of Excitotoxicity in Neurodegenerative Disease: Implications for Therapy. Pharmacol Ther 81:163–221.

Donnelly DJ, Popovich PG (2008a) Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Exp Neurol 209:378–388.

Donnelly DJ, Popovich PG (2008b) Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Exp Neurol 209:378–388.

Dumont RJ, Okonkwo DO, Verma S, Hurlbert RJ, Boulos PT, Ellegala DB, Dumont AS (2001) Acute Spinal Cord Injury, Part I: Pathophysiologic Mechanisms. Clin Neuropharmacol 24.

Elmes SJR, Millns PJ, Smart D, Kendall DA, Chapman V (2004) Evidence for biological effects of exogenous LPA on rat primary afferent and spinal cord neurons. Brain Res 1022:205–213.

Estivill-Torrús G, Llebrez-Zayas P, Matas-Rico E, Santín L, Pedraza C, De Diego I, Del Arco I, Fernández-Llebrez P, Chun J, De Fonseca FR (2008) Absence of LPA1 signaling results in defective cortical development. Cereb Cortex 18:938–950.

Fawcett JW (1997) Neuron-glia interactions in axogenesis and regeneration. Astrocytic and neuronal factors affecting axon regeneration in the damaged central nervous system. Cell Tissue Res 290:371–377.

Fink KL, López-Giráldez F, Kim I-J, Strittmatter SM, Cafferty WBJ (2017) Identification of Intrinsic Axon Growth Modulators for Intact CNS Neurons after Injury. Cell Rep 18:2687–2701.

Fleming JC, Norenberg MD, Ramsay DA, Dekaban GA, Marcillo AE, Saenz AD, Pasquale-styles M, Dietrich WD, Weaver LC (2006) The cellular inflammatory response in human spinal cords after injury. :3249–3269.

Francos-Quijorna I, Amo-Aparicio J, Martinez-Muriana A, L??pez-Vales R (2016) IL-4 drives microglia and macrophages toward a phenotype conducive for tissue repair and functional recovery after spinal cord injury. Glia 64:2079–2092.

Fu Q, Hue J, Li S (2007) Nonsteroidal Anti-Inflammatory Drugs Promote Axon Regeneration via RhoA Inhibition.

J Neurosci 27:4154 LP-4164.

Fujita R, Ma Y, Ueda H (2008) Lysophosphatidic acid-induced membrane ruffling and brain-derived neurotrophic factor gene expression are mediated by ATP release in primary microglia. J Neurochem 107:152–160.

Fukushima N, Weiner J a, Kaushal D, Contos JJ a, Rehen SK, Kingsbury M a, Kim KY, Chun J (2002) Lysophosphatidic acid influences the morphology and motility of young, postmitotic cortical neurons. Mol Cell Neurosci 20:271–282.

Funk CD (2001) Prostaglandins and Leukotrienes: Advances in Eicosanoid Biology. Science (80-) 294:1871–1875.

García-Barcina JM, Matute C (1998) AMPA-selective glutamate receptor subunits in glial cells of the adult bovine white matter. Mol Brain Res 53:270–276.

Ghasemlou N, Youngjeong S, Lacroix S, Samuel David (2007) T Cells Contribute to Lysophosphatidylcholine-Induced Macrophage Activation and Demyelination in the CNS. Glia 55:294–302.

Gierse J et al. (2010) Levels in Plasma and the Site of Inflammation. J Pharmacol Exp Ther 2:310–317.

Goldberg JL, Klassen MP, Hua Y, Barres B a (2002) Amacrine-signaled loss of intrinsic axon growth ability by retinal ganglion cells. Science (80-) 296:1860–1864.

Goldshmit Y, Matteo R, Sztal T, Ellett F, Frisca F, Moreno K, Crombie D, Lieschke GJ, Currie PD, Sabbadini R a., Pébay A (2012) Blockage of lysophosphatidic acid signaling improves spinal cord injury outcomes. Am J Pathol 181:978–992.

GrandPre T, Nakamura F, Vartanian T, Strittmatter SM (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 403:439–444.

Gris D, Marsh DR, Oatway MA, Chen Y, Hamilton EF, Dekaban GA, Weaver LC (2004) Transient Blockade of the CD11d/CD18 Integrin Reduces Secondary Damage after Spinal Cord Injury, Improving Sensory, Autonomic, and Motor Function. J Neurosci 24:4043 LP-4051.

Guha AB, Tator CH (1988) Acute Cardiovascular Effects of Experimental Spinal Cord Injury. J Trauma Acute Care Surg 28.

Hall SM (1972) The effect of injections of lysophosphatidyl choline into white matter of the adult mouse spinal cord. J Cell Sci 10:535–546.

Hammack BN, Fung KYC, Hunsucker SW, Duncan MW, Burgoon MP, Owens GP, Gilden DH (2004) Proteomic analysis of multiple sclerosis cerebrospinal fluid. Mult Scler J 10:245–260.

Hausmann ON (2003) Post-traumatic inflammation following spinal cord injury. Spinal Cord 41:369–378.

Holtsberg FW, Steiner MR, Keller JN, Mark RJ, Mattson MP, Steiner SM (1998) Lysophosphatidic acid induces necrosis and apoptosis in hippocampal neurons. J Neurochem 70:66–76.

Hurlbert RJ (2000) Methylprednisolone for acute spinal cord injury: an inappropriate standard of care. J Neurosurg Spine 93:1–7.

Hurlbert RJ (2006) Strategies of Medical Intervention in the Management of Acute Spinal Cord Injury. Spine (Phila Pa 1976) 31.

Kehlen A, Lauterbach R, Santos AN, Thiele K, Kabisch U, Weber E, Riemann D, Langner J (2001) IL-1beta- and IL-4-induced down-regulation of autotaxin mRNA and PC-1 in fibroblast-like synoviocytes of patients with rheumatoid arthritis (RA). Clin Exp Immunol 123:147–154.

Keller JN, Steiner MR, Mattson MP, Steiner SM (1996) Lysophosphatidic acid decreases glutamate and glucose uptake by astrocytes. J Neurochem 67:2300–2305.

Kigerl KA, Gensel JC, Ankeny DP, Alexander JK, Donnelly DJ, Popovich PG (2009) Identification of Two Distinct Macrophage Subsets with Divergent Effects Causing either Neurotoxicity or Regeneration in the Injured

Mouse Spinal Cord. 29:13435–13444.

Kirshblum SC, Biering-Sorensen F, Betz R, Burns S, Donovan W, Graves DE, Johansen M, Jones L, Mulcahey MJ, Rodriguez GM, Schmidt-Read M, Steeves JD, Tansey K, Waring W (2014) International Standards for Neurological Classification of Spinal Cord Injury: Cases with classification challenges. J Spinal Cord Med 37:120–127.

Kirshblum SC, Burns SP, Biering-sorensen F, Donovan W, Graves DE, Jha A, Johansen M, Jones L, Krassioukov A, Mulcahey MJ, Schmidt-read M, Waring W (2011) International standards for neurological classification of spinal cord injury (Revised 2011). 34:535–546.

Kishi Y, Okudaira S, Tanaka M, Hama K, Shida D, Kitayama J, Yamori T, Aoki J, Fujimaki T, Arai H (2006) Autotaxin is overexpressed in glioblastoma multiforme and contributes to cell motility of glioblastoma by converting lysophosphatidylcholine TO lysophosphatidic acid. J Biol Chem 281:17492–17500.

Kroner A, Greenhalgh AD, Zarruk JG, PassosdosSantos R, Gaestel M, David S (2014) TNF and Increased Intracellular Iron Alter Macrophage Polarization to a Detrimental M1 Phenotype in the Injured Spinal Cord. Neuron 83:1098–1116.

Lee CW, Rivera R, Dubin AE, Chun J (2007) LPA4/GPR23 is a lysophosphatidic acid (LPA) receptor utilizing Gs-, Gq/Gi-mediated calcium signaling and G12/13-mediated Rho activation. J Biol Chem 282:4310–4317.

Lee SM, Rosen S, Weinstein P, van Rooijen N, Noble-Haeusslein LJ (2011) Prevention of both neutrophil and monocyte recruitment promotes recovery after spinal cord injury. J Neurotrauma 28:1893–1907.

Li X, Shridas P, Forrest K, Bailey W, Webb NR (2010) Group X secretory phospholipase A2 negatively regulates adipogenesis in murine models. FASEB J 24:4313–4324.

Lin M-E, Rivera RR, Chun J (2012) Targeted Deletion of LPA5 Identifies Novel Roles for Lysophosphatidic Acid Signaling in Development of Neuropathic Pain. J Biol Chem 287:17608–17617.

Liu NK, Zhang YP, Titsworth WL, Jiang X, Han S, Lu PH, Shields CB, Xu XM (2006) A novel role of phospholipase A2 in mediating spinal cord secondary injury. Ann Neurol 59:606–619.

Llona-Minguez S, Ghassemian A, Helleday T (2015) Lysophosphatidic acid receptor (LPAR) modulators: The current pharmacological toolbox. Prog Lipid Res 58:51–75.

López-Vales R, Ghasemlou N, Redensek A, Kerr BJ, Barbayianni E, Antonopoulou G, Baskakis C, Rathore KI, Constantinou-Kokotou V, Stephens D, Shimizu T, Dennis E a, Kokotos G, David S (2011) Phospholipase A2 superfamily members play divergent roles after spinal cord injury. FASEB J 25:4240–4252.

Ma L, Nagai J, Ueda H (2010) Microglial activation mediates de novo lysophosphatidic acid production in a model of neuropathic pain. J Neurochem 115:643–653.

Maier K, Merkler D, Gerber J, Taheri N, Kuhnert A V., Williams SK, Neusch C, Bähr M, Diem R (2007) Multiple neuroprotective mechanisms of minocycline in autoimmune CNS inflammation. Neurobiol Dis 25:514–525.

Matute C, Domercq M, Sanchez-Gomez M-V (2006) Glutamate-mediated glial injury: mechanisms and clinical importance. Glia 53:212–224.

Matute C, Torre I, Perez-Cerda F, Perez-Samartin A, Alberdi E, Etxebarria E, Arranz AM, Ravid R, Rodriguez-Antiguedad A, Sanchez-Gomez M, Domercq M (2007) P2X7 Receptor Blockade Prevents ATP Excitotoxicity in Oligodendrocytes and Ameliorates Experimental Autoimmune Encephalomyelitis. J Neurosci 27:9525–9533.

Mautes AEM, Weinzierl MR, Donovan F, Noble LJ (2000) Vascular Events After Spinal Cord Injury: Contribution to Secondary Pathogenesis. Phys Ther 80:673 LP-687.

Maxwell W. (1996) Histopathological changes at central nodes of Ranvier after stretch-injury. Micros Res Tech 34:522–535.

Mazaira J, Labanda F, Romero J, García ME, Gambarruta C, Sanchez A, Alcaraz MA, Arroyo O, Esclarín A, Arzoz T, Artime C, Labarta C (1998) Epidemiology and other aspects of spinal cord injury. Rehabilitación 32:365–372.

McDonald J, Althomsons S, KL H, DW C, Goldberg M (1998) Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. Nat Med 4:291–297.

Mekhail M, Almazan G, Tabrizian M (2012) Oligodendrocyte-protection and remyelination post-spinal cord injuries: A review. Prog Neurobiol 96:322–339.

Merrill JE, Ignarro LJ, Sherman MP, Melinek J, Lane TE (1993) Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. J Immunol 151:2132–2141.

Möller T, Contos JJ, Musante DB, Chun J, Ransom BR (2001) Expression and function of lysophosphatidic acid receptors in cultured rodent microglial cells. J Biol Chem 276:25946–25952.

Mothe AJ, Tator CH (2013) Review of transplantation of neural stem/progenitor cells for spinal cord injury. Int J Dev Neurosci 31:701–713.

Murakami M, Nakatani Y, Atsumi G, Inoue K, Kudo I (1997) Regulatory functions of phospholipase A2. Crit Rev Immunol 17:225–283.

Nakamura K, Kishimoto T, Ohkawa R, Okubo S, Tozuka M, Yokota H, Ikeda H, Ohshima N, Mizuno K, Yatomi Y (2007) Suppression of lysophosphatidic acid and lysophosphatidylcholine formation in the plasma in vitro: Proposal of a plasma sample preparation method for laboratory testing of these lipids. Anal Biochem 367:20–27.

Nave K-A (2010) Myelination and support of axonal integrity by glia. Nature 468:244–252.

Nelson E, Gertz S, ML R, TB D, OR B (1977) Spinal cord injury: The role of vascular damage in the pathogenesis of central hemorrhagic necrosis. Arch Neurol 34:332–333.

Nishikawa T, Tomori Y, Yamashita S, Shimizu S (1989) Inhibition of Na+,K+-ATPase activity by phospholipase A2 and several lysophospholipids: possible role of phospholipase A2 in noradrenaline release from cerebral cortical synaptosomes. J Pharm Pharmacol 41:450–458.

Nogaroli L, Yuelling LM, Dennis J, Gorse K, Payne SG, Fuss B (2009) Lysophosphatidic acid can support the formation of membranous structures and an increase in MBP mRNA levels in differentiating oligodendrocytes. Neurochem Res 34:182–193.

Noguchi K, Herr D, Mutoh T, Chun J (2009) Lysophosphatidic acid (LPA) and its receptors. Curr Opin Pharmacol 9:15–23.

Ohuchi H, Hamada A, Matsuda H, Takagi A, Tanaka M, Aoki J, Arai H, Noji S (2008) Expression patterns of the lysophospholipid receptor genes during mouse early development. Dev Dyn 237:3280–3294.

Oka A, Belliveau MJ, Rosenberg PA, Volpe JJ (1993) Vulnerability of oligodendroglia to glutamate: pharmacology, mechanisms, and prevention. J Neurosci 13:1441 LP-1453.

Okudaira S, Yukiura H, Aoki J (2010) Biological roles of lysophosphatidic acid signaling through its production by autotaxin. Biochimie 92:698–706.

Ondarza AB, Ye Z, Hulsebosch CE (2003) Direct evidence of primary afferent sprouting in distant segments following spinal cord injury in the rat: colocalization of GAP-43 and CGRP. Exp Neurol 184:373–380.

Ousman SS, David S (2000) Lysophosphatidylcholine induces rapid recruitment and activation of macrophages in the adult mouse spinal cord. Glia 30:92–104.

Pandya K, Weant K, Cook A (2010) High-Dose Methylprednisolone in Acute Spinal Cord Injuries: Proceed With Caution. Orthopedics 33:327–331.

Park E, Velumian A a, Fehlings MG (2004) The role of excitotoxicity in secondary mechanisms of spinal cord injury : a review with an emphasis on the implications for white matter degeneration. J Neurotrauma

21:754–774.

Peng W, Cotrina ML, Han X, Yu H, Bekar L, Blum L, Takano T, Tian G-F, Goldman SA, Nedergaard M (2009) Systemic administration of an antagonist of the ATP-sensitive receptor P2X7 improves recovery after spinal cord injury. Proc Natl Acad Sci U S A 106:12489–12493.

Pérez K, Novoa AM, Santamari E, Narvaez Y, Arrufat V, Borrell C, Cabeza E, Cirera E, Ferrando J, García-altés A, Gonzalez-luque JC, Lizarbe V, Martin-cantera C, Seguí-gómez M (2012) Incidence trends of traumatic spinal cord injury and traumatic brain injury in. 46:37–44.

Piani D, Frei K, Do KQ, Cuénod M, Fontana A (1991) Murine brain macrophages induce NMDA receptor mediated neurotoxicity in vitro by secreting glutamate. Neurosci Lett 133:159–162.

Plastira I, Bernhart E, Goeritzer M, Reicher H, Kumble VB, Kogelnik N, Wintersperger A, Hammer A, Schlager S, Jandl K, Heinemann A, Kratky D, Malle E, Sattler W (2016) 1-Oleyl-lysophosphatidic acid (LPA) promotes polarization of BV-2 and primary murine microglia towards an M1-like phenotype. J Neuroinflammation 13:205.

Profyris C, Cheema SS, Zang D, Azari MF, Boyle K, Petratos S (2004) Degenerative and regenerative mechanisms governing spinal cord injury. Neurobiol Dis 15:415–436 Available at: http://www.ncbi.nlm.nih.gov/pubmed/15056450 [Accessed July 29, 2014].

Qin X, Qiu C, Zhao L (2014) Lysophosphatidylcholine perpetuates macrophage polarization toward classically activated phenotype in inflammation. Cell Immunol 289:185–190.

Ramakers GJ, Moolenaar WH (1998) Regulation of astrocyte morphology by RhoA and lysophosphatidic acid. Exp Cell Res 245:252–262.

Rao TS, Lariosa-Willingham KD, Lin F-F, Palfreyman EL, Yu N, Chun J, Webb M (2003) Pharmacological characterization of lysophospholipid receptor signal transduction pathways in rat cerebrocortical astrocytes. Brain Res 990:182–194.

Rathore KI, Kerr BJ, Redensek A, López-Vales R, Jeong SY, Ponka P, David S (2008) Ceruloplasmin protects injured spinal cord from iron-mediated oxidative damage. J Neurosci 28:12736–12747.

Rieske P, Azizi S., Augelli B, Gaughan, Krynska B (2007) A population of human brain parenchymal cells express markers of glial, neuronal and early neural cells and differentiate into cells of neuronal and glial lineages. Eur J Neurosci 25:31–37.

Rolls A, Shechter R, Schwartz M (2009) The bright side of the glial scar in CNS repair. Nat Rev Neurosci 10:235–241.

Rossignol S, Schwab M, Schwartz M, Fehlings MG (2007) Spinal Cord Injury: Time to Move? J Neurosci 27:11782 LP-11792.

Rothman SM (1985) The neurotoxicity of excitatory amino acids is produced by passive chloride influx. J Neurosci 5:1483 LP-1489.

Rowland JW, Hawryluk GWJ, Kwon B, Fehlings MG (2008) Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon. Neurosurg Focus 25:E2.

Saito S (1997) Effects of lysophosphatidic acid on primary cultured chick neurons. Neurosci Lett 229:73–76.

Sánchez-Gómez MV, Alberdi E, Ibarretxe G, Torre I, Matute C (2003) Caspase-dependent and caspase-independent oligodendrocyte death mediated by AMPA and kainate receptors. J Neurosci 23:9519–9528.

Santos-Nogueira E, López-Serrano C, Hernández J, Lago N, Astudillo AM, Balsinde J, Estivill-Torrús G, de Fonseca FR, Chun J, López-Vales R (2015) Activation of Lysophosphatidic Acid Receptor Type 1 Contributes to Pathophysiology of Spinal Cord Injury. J Neurosci 35:10224–10235.

Schwartz G, Fehlings MG (2001) Evaluation of the neuroprotective effects of sodium channel blockers after spinal cord injury: improved behavioral and neuroanatomical recovery with riluzole. J Neurosurg Spine

94:245–256.

Selzer ME (2010) Spinal Cord Injury: a guide for patients and families.

Sevastou I, Kaffe E, Mouratis MA, Aidinis V (2013) Lysoglycerophospholipids in chronic inflammatory disorders: The PLA 2/LPC and ATX/LPA axes. Biochim Biophys Acta - Mol Cell Biol Lipids 1831:42–60.

Silva NA, Sousa N, Reis RL, Salgado AJ (2014) From basics to clinical: A comprehensive review on spinal cord injury. Prog Neurobiol 114:25–57.

Silver J, Miller J (2004) Regeneration beyond the glial scar. Nat Rev Neurosci 5:146–156.

Spohr TCLDSE, Dezonne RS, Rehen SK, Gomes FCA (2011) Astrocytes treated by lysophosphatidic acid induce axonal outgrowth of cortical progenitors through extracellular matrix protein and epidermal growth factor signaling pathway. J Neurochem 119:113–123.

Spohr TCLDSE, Dezonne RS, Rehen SK, Gomes FCA (2014) LPA-primed astrocytes induce axonal outgrowth of cortical progenitors by activating PKA signaling pathways and modulating extracellular matrix proteins. Front Cell Neurosci 8:296.

Stankoff B, Barron S, Allard J, Barbin G, Noël F, Aigrot MS, Premont J, Sokoloff P, Zalc B, Lubetski C (2002) Oligodendroglial Expression of Edg-2 Receptor: Developmental Analysis and Pharmacological Responses to Lysophosphatidic Acid. Mol Cell Neurosci 20:415–428.

Stirling DP, Liu S, Kubes P, Yong VW (2009) Depletion of Ly6G/Gr-1 Leukocytes after Spinal Cord Injury in Mice Alters Wound Healing and Worsens Neurological Outcome. J Neurosci 29:753 LP-764.

Sun GY, Xu J, Jensen MD, Simonyi A (2004) Phospholipase A $_2$ in the central nervous system. J Lipid Res 45:205–213.

Tabuchi S, Kume K, Aihara M, Shimizu T (2000) Expression of lysophosphatidic acid receptor in rat astrocytes: Mitogenic effect and expression of neurotrophic genes. Neurochem Res 25:573–582.

Takahashi JL, Giuliani F, Power C, Imai Y, Yong VW (2003) Interleukin-1β promotes oligodendrocyte death through glutamate excitotoxicity. Ann Neurol 53:588–595.

Taoka Y, Okajima K, Uchiba M, Murakami K, Kushimoto S, Johno M, Naruo M, Okabe H, Takatsuki K (1997) Role of neutrophils in spinal cord injury in the rat. Neuroscience 79:1177–1182.

Tator CH, Fehlings MG (1991) Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. J Neurosurg 75:15–26.

Tekkök SB, Goldberg MP (2001) Ampa/kainate receptor activation mediates hypoxic oligodendrocyte death and axonal injury in cerebral white matter. J Neurosci 21:4237–4248.

Terayama R, Bando Y, Murakami K, Kato K, Kishibe M, Yoshida S (2007) Neuropsin promotes oligodendrocyte death, demyelination and axonal degeneration after spinal cord injury. Neuroscience 148:175–187.

Thuret S, Moon LD, Gage FH (2006) Therapeutic interventions after spinal cord injury. Nat Rev Neurosci 7:628–643.

Tikka TM, Koistinaho JE (2001) Minocycline Provides Neuroprotection Against N-Methyl-d-aspartate Neurotoxicity by Inhibiting Microglia. J Immunol 166:7527–7533.

Tsuda S, Okudaira S, Moriya-Ito K, Shimamoto C, Tanaka M, Aoki J, Arai H, Murakami-Murofushi K, Kobayashi T (2006) Cyclic phosphatidic acid is produced by autotaxin in blood. J Biol Chem 281:26081–26088.

Van Brocklyn JR, Gräler MH, Bernhardt G, Hobson JP, Lipp M, Spiegel S (2000) Sphingosine-1-phosphate is a ligand for the G protein-coupled receptor EDG-6. Blood 95:2624 LP-2629.

Vessal M, Aycock A, Garton MT, Ciferri M, Darian-Smith C (2007) Adult neurogenesis in primate and rodent spinal cord: Comparing a cervical dorsal rhizotomy with a dorsal column transection. Eur J Neurosci 26:2777–2794.

Walker CL, Xu X-M (2014) PTEN inhibitor bisperoxovanadium protects oligodendrocytes and myelin and

prevents neuronal atrophy in adult rats following cervical hemicontusive spinal cord injury. Neurosci Lett 573:64–68.

Watanabe N1, Ikeda H, Nakamura K, Ohkawa R, Kume Y, Aoki J, Hama K, Okudaira S, Tanaka M, Tomiya T, Yanase M, Tejima K, Nishikawa T, Arai M, Arai H, Omata M, Fujiwara K YY (2007) Both plasma lysophosphatidic acid and serum autotaxin levels are increased in chronic hepatitis C. J Clin Gastroenterol 41:616–623.

Watson C, Paxinos G, Kayalioglu G (2008) The Spinal Cord, 1st editio. Academic Press.

Wells JEA, Hurlbert RJ, Fehlings MG, Yong VW (2003) Neuroprotection by minocycline facilitates significant recovery from spinal cord injury in mice. Brain 126:1628 LP-1637.

Wen-Ta C, Hsiao-Chiao L, Carlos L, Shu-Fen C, Yung-Hsiao C, Shin-Han T (2016) Epidemiology of Traumatic Spinal Cord Injury : Comparisons Between Developed and Developing Countries. Asia-Pacific J Public Heal 22:9–18.

Whitaker CM, Beaumont E, Wells MJ, Magnuson DSK, Hetman M, Onifer SM (2008) Rolipram attenuates acute oligodendrocyte death in the adult rat ventrolateral funiculus following contusive cervical spinal cord injury. Neurosci Lett 438:200–204.

Xu J, Qu ZX, Hogan EL, Perot PL (1992) Protective Effect of Methylprednisolone on Vascular Injury in Rat Spinal Cord Injury. J Neurotrauma 9:245–253.

Yanagida K, Masago K, Nakanishi H, Kihara Y, Hamano F, Tajima Y, Taguchi R, Shimizu T, Ishii S (2009) Identification and characterization of a novel lysophosphatidic acid receptor, p2y5/LPA6. J Biol Chem 284:17731–17741.

Yang H, Lu P, McKay HM, Bernot T, Keirstead H, Steward O, Gage FH, Edgerton VR, Tuszynski MH (2006) Endogenous Neurogenesis Replaces Oligodendrocytes and Astrocytes after Primate Spinal Cord Injury. J Neurosci 26:2157 LP-2166.

Yiu G, He Z (2006) Glial inhibition of CNS axon regeneration. Nat Rev Neurosci 7:617–627 Available at: http://dx.doi.org/10.1038/nrn1956.

Yoshioka A, Hardy M, Younkin DP, Grinspan JB, Stern JL, Pleasure D (2002) α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionate (AMPA) Receptors Mediate Excitotoxicity in the Oligodendroglial Lineage. J Neurochem 64:2442–2448.

Yung YC, Stoddard NC, Mirendil H, Chun J (2015) Lysophosphatidic Acid Signaling in the Nervous System. Neuron 85:669–682.

Annex

Development/Plasticity/Repair

Activation of Lysophosphatidic Acid Receptor Type 1 Contributes to Pathophysiology of Spinal Cord Injury

Eva Santos-Nogueira,[1] Clara López-Serrano,[1] Joaquim Hernández,[1] Natalia Lago,[2] Alma M. Astudillo,[3] Jesús Balsinde,[3] Guillermo Estivill-Torrús,[4] Fernando Rodriguez de Fonseca,[4] Jerold Chun,[5] and Rubèn López-Vales[1]

[1]Department of Cellular Biology, Physiology, and Immunology, Institute of Neurosciences, Center for Biomedical Research in Neurodegenerative Diseases Network (CIBERNED), Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain, [2]Neuroinflammation and Gene Therapy Laboratory, Pasteur Institute of Montevideo, 11400 Montevideo, Uruguay, [3]Institute of Biology and Molecular Genetics, Spanish National Research Council, 47003 Valladolid, Spain, [4]Research Laboratories, Interdepartmental Neuroscience and Mental Health Clinical Management Units, Institute for Biomedical Research of Málaga, Regional University Hospital of Málaga and Virgen de la Victoria, 29009 Málaga, Spain, and [5]Molecular and Cellular Neuroscience Department, Dorris Neuroscience Center, Scripps Research Institute, La Jolla, California 92037

Lysophosphatidic acid (LPA) is an extracellular lipid mediator involved in many physiological functions that signals through six known G-protein-coupled receptors (LPA_1–LPA_6). A wide range of LPA effects have been identified in the CNS, including neural progenitor cell physiology, astrocyte and microglia activation, neuronal cell death, axonal retraction, and development 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. LPA levels increase in the contused spinal cord parenchyma during the first 14 d. To model this potential contribution of LPA in the spinal cord, we injected LPA into the normal spinal cord, revealing that LPA induces microglia/macrophage activation and demyelination. Use of a selective LPA_1 antagonist or mice lacking LPA_1 linked receptor-mediated signaling to demyelination, which was in part mediated by microglia. 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 support LPA–LPA_1 signaling as a novel pathway that contributes to secondary damage after spinal cord contusion in mice and suggest that LPA_1 antagonism might be useful for the treatment of acute spinal cord injury.

Key words: **demyelination; lysophosphatidic acid; microglia; neuroprotection; oligodendrocytes; spinal cord injury**

Significance Statement

This study reveals that LPA signaling via LPA receptor type 1 activation causes demyelination and functional deficits after spinal cord injury.

Introduction

Spinal cord injury (SCI) causes functional deficits produced by the loss of axons and neurons and contributes to the death of oligodendrocytes and the limited ability of the CNS to regenerate axons (Rowland et al., 2008; David et al., 2012a). The pathophysiology of SCI involves two stages of tissue degeneration known as primary and secondary injury. Primary injury results from the direct mechanical trauma to the spinal cord, which is followed by secondary injury, consisting of a wave of tissue degeneration that occurs over a period of several weeks, and is associated with inflammation and other mechanisms triggered by injury (David et al., 2012a,b; Popovich, 2014). Secondary injury is thought to contribute importantly to the functional deficits seen after SCI and may 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. Hence, there is 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.

Received Nov. 14, 2014; revised May 19, 2015; accepted June 9, 2015.

Author contributions: C.L.-S., N.L., G.E.-T., and R.L.-V. designed research; E.S.-N., C.L.-S., J.H., N.L., A.M.A., J.B., F.R.d.F., J.C., and R.L.-V. performed research; R.L.-V. contributed unpublished reagents/analytic tools; E.S.-N., C.L.-S., J.H., N.L., and R.L.-V. analyzed data; E.S.-N., G.E.-T., F.R.d.F., J.C., and R.L.-V. wrote the paper.

This work was supported by National Institutes of Health Grant NS084398 (J.C.), Wings for Life Foundation, Marie-Curie International Reintegration Program Grant MC IRG 249274, Spanish Ministry of Economy and Competitiveness Grant SAF2013-48431-R, and the Health Research Fund of Spain [Cell Therapy Network and Center for Biomedical Research in Neurodegenerative Diseases Network (CIBERNED)] (R.L-V.) E.S.-N. is a recipient from a FPU fellowship. We thank Dr. Samuel David for critical review of this manuscript. We also acknowledge Marta Morell and Jessica Jaramillo for excellence technical assistance and Margaret Attiwell for help in electron microscopy. We thank Bristol-Myers Squibb for the gift of AM095.

Correspondence should be addressed to Dr. Rubèn López-Vales, Faculty of Medicine, M4-114, Autonomous University of Barcelona, 08193 Bellaterra, Catalonia, Spain. E-mail: ruben.lopez@uab.cat.

DOI:10.1523/JNEUROSCI.4703-14.2015

 0270-6474/15/3510224-12$15.00/0

Lysophosphatidic acid (LPA; 1-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; Yung et al., 2014). LPA exerts this wide variety of effects by binding to specific G-protein-coupled receptors, such as LPA receptors (LPARs) LPA_1–LPA_3, which belong to the endothelial differentiation gene family, and the genetically more distant LPA_4–LPA_6, which belong to the P2Y purinergic receptor family (Choi et al., 2010; Choi and Chun, 2013; Kihara et al., 2014). These receptors differ in their ability to alter downstream signaling pathways, including intracellular Rho levels, mobilize Ca^{2+}, contribute to 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 (Yung et al., 2011), fetal hypoxic brain damage (Herr et al., 2011), and the development of neuropathic pain after sciatic nerve injury and cerebral ischemia (Inoue et al., 2004; Frisca et al., 2012; Lin et al., 2012). Because of the wide variety of LPAR subtypes, it is likely that LPA may exert helpful or harmful effects in the CNS depending on the receptors it signals through. Thus, there is a need to know which LPARs contribute to neurodegeneration and/or neuroprotection. Here we show that LPA levels increase in the spinal cord parenchyma after contusion injury. We provide evidence suggesting that LPA leads to demyelination via activation of microglia LPA_1. Moreover, we demonstrate that selective blockade of LPA_1 after SCI reduces functional deficits and demyelination, altogether revealing important contributions of LPA–LPA_1 signaling in secondary damage after SCI.

Materials and Methods

Surgical procedure

All surgical procedures were approved by the Autonomous University of 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$_1$-null; Estivill-Torrús et al., 2008), or wild-type littermates (C57BL/6J × 129X1/SvJ background) were anesthetized with ketamine (90 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.). After performing a laminectomy at the 11th thoracic vertebrae, the exposed spinal cord was either injected intraspinally or contused.

Intraspinal injections were performed using a glass needle (30 μm internal diameter; Eppendorf) coupled to a 10 ml Hamilton syringe (catalog #701; Hamilton). One microliter of saline, saline containing 5 nmol of LPA (18:1 LPA; Sigma-Aldrich), or 5 nmol of LPA plus the selective LPA_1 antagonist AM095 sodium, {4'-[3-methyl-4-((*R*)-1-phenyl-ethoxycarbonylamino)-isoxazol-5-yl]-biphenyl-4-yl}-acetate 0.22 nmol; kindly provided by Bristol-Myers Squibb; 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), and the tip of the needle was maintained inside the cord tissue 3 min after each injection to avoid liquid reflux.

SCIs were performed using the Infinite Horizon Impactor device (Precision Scientific), using a force of 50 kdyn and tissue displacement ranging from 400 to 600 μm (Klopstein et al., 2012). When indicated, AM095 or saline was administered orally at a dose of 30 mg/kg. Treatment was initiated 1 h after SCI and then given every 12 h for 1 week.

Functional assessment

Locomotor recovery was evaluated at 1, 3, 5, 7, 10, 14, 21, and 28 d post-injury (dpi) in an open-field test using the nine-point Basso Mouse Scale (BMS; Basso et al., 2006), which was developed specifically for locomotor testing after contusion injuries in mice. The BMS analysis of hindlimb movements and coordination was performed by two independent assessors who were blinded to the experimental groups and the consensus score taken. In addition, at the end of the follow-up (28 dpi), 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 the maximum speed at which each mouse was to perform for at least 5 s was recorded.

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 (GM) 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, with the cathode over the skull overlying the sensorimotor cortex and the anode at the nose (García-Alías et al., 2003). Compound muscle action potential from GM 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; Grass Instruments) 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 cultures

Mouse oligodendrocyte cultures. Primary oligodendrocyte progenitor cells (OPCs) were isolated from mouse cerebral cortex at postnatal day 2 (P2) to P4 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-cysteine (Sigma), and 1 mg/ml DNase I (Roche) in MEM (Gibco) for 20 min at 37°C. Mixed glial culture media [MGCM; 10% inactivated and filtered fetal bovine serum (FBS; Sigma), 0.33% penicillin–streptomycin (P/S; Sigma), and 1% Glutamax 100× (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 h at 37°C and cultured at 37°C in an humidified incubator with 5% CO_2 supplementation. Three hours after plating, the floating cells were discarded by replacing the medium. Two thirds of the MGCM was replaced every 3 d 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 100×, 2% B27 (Gibco), 0.5% FBS, 50 pg/ml recombinant mouse ciliary neurotrophic factor (BioTrend), and 1% OL supplement (10 μl/ml N-2 supplement 100× (Gibco), 10 mg/ml bovine serum albumin (Sigma), and 40 μg/ml 3,3′,5-triiodo-L-thyronine (Sigma). OPC maturation in oligodendrocytes was achieved after 7 d *in vitro*.

Mouse microglia 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/F-12 (Gibco) with 10% FBS and 5% P/S and cultured at 37°C in a humidified incubator with 5% CO_2 supplementation. Medium was replaced every 4–5 d, and confluence was achieved after 10–12 d. At this point, mixed cultures were incubated with 0.25% trypsin–EDTA (Gibco) diluted 1:4 in DMEM/F-12 for 30 min at 37°C. This trypsinization resulted in the detachment of the upper layer of cells in one piece, and the remaining adherent microglial cells were cultured for 24 h before stimulation. Microglial cells were stimulated with LPA (1 μM) or LPA plus AM095 (12.5 μM) or 0.025% DMSO (vehicle for AM095) as control for 24 h. Afterward, cells were washed to remove the LPA from the medium, and microglia were then incubated with oligodendrocyte conditioned media for 24 h and then used for cytotoxicity assays in oligodendrocyte cell cultures.

Table 1. Real-time PCR primer sequences

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
LPA_6	ACTGCTGCTTTGACCCTATTG	AAGGTCTGTAGGTTGTGTTGG
GAPDH	TCAACAGCAACTCCCACTCTTCCA	ACCCTGTTGCTGTAGCCGTATTCA

Assessment of oligodendrocyte cell death

Oligodendrocytes were stimulated with LPA (0.01, 0.1, or 1 μM), LPA plus AM095 (12.5 μM), and DMSO (0.025%) as control, or with conditioned media of microglia harvested from C57BL/6 mice, $maLPA_1$ null mice, or wild-type littermates, stimulated with DMSO, LPA, (1 μM), or LPA (1 μM) plus AM095 (12.5 μM) for 24 h. Afterward, coverslips were fixed in 4% paraformaldehyde for 20 min, washed in PBS, and incubated overnight with rat anti-MBP (1:300; Abcam), mouse anti-A2B5–Alexa Fluor 488 (1:100; Millipore), and rabbit anti-NG2 (1:100; Millipore) in 0.3% Triton X-100/PBS with 5% fetal calf serum at 4°C. After several washes in PBS, coverslips were incubated for 1 h at room temperature (RT) with anti-rat or anti-rabbit Alexa Fluor 594-conjugated antibody (1:500; Invitrogen) and DAPI (Sigma), and the total number of oligodendrocytes (MBP^+ cells) or oligodendrocytes precursor cells (A2B5 and NG2) were counted under fluorescence microscope (Olympus BX51). Four independent replicates were done for each of the experiments.

Histology

At 4 d after intraspinal injection and 28 d 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, postfixed with 4% paraformaldehyde in 0.1 M PB for 1 h, and cryoprotected with 30% sucrose in 0.1 M PB at 4°C for a minimum of 48 h. The samples were fast-frozen at −60°C 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 fluoromyelin (Invitrogen). After graded dehydration, sections were placed in a 1 mg/ml LFB solution in 95% ethanol and 0.05% acetic acid overnight at 37°C. Sections were then washed in 95% ethanol and distilled water before being placed in a solution of 0.5 mg/ml Li_2CO_3 in distilled water for 1 min at RT. 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 PBS with Tween 20 (PBST) for 1 h at RT. The sections were then incubated overnight at 4°C with primary antibodies against NeuN (for neurons; 1:200; Millipore), neurofilament-200 (NF-200; for axons; 1:1000; Millipore), GFAP (for astrocytes; 1:500; Invitrogen), ionized calcium-binding adapter molecule 1 (Iba1; for macrophage/microglia; 1:400; Abcam), and MBP (for myelin; 1:100; Abcam). After several washes in PBST, sections were incubated for 1 h at RT with the appropriate Alexa Fluor 594 or 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 an Olympus BX51 microscope, and images were captured with an Olympus DP50 digital camera attached to it and using the CellÂ 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. For experiments injecting LPA, demyelination was measured by delineating LFB nonstained white matter in the dorsal column, whereas microglia activation was assessed by calculating the percentage of $Iba1^+$ immunoreactivity area within a 0.025 mm^2 box placed within the dorsal column. For SCI experiments, 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. Moreover, axonal and demyelination in the dorsal column was assessed by counting the NF^+ structures as well as fibers double stained for NF and MBP at the lesion epicenter.

Electron microscopy

Four and 21 d after intraspinal injection of LPA or saline, mice were perfused with 0.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PB, 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 postfixation in 2% osmium tetroxide for 2 h at RT and then processed for embedding in Epon. One-millimeter-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.

Flow cytometry

Immune cells from the injured spinal cord were analyzed by flow cytometry at 7 dpi, as described previously (Klopstein et al., 2012). Briefly, spinal cords were cut in little pieces and passed through a cell strainer of 70 μm (Falcon; BD Bioscience Discovery Labware), and the cell suspension was centrifuged twice at 300 × *g* for 10 min at 4°C. Samples were divided, and cells alone and isotype-matched control samples were generated to control for nonspecific binding of antibodies and for autofluorescence. Isotype control antibodies were purchased from eBioscience and included phycoerythrin–cyanine 7 (PE–Cy7)-labeled rat IgG2b, adenomatous polyposis coli (APC)-labeled rat IgG2b, peridinin chlorophyll-a protein cyanine 5.5 (PerCP–Cy5.5)-conjugated rat IgG2b, and fluorescein isothiocyanate-conjugated rat IgG2b and IgG2M. The following antibodies were also purchased from eBioscience: CD45–PerCP–Cy5.5, CD11b–PE–Cy7, and F4/80–APC. After 30 min of incubation with combinations of antibodies at 4°C, the samples were washed and fixed in 1% paraformaldehyde. Microglial cells were identified as $CD45^{low}$ and $CD11b^+$ cells, whereas myeloid cells were identified as $CD45^{high}$ and $CD11b^+$ cells (Stirling and Yong, 2008). Macrophages were identified from myeloid cells based on F4/80 expression ($CD45^{high}$, $CD11b^+$, $F4/80^+$). At least 3000 $CD45^+$ cells were analyzed per spinal cord sample using FlowJo software on a FACSCanto flow cytometer (BD Biosciences). Four contused mice treated with saline or AM095 were used in this experiment.

Isolation of microglia from CNS tissue

Briefly, spinal cord and brain from adult C57BL/6 mice (8–10 weeks old) were removed and digested enzymatically with 0.2% collagenase B (Roche Diagnostics) and 0.2% trypsin–EDTA at 37°C for 30 min and then passed through a cell strainer of 40 μm (Falcon; BD Bioscience Discovery Labware). Cell suspension was centrifuged twice at 300 × *g* for 10 min at 4°C, and microglial cells were first isolated by magnetic sorting using a CD11b antibody (Miltenyi Biotec) and then stained with PerCP–Cy5.5-conjugated CD45 and PE–Cy7-conjugated CD11b antibodies for additional purification on a cell sorter (FACSARIA III; BD Bioscience). Microglia cells were assessed on a flow cytometer (FACSCalibur; BD Biosciences), and only populations presenting >90% purity were used for gene expression analysis.

RNA isolation, reverse transcription, and real-time PCR

Mice were perfused with sterile saline, and a 5-mm-length of uninjured spinal cord was removed. Tissue was homogenized with QIAzol lysis reagent (Qiagen) and RNA extracted using the RNeasy Lipid Tissue kit (Qiagen), according to the protocol of the manufacturer. RNA was treated with DNaseI (Qiagen) to eliminate genomic DNA contamination. mRNA from cultured and *in vivo* sorted microglia was extracted using the RNeasy Micro kit following the guidelines of the manufacturer. One and 0.5 μg of RNA obtained from tissue or microglia, respectively, was primed with random hexamers (Promega) and reverse transcribed using the Omniscript RT kit (Qiagen). RNase inhibitor (Roche) was added (1 U/μl final concentration) to avoid RNA degradation. 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

Table 2. Quantification of the different LPA species in SCI

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 h	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 d	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 d	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 d	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 d	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

*$p < 0.01$ versus intact spinal cord.

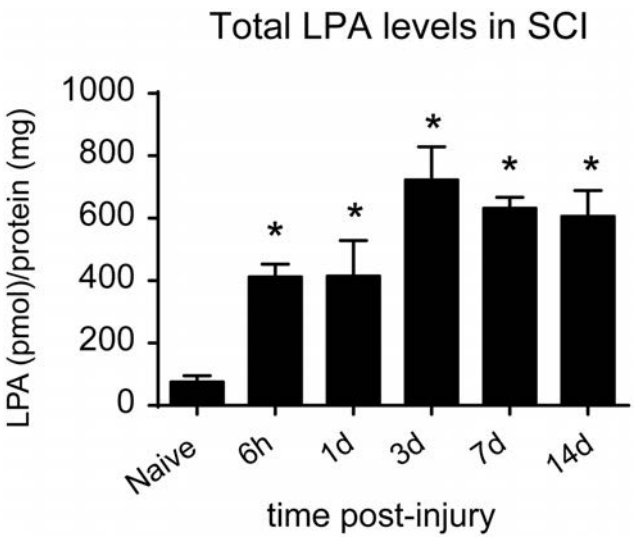


Figure 1. Increased LPA levels in the contused spinal cord. Mass spectrometry analyses of LPA in spinal cord tissue from uninjured and contused spinal cord at 6 h and 1, 3, 7, and 14 d after SCI ($n = 4$ time point). *$p < 0.05$. Error bars indicate SEM.

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

Mass spectrometry analysis of LPA in the spinal cord

Mice were perfused with sterile saline, and a 5-mm-length of uninjured or injured spinal cord centered at the impact site was harvested at 6 h and 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).

The volume corresponding to 300 μg of protein was taken of every sample. Two hundred picomoles 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 H_2O/HCl at 19:1, and LPA species were extracted with 500 μl of water-saturated butanol twice. Organic phase was recollected, evaporated until dryness, and resuspended in 100 μl of $CHCl_3$. Fifty microliters of this extract were injected in a high-performance liquid chromatograph equipped with a 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). The column was a Supelcosil LC-Si 3 μm 150 × 3 mm column (Sigma-Aldrich) protected with a Supelguard LC-Si 20 × 3 mm guard cartridge (Sigma-Aldrich). Mobile phase was a gradient of solvent A (chloroform/methanol/32% ammonium hydroxide at 75:24.5:0.5 v/v/v) and solvent B (chloroform/methanol/water/32% ammonium hydroxide at 55:39:5.5:0.5 v/v/v/v). The gradient was started at 100% solvent A; it was decreased linearly to 50% solvent A in 2 min, maintained for 4 min, to 0% solvent A in 6 min, and maintained there for an additional 18 min. The flow rate was 0.5 ml/min in the chromatographic step and 0.2 ml/min entered into the electrospray interface of the mass spectrometer. Nebulizer gas was set to 30 lb 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 deprotonated molecular ions $[M\text{-}H]^-$.

Statistical analysis

All analyses were conducted through IBM SPSS Statistics version 19. 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 were analyzed using two-way repeated-measures (RM) ANOVA. The rest of the data were analyzed using one-way or two-way ANOVA depending on the appropriate design. *Post hoc* comparisons were performed 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 SE. Differences were considered significant at $p < 0.05$.

Results

LPA levels are increased in the injured mouse spinal cord

We first assessed whether LPA levels increased in the spinal cord after contusion injury. Using mass spectrometry, we detected the presence of three 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 elevated significantly at all time points analyzed (one-way ANOVA; *post hoc* Bonferroni's test, $p < 0.01$; $n = 4$ 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 (Fig. 1), being approximately fivefold higher at 6 h, rising to ~10-fold at day 3, and the remaining approximately eightfold increased from 7 to 14 dpi compared with intact spinal cord (one-way ANOVA; *post hoc* Bonferroni's test, $p < 0.01$; $n = 4$ per time point). These results demonstrate that LPA levels increase in the spinal cord after contusion injury.

Exogenous LPA exposure 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 nmol of LPA into the dorsal column of uninjured spinal cord or saline alone as a control group. The LPA variant used in these experiments was the 18:1 oleoyl LPA, because our mass spectrometry results revealed that this LPA species was the most abundant in the contused spinal cord. Microglia/macrophage activation in the spinal cord parenchyma was assessed by measuring the presence of reactive $Iba1^+$ cells, 4 d after injection. Microglia/macrophage activation was restricted to the injection area in saline-injected mice, probably because of the mechanical injury caused by the needle insertion (Fig. 2*A–C*). By comparison, spinal cord sections from mice injected with LPA showed a marked activation of microglia/macrophages 4 d after injection (Fig. 2*D*). The presence of these inflammatory cells was not limited to the injection site but were also distributed within the whole dorsal columns (Fig. 2*D*). In addition, the immunoreactivity for $Iba1^+$

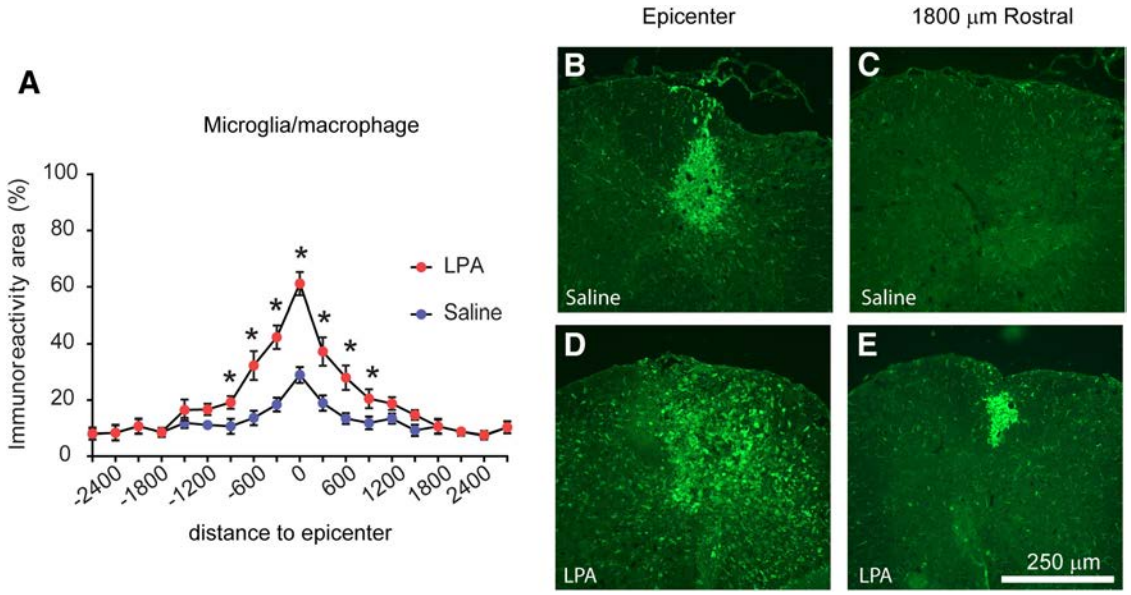


Figure 2. Intraspinal injection of LPA leads to microglia/macrophage activation. ***A***, Quantification of microglia/macrophage immunoreactivity at 4 d after intraspinal injection of saline or LPA. ***B–E***, Representative images of spinal cords stained against Iba1 at the epicenter (***B***, ***D***)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

cells was also increased in rostral and caudal areas from the injection site in LPA-injected compared with saline-injected mice (Fig. 2; two-way RM-ANOVA; *post hoc* Bonferroni's test, $p < 0.05$; $n = 4$ per group). Mild activation of astrocytes was also observed but were only restricted to areas close to the LPA injection (data not shown), indicating that LPA activates both microglia and astrocytes but has a greater preference for microglia.

We then assessed whether activation of microglia/macrophages triggered by LPA led to demyelination. At 4 dpi, LFB staining revealed the presence of a small demyelinating lesion in the dorsal column of saline-injected spinal cords (Fig. 3), which was restricted to the area of the needle insertion (Fig. 3*B*). We also observed a demyelinating lesion in the spinal cords injected with LPA (Fig. 3*D*). The area of this lesion was significantly increased in LPA-injected compared with PBS-injected mice at the injection epicenter and in adjacent areas (Fig. 3*A–E*; two-way RM-ANOVA; *post hoc* Bonferroni's test, $p < 0.05$; $n = 4$ per group). To assess whether demyelination induced by LPA was caused by axonal or myelin damage, we double stained spinal cord sections with fluoromyelin and NF. Double immunofluorescence revealed the presence of NF^+ structures within the demyelinating lesion (Fig. 3*F*), suggesting that LPA led to demyelination. To further confirm these observations, we used electron microscopy (Fig. 3*G–J*). This technique confirmed the presence of fibers with degenerating myelin (Fig. 3*H*,*I*) and naked axons (Fig. 3*J*) within the demyelinating lesion. Therefore, these results indicate that the increased levels of LPA in the spinal cord leads to neuroinflammation and demyelination.

Exogenous LPA exposure mediates demyelination via LPA_1

Because LPA signals through six different LPARs, we studied which LPARs were expressed constitutively in the normal spinal cord. Real-time PCR analysis revealed that all six LPARs are expressed constitutively in the uninjured spinal cord, with LPA_1 being the most highly expressed (Fig. 4; $n = 4$ per time point). To assess whether the demyelination triggered by intraspinal injection of LPA was mediated via 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 4 d after LPA administration, the demyelinating lesion was reduced substantially when LPA_1 was blocked (Fig. 5*A–C*). 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 600 μm rostral and caudal to the epicenter (two-way ANOVA; *post hoc* Bonferroni's test, $p < 0.05$; $n = 4$ per group). Spinal cord tissue sections from LPA_1 null mice ($maLPA_1$ variant) also displayed smaller demyelinating lesions (Fig. 5*D–F*) after intraspinal injection of LPA, further implicating LPA_1 signaling in LPA-induced demyelination.

Because LPA_1 is expressed in mature oligodendrocytes (Weiner et al., 1998), we assessed whether LPA-induced demyelination was attributable to a toxic effect of LPA on oligodendrocytes. Cell culture work indicated that LPA treatment led to a mild reduction in the viability of oligodendrocytes (~20%) at concentrations of 1 μM (Fig. 6*A*; $n = 4$ per group). However, LPA did not have an effect on oligodendrocyte precursor cell survival (Fig. 6*B*). Administration of AM095 failed to rescue cultured oligodendrocytes from cell death, suggesting that the LPA_1 signaling does not mediate a direct cytotoxic effect in cultured oligodendrocytes (Fig. 6*C*; $n = 4$ per group), albeit with the caveat that these cells only approximate what occurs *in vivo*. Because LPA also triggers microglia activation when injected into the spinal cord and activated microglial cells were associated with areas of demyelination (Fig. 6*D*), we also assessed whether microglial LPA_1 was responsible for the LPA-induced demyelination. In agreement with previous work (Möller et al., 2001; Tham et al., 2003) real-time PCR analysis identified LPA_1 gene expression in cultured microglia (Fig. 6*F*; $n = 4$). Moreover, microglial cells *in vivo* have constitutive expression of LPA_1, showing similar levels of LPA_1 relative to cultured microglia (Fig. 6*E*,*F*; $n = 4$). Interestingly, we observed that conditioned medium of primary microglial cells stimulated with LPA led to a marked reduction (~85%) in oligodendrocyte survival (Fig. 6*G*,*I–F*; one-way ANOVA; *post hoc* Bonferroni's test, $p < 0.001$; $n = 4$ per group), suggesting that microglia become cytotoxic after LPA stimulation., Oligodendrocyte cell death was reduced approximately

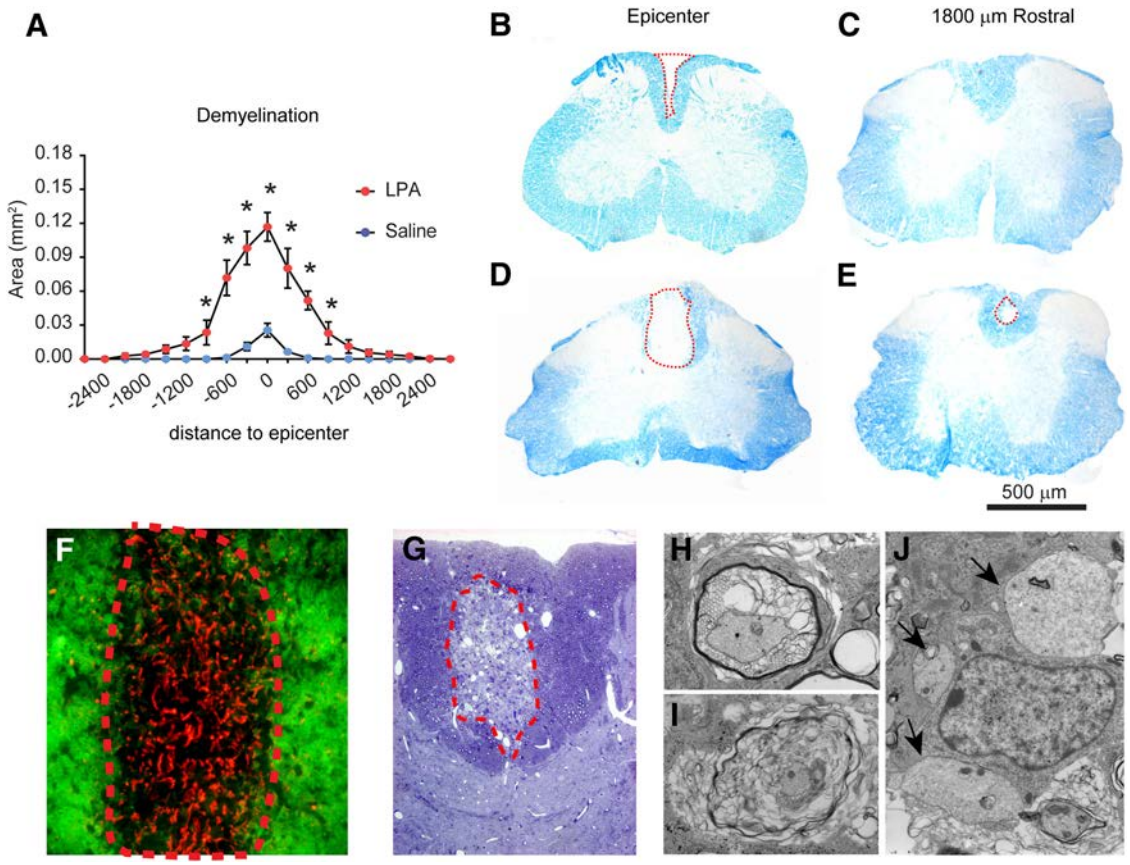


Figure 3. Intraspinal injection of LPA leads to demyelination. ***A***, Quantification of demyelination at 4 d after intraspinal injection of saline or LPA. ***B–E***, Representative images of spinal cords stained with LFB at the epicenter (***B***, ***D***) and 1800 μm rostral to the injection site (***C***, ***E***) in saline-injected (***B***, ***C***) and LPA-injected (***D***, ***E***) mice. ***F***, High-magnification image of spinal cord tissue section stained against NF (red) and fluoromyelin (green) showing the presence of NF^+ structures within the demyelinating lesion at 4 d after intraspinal injection of LPA. ***G***, Toluidine blue-stained section from spinal cord at 4 d after intraspinal injection of LPA. ***H–J***, Electron microscopy images taken from the demyelinating area showing fibers with myelin breakdown (***H***, ***I***) and demyelinated axons (***J***; see arrows; $n = 4$ per group). *$p = 0.001$. Error bars indicate SEM. Scale bar, 500 μm.

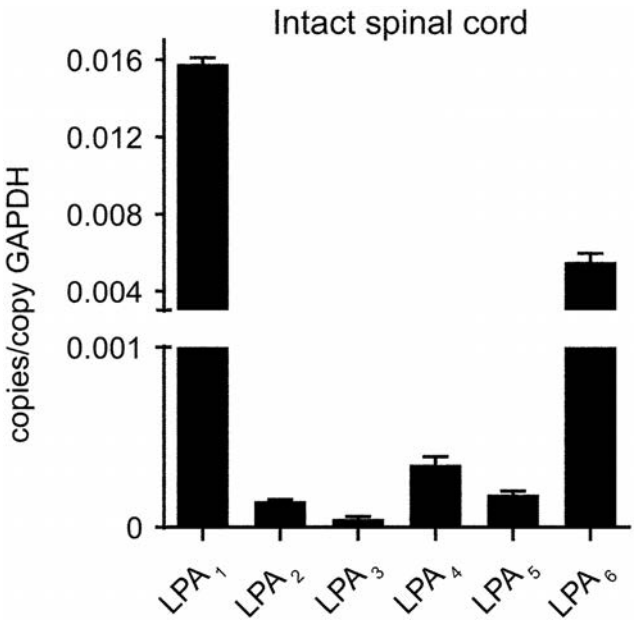


Figure 4. Expression of LPA_1–LPA_5 in the spinal cord parenchyma. Note that LPA_1 is the LPAR most highly expressed in the spinal cord. Data are presented as copies of receptor mRNA relative to copies of GAPDH mRNA ($n = 4$ per time point). Error bars indicate SEM.

three times when microglia LPA_1 was blocked with AM095 (Fig. 6*G*,*I–F*; one-way ANOVA; *post hoc* Bonferroni's test, $p = 0.017$; $n = 4$ per group). Similar results were obtained from conditioned medium of LPA-stimulated microglia cells isolated from $maLPA_1$ mice (Fig. 6*H*; two-way ANOVA; *post hoc* Bonferroni's test, $p < 0.05$; $n = 4$ per group). These data indicate that the activation of LPA_1 in microglial cells is responsible, in part, for the cytotoxic effects mediated by LPA-stimulated microglia. Overall, our results suggest that demyelination triggered by intraspinal injection of LPA is mediated, in part, by the activation of microglial LPA_1, with unclear participation of oligodendrocyte LPA signaling *in vivo*.

LPA_1 contributes to SCI pathophysiology

LPA–LPA_1 signaling was assessed for its role in secondary damage and functional impairment after SCI in mice. Because $maLPA_1$ null mice showed locomotor deficits (data not shown), probably attributable to its developmental brain defects (Santin et al., 2009), we used AM095 to assess the role of LPA_1 in SCI. Oral administration of AM095 significantly reduced locomotor impairments based on the BMS assessment (Fig. 7*A*; $p < 0.001$, two-way RM-ANOVA; $n = 8$ per group). At the end of the follow up (28 dpi), the BMS score was improved by 1.5 points after AM095 treatment (Fig. 7*A*). Mice treated with saline showed occasional plantar stepping and no coordination, whereas AM095 showed frequent/consistent plantar stepping and most of them (87.5%) had coordination. In addition, the BMS subscore, which

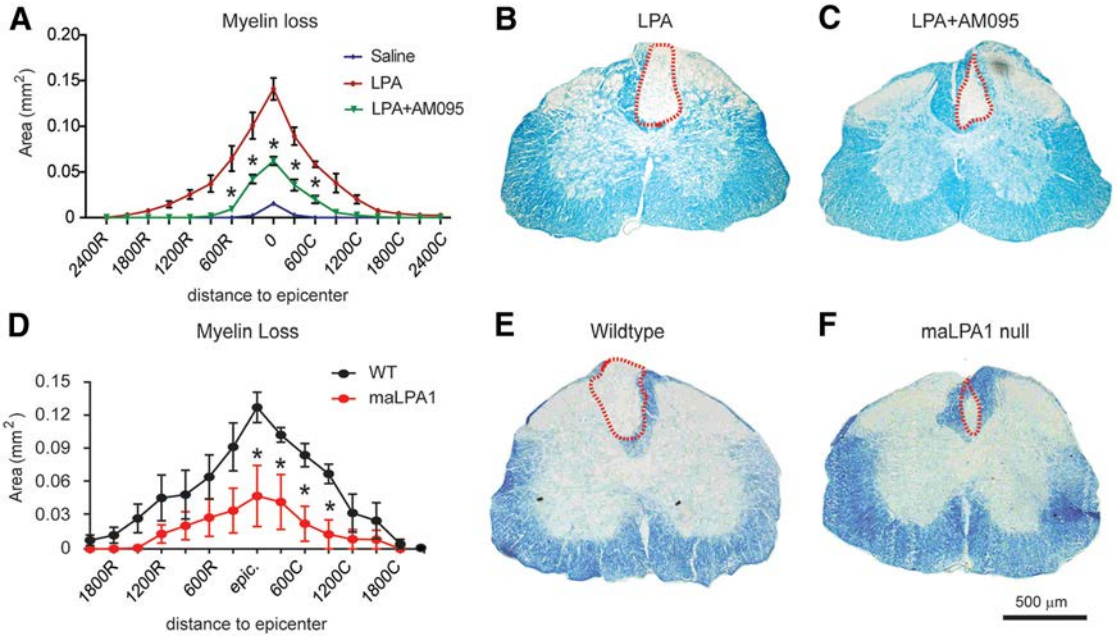


Figure 5. LPA$_1$ signaling mediates demyelination in the spinal cord. ***A***, Quantification of the demyelinating injury in the spinal cord of animals injected with saline, LPA, or LPA1 plus AM095. ***B***, ***C***, Representative images of spinal cords stained with LFB at the epicenter of the injection site in mice administered with LPA (***B***) or LPA plus AM095 (***C***). Note that demyelination was markedly reduced in the absence of LPA$_1$ activity. ***D***, Quantification of the demyelinating injury after intraspinal injection of LPA in wild-type or maLPA$_1$ null mice. ***E***, ***F***, Representative images of spinal cords stained with LFB at the epicenter of the injection site in wild-type (***E***) and maLPA$_1$ null (***F***) mice. Note that demyelination was reduced in the absence of LPA$_1$ ($n = 4$ per group). Scale bar, 500 μm. *$p < 0.05$ saline versus LPA; *$p < 0.05$ wild-type versus maLPA$_1$ null mice. Error bars indicate SEM.

assess fine aspects of locomotion, was markedly improved after LPA$_1$ blockade (Fig. 7*B*; $p < 0.001$, two-way RM-ANOVA; $n = 8$ per group), whereas mice treated with AM095 were able to run at significantly higher speeds on a treadmill (Fig. 7*C*; $p = 0.011$, Mantel–Cox test; $n = 8$ per group).

Electrophysiological tests designed to assess the preservation of descending pathways of the spinal cord based on MEP analysis were used on 28 dpi mice treated with AM095: treated animals showed significantly greater MEP amplitudes for the GM compared with controls ($p = 0.018$, *t* test; $n = 8$ per group), suggesting an increase in spared functional descending tracts in the spinal cord after the lesion (Fig. 7*D*,*E*). Mice administered AM095 were further assessed for amelioration of secondary tissue damage. Histological sections of the spinal cord stained with fluoromyelin revealed that animals treated with the LPA$_1$ antagonist showed less demyelination at the injury epicenter and in adjacent regions (Fig. 7*F–H*; $p < 0.001$, two-way ANOVA; $n = 8$ per group). We also assessed whether the greater myelin sparing observed after AM095 treatment was attributable to reduced axonal loss or reduced demyelination. Spinal cord tissue section at the injury epicenter, the most damaged area of the spinal cord, revealed that AM095 did not enhance the number of NF$^+$ fibers but increased the percentage of axons labeled with MBP (Fig. 7*I–K*; $p = 0.034$, *t* test; $n = 8$ per group). However, AM095 treatment led to only minor improvement in neuronal sparing, seen only at 400 μm caudal to the injury epicenter (Fig. 7*L–N*). These results suggest that the increased levels of LPA in the injured spinal cord contributes to demyelination and loss of function by signaling via LPA$_1$.

We finally assessed whether AM095 attenuated the inflammatory response after SCI. FACS analysis of injured spinal cord harvested at 7 dpi, when microglial cell and macrophages peak in numbers after spinal cord contusion, revealed that AM095 did not reduce microglia counts (Fig. 8). Similarly, AM095 did not attenuate the infiltration of macrophages into the injured spinal cord (Fig. 8). Overall, our data suggest that AM095 confers protection against demyelination by reducing the cytotoxic actions of microglia LPA1 signaling but not its density.

Discussion

The results of the present study provide clear evidence that the LPA–LPA$_1$ pathway contributes to secondary damage after SCI. LPA levels increase in the spinal cord after contusion injury, contributing to activation of microglia/macrophages and demyelination that are, in part, mediated by LPA$_1$ signaling. Moreover, our *in vitro* experiments suggest that detrimental actions of LPA$_1$ involve signaling in microglial cells. Finally, 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.

Lysophospholipids, which include LPA and sphingosine 1-phosphate (S1P), have emerged as key modulators of inflammation, including in nervous system disorders (Chun and Brinkmann, 2011; David et al., 2012c; Choi and Chun, 2013). The contribution of lysophospholipids to CNS pathology includes experiments identifying lysolecithin (also known as lysophosphatidylcholine) as a potent demyelinating and inflammation-inducing agent in the CNS (Ousman and David, 2000, 2001). S1P has been demonstrated to play a key role in multiple sclerosis (Brinkmann et al., 2010) through the actions of fingolimod (FTY720; Chun and Brinkmann, 2011), a nonselective S1P receptor modulator, that was the first worldwide oral treatment for relapsing forms of multiple sclerosis (Brinkmann et al., 2010).

LPA is a lipid signaling molecule capable of evoking multiple physiological responses in a wide variety of cells (Choi et al., 2010;

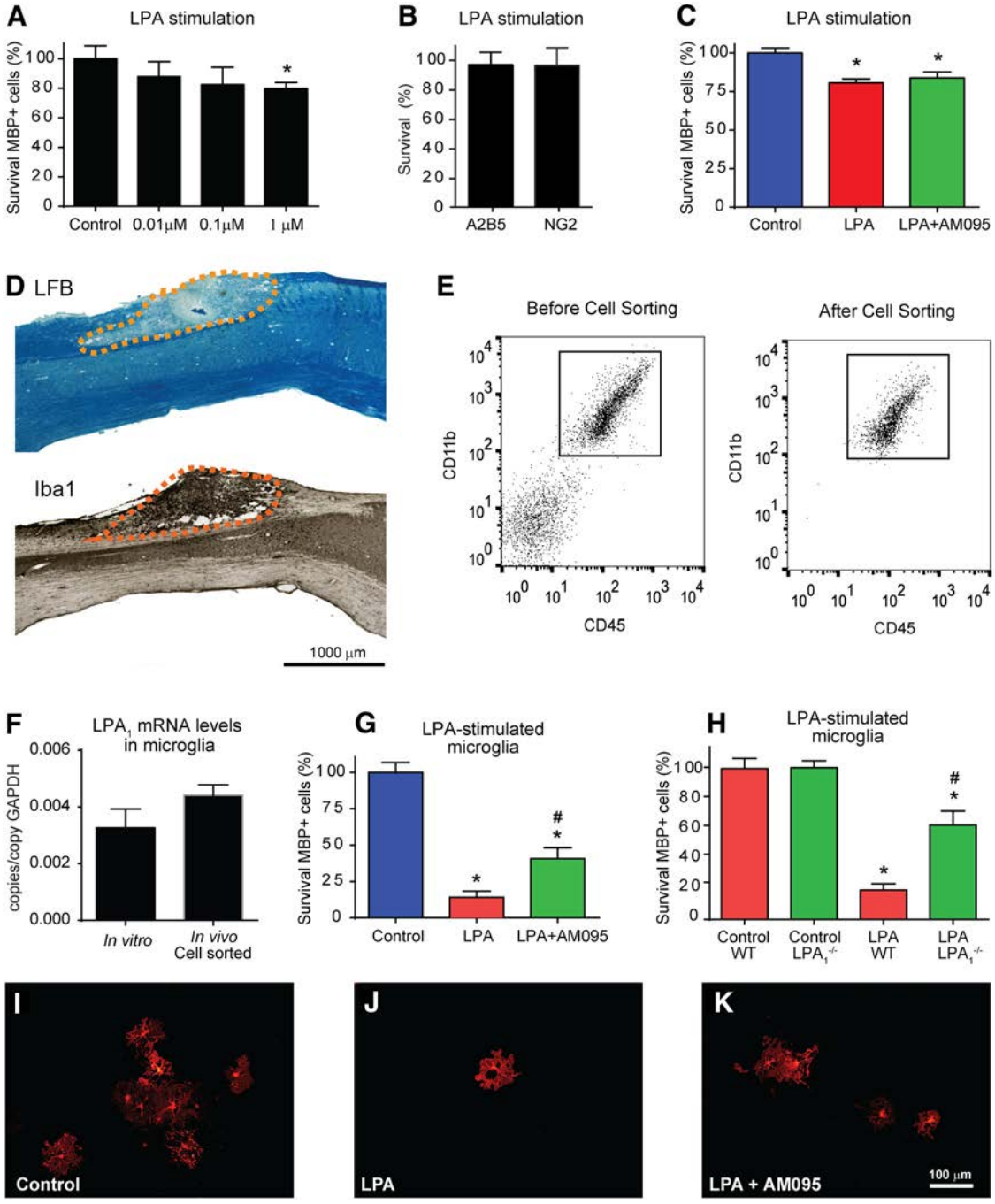


Figure 6. Microglia LPA_1 mediates oligodendrocyte cell death. ***A***, Effects of several concentrations of LPA on oligodendrocyte survival. ***B***, Effects of LPA (1 μM) on OPC survival. ***C***, Quantification of oligodendrocyte cell death after stimulation with LPA or LPA plus AM095. ***D***, Representative spinal cord sections from mice that received intraspinal injection of LPA showing that the demyelinating lesion is associated with areas enriched in microglia. ***E***, Representative FACS analysis of microglia immunopanned with the CD11b antibody from adult CNS before and after being cell sorted. ***F***, Quantification of LPA_1 expression in cultured and cell-sorted microglial cells. Note the microglia show similar levels of LPA_1 in both conditions. ***G***, Assessment of conditioned medium of unstimulated (control) microglia or conditioned medium of microglia stimulated with LPA or LPA plus AM095 on oligodendrocyte survival. ***H***, Assessment of conditioned medium of unstimulated (DMSO; control) or LPA-stimulated microglial cells harvested from wild-type or LPA_1 null mice. ***I***, ***K***, Representative images of oligodendrocytes (MBP^+) from cultures treated with conditioned medium from untreated microglia (DMSO; control) or microglia treated with LPA or LPA plus AM095 stimulated microglia. Note that gene deletion of pharmacological blockade of microglia LPA_1 significantly reduces oligodendrocyte cell death. Scale bar, 100 μm. $^*p < 0.05$ versus control; $^{\#}p < 0.05$ versus LPA or LPA wild type). Error bars indicate SEM ($n = 4$ per group).

Choi and Chun, 2013). LPA is present in low concentrations in mammalian cells and tissues, but its concentrations in the blood can range from 0.1 μM in plasma to >10 μM in serum. LPA is synthesized *in vivo* from membrane phospholipids by multiple pathways (Aoki et al., 2002; Choi et al., 2010; Choi and Chun, 2013). Here, we observed that LPA levels rise rapidly in the spinal cord parenchyma after injury. This is likely attributable to a combination of its *de novo* production in the contused spinal cord, including through phospholipase A_2 enzymes that are upregulated in SCI (Titsworth et al., 2009; López-Vales et al., 2011; David et al., 2012c). However, plasma leakage and blood vessel disruption, as well as serum production during the clotting process, likely contribute to increases in LPA levels in SCI, as has been modeled for post-hemorrhagic hydrocephalus wherein LPA

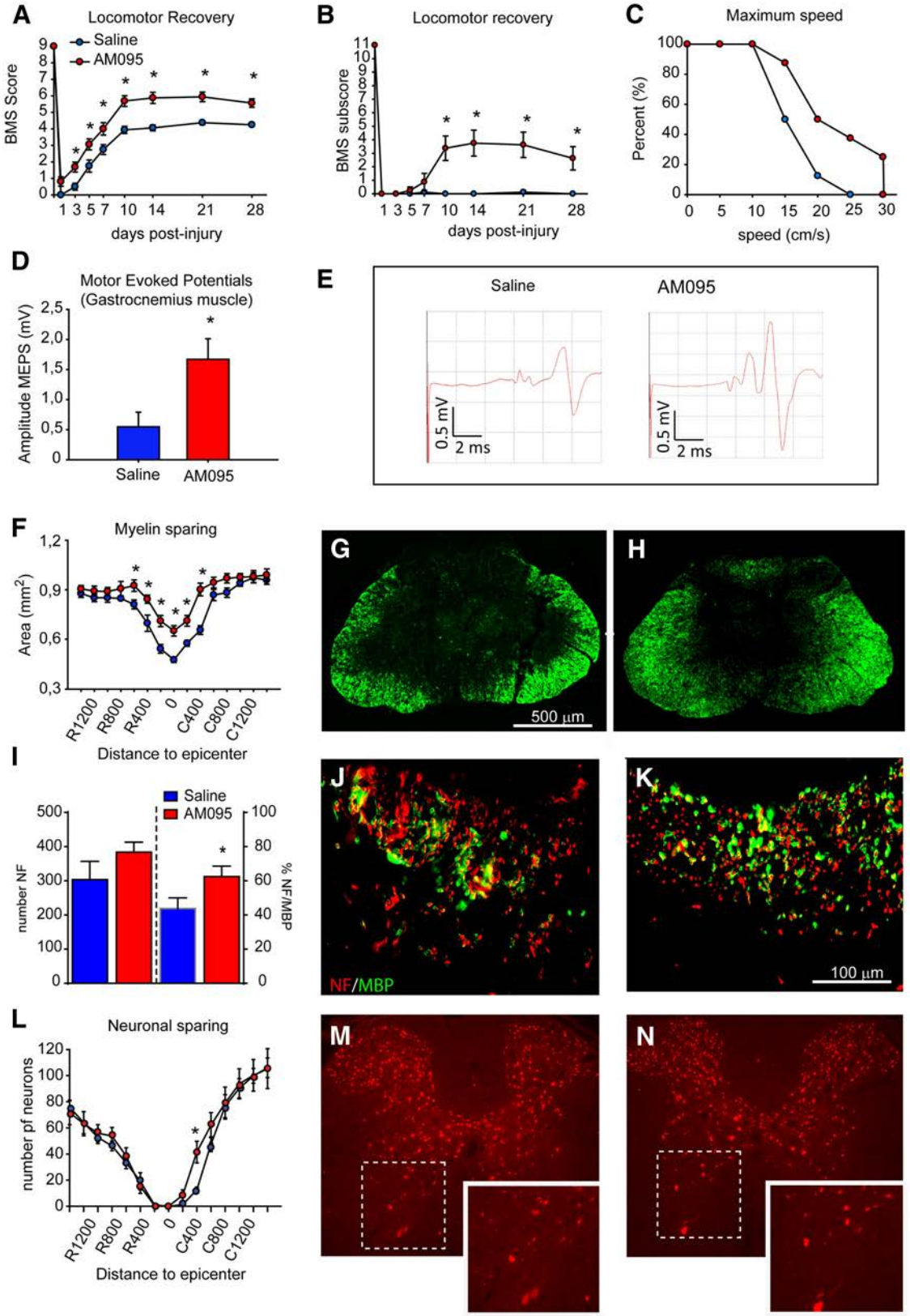


Figure 7. LPA_1 blockade enhances functional outcomes and reduces demyelination after SCI. ***A***, ***B***, Animals treated with AM095 show significant improvement in locomotor performance compared with saline-treated mice using the nine-point BMS (***A***) and the 11-point BMS subscore (***B***), as well as faster locomotion on a treadmill (***C***). ***D***, ***E***, Mice treated with AM095 show greater preservation of MEPs. ***D***, Quantification of MEP recordings from GM at 28 d after SCI. ***E***, 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 with 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. *$p < 0.05$. ***I***, Quantification of axon and myelin sparing in the dorsal column at the epicenter of the injury. Treatment with AM095 resulted in greater myelin but not axonal preservation. ***J***, ***K***, Representative micrographs of the dorsal columns stained against NF and MBP from mice treated with saline (***J***) and AM095 (***K***). ***L***, Quantification of ventral (*Figure legend continues.*)

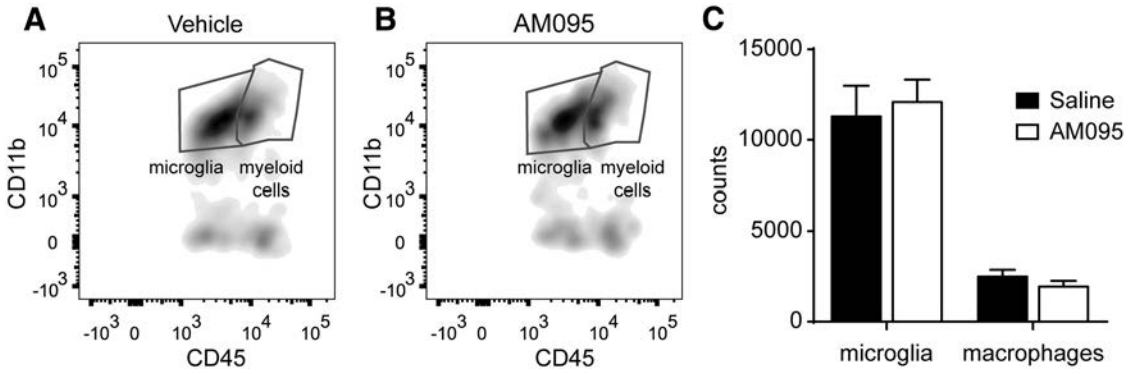


Figure 8. AM095 does not attenuate inflammatory response. ***A***, ***B***, Representative density plots of FACS analysis showing microglia and myeloid cells in the injured spinal cord of saline-treated (***A***) or AM095-treated (***B***) mice at 7 dpi. Myeloid cell were further discriminated in macrophages based on F4/80 expression. ***C***, Graph showing microglia and macrophage counts in the injured spinal cord after LPA_1 blockade. Note that treatment with AM095 did not reduce the numbers of microglia and macrophages. Error bars indicate SEM. $n = 4$ per group.

is an active factor in hemorrhagic fluids (Yung et al., 2011). Indeed, the presence of the 20:4 LPA in the injured spinal cord, which is a major form of LPA in plasma (Scherer et al., 2009) but that was not found in the intact cord, supports hemorrhagic blood fractions as a source of LPA in SCI.

Exogenous LPA can initiate a large range of neural sequelae within the nervous system that includes effects on most cell types and activities in disease models. Cell types include developing neural cells (Dubin et al., 1999, 2010; Kingsbury et al., 2003), astrocytes (Shano et al., 2008; Spohr et al., 2008), microglia (Möller et al., 2001; Ma et al., 2013), oligodendrocytes and Schwann cells (Weiner et al., 1998, 2001), and neurons (Fukushima et al., 2002; Trimbuch et al., 2009; Lin et al., 2012). Disease models include pain (Inoue et al., 2004; Lin et al., 2012; Ma et al., 2013), hypoxia and ischemia (Herr et al., 2011; Halder et al., 2013), demyelination (Inoue et al., 2004, 2008a,b; Nagai et al., 2010), and behavioral disorders (Contos et al., 2000, 2002; Santin et al., 2009; Castilla-Ortega et al., 2012; Pedraza et al., 2014). These physiological responses linked to LPA activation are triggered by a variety of downstream pathways, including Ca^{2+} mobilization, adenylyl cyclase inhibition, activation of Rho, mitogen-activated protein kinase, phospholipase C, and Akt (Yung et al., 2014, 2015; Sheng et al., 2015). The findings here identify LPA as a new trigger of secondary damage in SCI. The mechanism involves LPA_1, which is the mostly highly expressed LPAR in the spinal cord, suggesting that the detrimental actions of LPA are likely to be mediated by binding to LPA_1. The $maLPA_1$ knock-out mice and 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 LPA_1 signaling is involved in the LPA-demyelinating lesion, consistent with previous studies within the peripheral nervous system (Inoue et al., 2004, 2008a,b; Xie et al., 2008; Nagai et al., 2010; Halder et al., 2013). SCI effects appear to involve microglia/macrophages. Although we also noticed mild astrogliosis in the spinal cord after LPA injection, the effects of the LPA were more pronounced in microglia/macrophages, suggesting that they are more susceptible to activation by LPA. Importantly, we also observed that intraspinal injection of LPA led to the development of a demyelinating lesion in the spinal cord. This is of crucial importance because myelin loss contributes to functional impairments after SCI and in other neural conditions, such as multiple sclerosis.

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. Our data indicate that LPA exerts mild toxicity in oligodendrocytes at doses ≥ 1 μM but not in oligodendrocyte precursor cells. The mechanism underlying LPA toxicity is unknown, but this effect is not mediated via LPA_1 because AM095 failed to rescue cell death, at least in cell culture. Hence, LPA-mediated demyelination is likely to be mediated by the activation of LPA_1 in other cell types rather than to direct activation of LPA_1 in oligodendrocytes, consistent with our data showing that intraspinal injection of LPA leads to activation of microglia/macrophages that express LPA_1 *in vivo* and *in vitro*, as we and other authors have demonstrated (Tham et al., 2003). Conditioned medium of microglia stimulated with LPA mediates marked oligodendrocyte cell death, indicating that microglial become cytotoxic during LPA stimulation of LPA_1 signaling. Interestingly, selective blockade or gene deletion of LPA_1 in LPA-stimulated microglial cells appears to increase oligodendrocyte cell survival. This result suggests 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 microglial LPARs may contribute to oligodendrocyte cell death. Similarly, myelin loss was not completely abrogated in the $maLPA_1$ null mice or in mice administered AM095, indicating that other LPARs or other non-LPA-mediated mechanisms also contribute to demyelination. However, LPA_1 blockade does not reduce microglia or macrophage accumulation in SCI, but it likely attenuates the harmful effects linked to LPA_1 activation. The soluble factors released by microglial cells during LPA_1 activation that mediate oligodendrocyte cell death are unidentified yet, but it can be attributed to cytokine and free radical production because LPA regulates the production of these factor in several conditions (Cummings et al., 2004; Fang et al., 2004; Sevastou et al., 2013).

Activation of Rho, which can be induced by LPA, is one of the most important intracellular pathways that inhibit axonal regen-

←

(*Figure legend continued.*) horn neuron survival at various distances rostral and caudal to the injury epicenter reveals significantly greater neuronal survival in mice treated with AM095. ***M***, ***N***, Representative micrographs showing sparing of ventral horn neurons in mice administered with saline (***M***) and AM095 (***N***) in sections stained against NeuN at 600 μm rostral to the injury epicenter. Error bars indicate SEM. $n = 8$ per group.

eration. Previous works revealed that LPA triggers axonal collapse and retraction (Tigyi et al., 1996; Birgbauer and Chun, 2006), in part by activating Rho (Fincher et al., 2014). However, the inhibitory effects of LPA on axonal growth are not triggered by LPA_1, at least *in vitro* (Birgbauer and Chun, 2010).

Reports that a "B3" antibody that can bind to LPA and other lysophospholipids and mediates protection after spinal cord hemisection and traumatic brain injury (Goldshmit et al., 2012; Crack et al., 2014) are consistent with increased LPA in CNS injuries. However, mechanistic validation, including demonstration that B3 specifically lowers pathogenic LPA availability to account for its effects, remain to be established. Nevertheless, possible therapies targeting LPA, its biosynthetic enzymes, and/or its receptors may represent viable strategies for improving clinical outcomes for SCI in the future. In particular, our results suggest that LPA_1 could be a novel therapeutic target for the treatment of acute SCI, as well as other CNS conditions in which demyelination contributes to the pathology.

References

Aoki J, Taira A, Takanezawa Y, Kishi Y, Hama K, Kishimoto T, Mizuno K, Saku K, Taguchi R, Arai H (2002) Serum lysophosphatidic acid is produced through diverse phospholipase pathways. J Biol Chem 277:48737–48744. CrossRef Medline

Basso DM, Fisher LC, Anderson AJ, Jakeman LB, McTigue DM, Popovich PG (2006) Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. J Neurotrauma 23:635–659. CrossRef Medline

Birgbauer E, Chun J (2006) New developments in the biological functions of lysophospholipids. Cell Mol Life Sci 63:2695–2701. CrossRef Medline

Birgbauer E, Chun J (2010) Lysophospholipid receptors LPA1–3 are not required for the inhibitory effects of LPA on mouse retinal growth cones. Eye Brain 2:1–13.

Bot M, Bot I, Lopez-Vales R, van de Lest CH, Saulnier-Blache JS, Helms JB, David S, van Berkel TJ, Biessen EA (2010) Atherosclerotic lesion progression changes lysophosphatidic acid homeostasis to favor its accumulation. Am J Pathol 176:3073–3084. CrossRef Medline

Brinkmann V, Billich A, Baumruker T, Heining P, Schmouder R, Francis G, Aradhye S, Burtin P (2010) Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. Nat Rev Drug Discov 9:883–897. CrossRef Medline

Castilla-Ortega E, Pedraza C, Chun J, de Fonseca FR, Estivill-Torrús G, Santín LJ (2012) Hippocampal c-Fos activation in normal and LPA(1)-null mice after two object recognition tasks with different memory demands. Behav Brain Res 232:400–405. CrossRef Medline

Choi JW, Chun J (2013) Lysophospholipids and their receptors in the central nervous system. Biochim Biophys Acta 1831:20–32. CrossRef Medline

Choi JW, Herr DR, Noguchi K, Yung YC, Lee CW, Mutoh T, Lin ME, Teo ST, Park KE, Mosley AN, Chun J (2010) LPA receptors: subtypes and biological actions. Annu Rev Pharmacol Toxicol 50:157–186. CrossRef Medline

Chun J, Brinkmann V (2011) A mechanistically novel, first oral therapy for multiple sclerosis: the development of fingolimod (FTY720, Gilenya). Discov Med 12:213–228. Medline

Contos JJ, Fukushima N, Weiner JA, Kaushal D, Chun J (2000) Requirement for the lpA1 lysophosphatidic acid receptor gene in normal suckling behavior. Proc Natl Acad Sci U S A 97:13384–13389. CrossRef Medline

Contos JJ, Ishii I, Fukushima N, Kingsbury MA, Ye X, Kawamura S, Brown JH, Chun J (2002) Characterization of lpa(2) (Edg4) and lpa(1)/lpa(2) (Edg2/Edg4) lysophosphatidic acid receptor knockout mice: signaling deficits without obvious phenotypic abnormality attributable to lpa(2). Mol Cell Biol 22:6921–6929. CrossRef Medline

Crack PJ, Zhang M, Morganti-Kossmann MC, Morris AJ, Wojciak JM, Fleming JK, Karve I, Wright D, Sashindranath M, Goldshmit Y, Conquest A, Daglas M, Johnston LA, Medcalf RL, Sabbadini RA, Pebay A (2014) Anti-lysophosphatidic acid antibodies improve traumatic brain injury outcomes. J Neuroinflammation 11:37. CrossRef Medline

Cummings R, Zhao Y, Jacoby D, Spannhake EW, Ohba M, Garcia JG, Watkins T, He D, Saatian B, Natarajan V (2004) Protein kinase Cdelta mediates lysophosphatidic acid-induced NF-kappaB activation and interleukin-8 secretion in human bronchial epithelial cells. J Biol Chem 279:41085–41094. CrossRef Medline

David S, López-Vales R, Wee Yong V (2012a) Harmful and beneficial effects of inflammation after spinal cord injury: potential therapeutic implications. Handb Clin Neurol 109:485–502. CrossRef Medline

David S, Zarruk JG, Ghasemlou N (2012b) Inflammatory pathways in spinal cord injury. Int Rev Neurobiol 106:127–152. CrossRef Medline

David S, Greenhalgh AD, López-Vales R (2012c) Role of phospholipase A2s and lipid mediators in secondary damage after spinal cord injury. Cell Tissue Res 349:249–267. CrossRef Medline

Dubin AE, Bahnson T, Weiner JA, Fukushima N, Chun J (1999) Lysophosphatidic acid stimulates neurotransmitter-like conductance changes that precede GABA and L-glutamate in early, presumptive cortical neuroblasts. J Neurosci 19:1371–1381. Medline

Dubin AE, Herr DR, Chun J (2010) Diversity of lysophosphatidic acid receptor-mediated intracellular calcium signaling in early cortical neurogenesis. J Neurosci 30:7300–7309. CrossRef Medline

Estivill-Torrús G, Llebrez-Zayas P, Matas-Rico E, Santín L, Pedraza C, De Diego I, Del Arco I, Fernández-Llebrez P, Chun J, De Fonseca FR (2008) Absence of LPA1 signaling results in defective cortical development. Cereb Cortex 18:938–950. CrossRef Medline

Fang X, Yu S, Bast RC, Liu S, Xu HJ, Hu SX, LaPushin R, Claret FX, Aggarwal BB, Lu Y, Mills GB (2004) Mechanisms for lysophosphatidic acid-induced cytokine production in ovarian cancer cells. J Biol Chem 279:9653–9661. CrossRef Medline

Fincher J, Whiteneck C, Birgbauer E (2014) G-protein-coupled receptor cell signaling pathways mediating embryonic chick retinal growth cone collapse induced by lysophosphatidic acid and sphingosine-1-phosphate. Dev Neurosci 36:443–453. CrossRef Medline

Frisca F, Sabbadini RA, Goldshmit Y, Pébay A (2012) Biological effects of lysophosphatidic acid in the nervous system. Int Rev Cell Mol Biol 296:273–322. CrossRef Medline

Fukushima N, Weiner JA, Kaushal D, Contos JJ, Rehen SK, Kingsbury MA, Kim KY, Chun J (2002) Lysophosphatidic acid influences the morphology and motility of young, postmitotic cortical neurons. Mol Cell Neurosci 20:271–282. CrossRef Medline

García-Alías G, Verdú E, Forés J, López-Vales R, Navarro X (2003) Functional and electrophysiological characterization of photochemical graded spinal cord injury in the rat. J Neurotrauma 20:501–510. CrossRef Medline

Garcia-Diaz B, Riquelme R, Varela-Nieto I, Jimenez AJ, de Diego I, Gomez-Conde AL, Matas-Rico E, Aguirre JA, Chun J, Pedraza C, Santin LJ, Fernandez O, Rodriguez de Fonseca F, Estivill-Torrus G (2014) Loss of lysophosphatidic acid receptor LPA alters oligodendrocyte differentiation and myelination in the mouse cerebral cortex. Brain Struct Funct. Advance online publication. Retrieved June 14, 2015. CrossRef Medline

Goldshmit Y, Matteo R, Sztal T, Ellett F, Frisca F, Moreno K, Crombie D, Lieschke GJ, Currie PD, Sabbadini RA, Pébay A (2012) Blockage of lysophosphatidic acid signaling improves spinal cord injury outcomes. Am J Pathol 181:978–992. CrossRef Medline

Halder SK, Yano R, Chun J, Ueda H (2013) Involvement of LPA1 receptor signaling in cerebral ischemia-induced neuropathic pain. Neuroscience 235:10–15. CrossRef Medline

Herr KJ, Herr DR, Lee CW, Noguchi K, Chun J (2011) Stereotyped fetal brain disorganization is induced by hypoxia and requires lysophosphatidic acid receptor 1 (LPA1) signaling. Proc Natl Acad Sci U S A 108:15444–15449. CrossRef Medline

Inoue M, Rashid MH, Fujita R, Contos JJ, Chun J, Ueda H (2004) Initiation of neuropathic pain requires lysophosphatidic acid receptor signaling. Nat Med 10:712–718. CrossRef Medline

Inoue M, Ma L, Aoki J, Chun J, Ueda H (2008a) Autotaxin, a synthetic enzyme of lysophosphatidic acid (LPA), mediates the induction of nerve-injured neuropathic pain. Mol Pain 4:6. CrossRef Medline

Inoue M, Xie W, Matsushita Y, Chun J, Aoki J, Ueda H (2008b) Lysophosphatidylcholine induces neuropathic pain through an action of autotaxin to generate lysophosphatidic acid. Neuroscience 152:296–298. CrossRef Medline

Kihara Y, Maceyka M, Spiegel S, Chun J (2014) Lysophospholipid receptor nomenclature review: IUPHAR Review 8. Br J Pharmacol 171:3575–3594. CrossRef Medline

Kingsbury MA, Rehen SK, Contos JJ, Higgins CM, Chun J (2003) Non-

proliferative effects of lysophosphatidic acid enhance cortical growth and folding. Nat Neurosci 6:1292–1299. CrossRef Medline

Klopstein A, Santos-Nogueira E, Francos-Quijorna I, Redensek A, David S, Navarro X, López-Vales R (2012) Beneficial effects of alphaB-crystallin in spinal cord contusion injury. J Neurosci 32:14478–14488. CrossRef Medline

Lin ME, Rivera RR, Chun J (2012) Targeted deletion of LPA5 identifies novel roles for lysophosphatidic acid signaling in development of neuropathic pain. J Biol Chem 287:17608–17617. CrossRef Medline

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408. CrossRef Medline

López-Vales R, Ghasemlou N, Redensek A, Kerr BJ, Barbayianni E, Antonopoulou G, Baskakis C, Rathore KI, Constantinou-Kokotou V, Stephens D, Shimizu T, Dennis EA, Kokotos G, David S (2011) Phospholipase A2 superfamily members play divergent roles after spinal cord injury. FASEB J 25:4240–4252. CrossRef Medline

Ma L, Nagai J, Chun J, Ueda H (2013) An LPA species (18:1 LPA) plays key roles in the self-amplification of spinal LPA production in the peripheral neuropathic pain model. Mol Pain 9:29. CrossRef Medline

Möller T, Contos JJ, Musante DB, Chun J, Ransom BR (2001) Expression and function of lysophosphatidic acid receptors in cultured rodent microglial cells. J Biol Chem 276:25946–25952. CrossRef Medline

Nagai J, Uchida H, Matsushita Y, Yano R, Ueda M, Niwa M, Aoki J, Chun J, Ueda H (2010) Autotaxin and lysophosphatidic acid1 receptor-mediated demyelination of dorsal root fibers by sciatic nerve injury and intrathecal lysophosphatidylcholine. Mol Pain 6:78. CrossRef Medline

O'Meara RW, Ryan SD, Colognato H, Kothary R (2011) Derivation of enriched oligodendrocyte cultures and oligodendrocyte/neuron myelinating co-cultures from post-natal murine tissues. J Vis Exp pii:3324. CrossRef Medline

Ousman SS, David S (2000) Lysophosphatidylcholine induces rapid recruitment and activation of macrophages in the adult mouse spinal cord. Glia 30:92–104. CrossRef Medline

Ousman SS, David S (2001) MIP-1alpha, MCP-1, GM-CSF, and TNF-alpha control the immune cell response that mediates rapid phagocytosis of myelin from the adult mouse spinal cord. J Neurosci 21:4649–4656. Medline

Pedraza C, Sánchez-López J, Castilla-Ortega E, Rosell-Valle C, Zambrana-Infantes E, García-Fernández M, Rodriguez de Fonseca F, Chun J, Santín LJ, Estivill-Torrús G (2014) Fear extinction and acute stress reactivity reveal a role of LPA(1) receptor in regulating emotional-like behaviors. Brain Struct Funct 219:1659–1672. CrossRef Medline

Popovich PG (2014) Neuroimmunology of traumatic spinal cord injury: a brief history and overview. Exp Neurol 258:1–4. CrossRef Medline

Rowland JW, Hawryluk GW, Kwon B, Fehlings MG (2008) Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon. Neurosurg Focus 25:E2. CrossRef Medline

Santin LJ, Bilbao A, Pedraza C, Matas-Rico E, López-Barroso D, Castilla-Ortega E, Sánchez-López J, Riquelme R, Varela-Nieto I, de la Villa P, Suardíaz M, Chun J, De Fonseca FR, Estivill-Torrús G (2009) Behavioral phenotype of maLPA1-null mice: increased anxiety-like behavior and spatial memory deficits. Genes Brain Behav 8:772–784. CrossRef Medline

Saura J, Tusell JM, Serratosa J (2003) High-yield isolation of murine microglia by mild trypsinization. Glia 44:183–189. CrossRef Medline

Scherer M, Schmitz G, Liebisch G (2009) High-throughput analysis of sphingosine 1-phosphate, sphinganine 1-phosphate, and lysophosphatidic acid in plasma samples by liquid chromatography-tandem mass spectrometry. Clin Chem 55:1218–1222. CrossRef Medline

Sevastou I, Kaffe E, Mouratis MA, Aidinis V (2013) Lysoglycerophospholipids in chronic inflammatory disorders: the PLA(2)/LPC and ATX/LPA axes. Biochim Biophys Acta 1831:42–60. CrossRef Medline

Shano S, Moriyama R, Chun J, Fukushima N (2008) Lysophosphatidic acid stimulates astrocyte proliferation through LPA1. Neurochem Int 52:216–220. CrossRef Medline

Sheng X, Yung YC, Chen A, Chun J (2015) Lysophosphatidic acid signalling in development. Development 142:1390–1395. CrossRef Medline

Spohr TC, Choi JW, Gardell SE, Herr DR, Rehen SK, Gomes FC, Chun J (2008) Lysophosphatidic acid receptor-dependent secondary effects via astrocytes promote neuronal differentiation. J Biol Chem 283:7470–7479. CrossRef Medline

Stirling DP, Yong VW (2008) Dynamics of the inflammatory response after murine spinal cord injury revealed by flow cytometry. J Neurosci Res 86:1944–1958. CrossRef Medline

Swaney JS, Chapman C, Correa LD, Stebbins KJ, Broadhead AR, Bain G, Santini AM, Darlington J, King CD, Baccei CS, Lee C, Parr TA, Roppe JR, Seiders TJ, Ziff J, Prasit P, Hutchinson JH, Evans JF, Lorrain DS (2011) Pharmacokinetic and pharmacodynamic characterization of an oral lysophosphatidic acid type 1 receptor-selective antagonist. J Pharmacol Exp Ther 336:693–700. CrossRef Medline

Tham CS, Lin FF, Rao TS, Yu N, Webb M (2003) Microglial activation state and lysophospholipid acid receptor expression. Int J Dev Neurosci 21:431–443. CrossRef Medline

Tigyi G, Fischer DJ, Sebök A, Yang C, Dyer DL, Miledi R (1996) Lysophosphatidic acid-induced neurite retraction in PC12 cells: control by phosphoinositide-Ca2+ signaling and Rho. J Neurochem 66:537–548. Medline

Titsworth WL, Cheng X, Ke Y, Deng L, Burckardt KA, Pendleton C, Liu NK, Shao H, Cao QL, Xu XM (2009) Differential expression of sPLA2 following spinal cord injury and a functional role for sPLA2-IIA in mediating oligodendrocyte death. Glia 57:1521–1537. CrossRef Medline

Trimbuch T, Beed P, Vogt J, Schuchmann S, Maier N, Kintscher M, Breustedt J, Schuelke M, Streu N, Kieselmann O, Brunk I, Laube G, Strauss U, Battefeld A, Wende H, Birchmeier C, Wiese S, Sendtner M, Kawabe H, Kishimoto-Suga M, et al. (2009) Synaptic PRG-1 modulates excitatory transmission via lipid phosphate-mediated signaling. Cell 138:1222–1235. CrossRef Medline

Weiner JA, Hecht JH, Chun J (1998) Lysophosphatidic acid receptor gene vzg-1/lpA1/edg-2 is expressed by mature oligodendrocytes during myelination in the postnatal murine brain. J Comp Neurol 398:587–598. CrossRef Medline

Weiner JA, Fukushima N, Contos JJ, Scherer SS, Chun J (2001) Regulation of Schwann cell morphology and adhesion by receptor-mediated lysophosphatidic acid signaling. J Neurosci 21:7069–7078. Medline

Xie W, Matsumoto M, Chun J, Ueda H (2008) Involvement of LPA1 receptor signaling in the reorganization of spinal input through Abeta-fibers in mice with partial sciatic nerve injury. Mol Pain 4:46. CrossRef Medline

Yung YC, Mutoh T, Lin ME, Noguchi K, Rivera RR, Choi JW, Kingsbury MA, Chun J (2011) Lysophosphatidic acid signaling may initiate fetal hydrocephalus. Sci Transl Med 3:99ra87. CrossRef Medline

Yung YC, Stoddard NC, Chun J (2014) LPA receptor signaling: pharmacology, physiology, and pathophysiology. J Lipid Res 55:1192–1214. CrossRef Medline

Yung YC, Stoddard NC, Mirendil H, Chun J (2015) Lysophosphatidic acid signaling in the nervous system. Neuron 85:669–682. CrossRef Medline