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FACULTAD DE BIOLOGÍA
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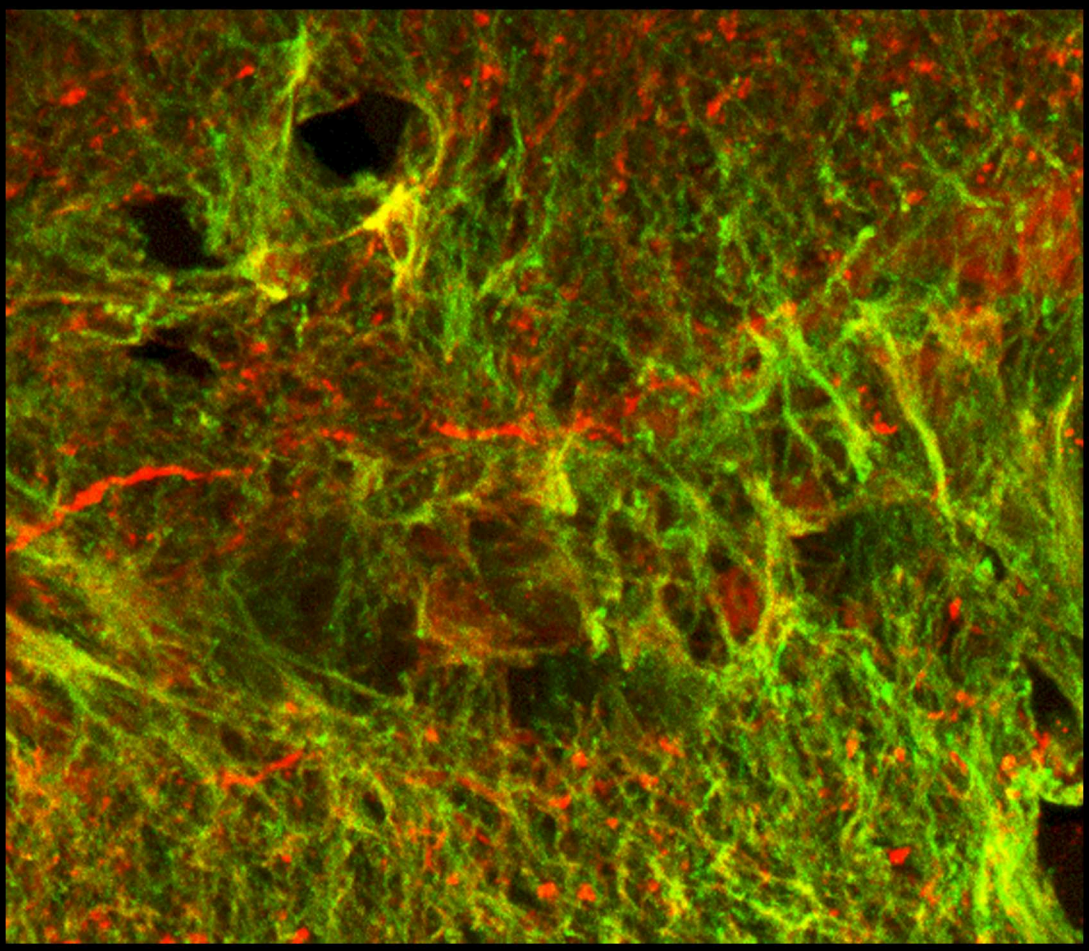
Glutamatergic spinal system in the sea lamprey. Changes after spinal cord injury and during regeneration



Doctoral Thesis

BLANCA FERNÁNDEZ LÓPEZ

Santiago de Compostela, 2014

Universidad de Santiago de Compostela

DEPARTAMENTO DE BIOLOGÍA CELULAR Y ECOLOGÍA

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Glutamatergic spinal system in the sea lamprey. Changes after spinal cord injury and during regeneration

MEMORIA

Que para optar al Grado de Doctor presenta

Blanca Fernández López

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MARÍA CELINA RODICIO RODICIO, CATEDRÁTICA DE BIOLOGÍA CELULAR DEL DEPARTAMENTO DE BIOLOGÍA CELULAR Y ECOLOGÍA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

CERTIFICA:

Que la presente memoria titulada "Glutamatergic spinal system in the sea lamprey. Changes after spinal cord injury and during regeneration ", (Sistema espinal glutamatérgico de la lamprea de mar. Cambios tras lesión medular y durante la regeneración) que para optar al Grado de Doctor en Biología presenta Dña. BLANCA FERNÁNDEZ LÓPEZ, ha sido realizada bajo su dirección, y considerando que constituye trabajo de TESIS DOCTORAL, autoriza su presentación a la Comisión Académica correspondiente.

Y para que así conste expide el presente certificado en Santiago de Compostela, a 14 de octubre de 2014

La Doctoranda

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Esta Tesis Doctoral forma parte de los Proyectos de Investigación titulados:

- "Estudio inmunohistoquímico de los sistemas aminoacidérgicos durante el desarrollo del sistema nervioso central de la lamprea de mar" del Ministerio de Ciencia y Tecnología (BFU2007-61056/BFI).

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General introduction

GENERAL INTRODUCTION

1. Spinal cord injury

Spinal cord injury (SCI) is a pathology that has been studied since ancient times, as it is revealed by some Egyptian documents and Hippocrates writings where some definitions, descriptions and treatments were outlined. However, the major advances in the study, treatments and rehabilitation therapies of SCI have happened from the Second World War onwards (Rueda and Aguado, 2003). In the last years, spinal injury research has greatly increased but, in spite of the effort of the researchers, an appropriate therapy for SCI does not exist so far.

SCI is defined as a lesion within the spinal cord that results in the disruption of nerve fibre bundles that convey ascending sensory and descending motor information (Kraus et al. 1975; Raineteau and Schwab 2001; Furlan et al., 2014). In mammals, including humans, SCI leads to permanent disability and an irreversible loss of sensitivity and motor function below the lesion site. Damaged axons do not regenerate through the lesion site and do not re-establish the connections with their appropriate targets, whereas death neurons are not replaced by new generated ones. In the pathophysiology of SCI, two phases can be distinguished: primary and secondary injury. The primary injury corresponds with the initial alterations of the tissue and is due to the lesion itself. It is characterised by disruption of the meninges and the blood brain barrier, haemorrhage and massive death of neurons, astrocytes and oligodendrocytes by necrosis. The primary injury spreads and triggers some events, such as recruitment of inflammatory cells which leads to the secondary injury. As a result, the tissue damage reaches distal regions

from the site of injury (Fig. 1). During the secondary injury process, the haemorrhage becomes bigger leading to edema, cellular necrosis and great amounts of neurotransmitters, including glutamate are released. Glutamate is excitotoxic and causes excessive calcium influx inside the cells which leads to neuronal, astrocyte and oligodendrocyte death by apoptosis. This results also in loss of myelin. In addition, astrocytes become reactive and constitute the glial scar which, in long term, results in a physical and chemical barrier that prevents the axonal growth. The inflammatory response leads to the activation and infiltration of neutrophils, macrophages and microglia. All together result in a massive alteration of the nervous tissue (for revision see Lee-Liu et al., 2013).

There are two types of SCI: complete and incomplete. Complete SCI refers to lesions that affect the whole width of the cord leading to a total loss of sensation and motor function below the lesion site (Fig. 2A). In an incomplete injury, the lesion does not spread across the whole spinal cord and some regions result intact (Fig. 2B-D). Thus, these patients maintain some sensation and/or motor function below the lesion site, based on the region of the cord affected. The effect of the lesion depends also on the spinal level injured. Consequently, a higher location of the injury in the spinal cord leads to a greater proportion of the body affected.

The estimated global annual incidence of SCI is 40 to 80 new cases per million population. This supposes between 250,000 – 500,000 people suffering SCI every year around the world (Data from the World Health Organization: http://www.who.int/mediacentre/factsheets/fs384/en/). The incidence of SCI in Western Europe is 16 new cases per million inhabitants per year (Furlan et al., 2014; Lee et al., 2014) whereas in Spain the incidence is estimated in 23.5 (Pérez et al., 2012). This means that about 1,000 people suffer SCI every year in our country. Most of the cases of SCI are due to road accidents and more than half of the lesions occur to young people between 16 – 30 years old (Lee et al., 2014). Apart from the

devastating physical effects that SCIs have on the people, it constitutes a social and economic problem of great magnitude. All these factors make SCI an interesting and really important field of research for the society.

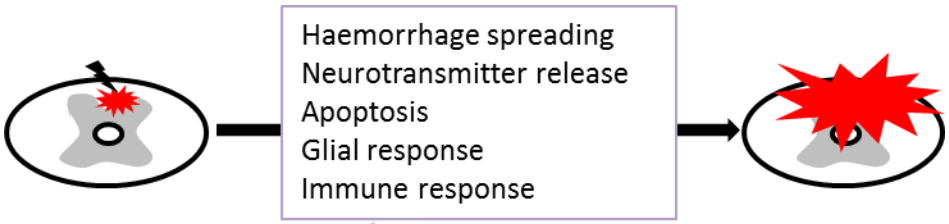

Fig. 1 Effects of secondary injury mechanisms on SCI

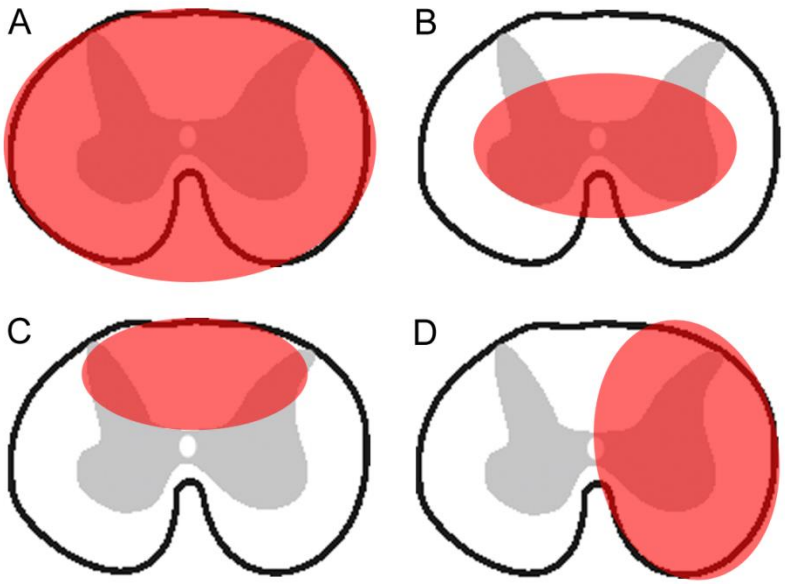

Fig.2 Transverse schemes of mammal spinal cord showing different types of spinal cord injury (red circles).

2. The sea lamprey

Lampreys belong to the cyclostomes or agnathans (jawless vertebrates), the most ancient group of extant vertebrates (Hubbs and Potter, 1971). They are aquatic organisms with elongated, eel-like body which lack paired fins. Instead of jaws they possess a round, sucking mouth with many horny teeth (Nieuwenhuys and Nicholson, 1998). They diverged from the gnathostomes (jawed vertebrates) early in evolution, about 535 – 462 million years, as it has been confirmed by DNA and RNA data (Osório and Rétaux, 2008), before the development of jaws and paired appendages (Shimeld and Donoghue, 2012). This makes lampreys to occupy a key phylogenetic position between urochordates, the immediately related invertebrates, and gnathostomes (Delsuc et al., 2006; Osório and Rétaux, 2008; Shimeld and Donoghue, 2012) (Fig. 3).

There are 38 species of lampreys widely distributed in the temperate regions of both hemispheres (Nieuwenhuys and Nicholson, 1998; Hardisty, 2006) which belong to three different families. One of them includes all the species of the Northern hemisphere (Petromyzontidae) and the other two comprise the species of the Southern hemisphere (Hubbs and Potter, 1971; Gill et al., 2003). Three species of lampreys live in the Iberian Peninsula: *Petromyzon marinus* L. (sea lamprey), *Lampetra fluviatilis* L. (river lamprey, which is only present in Portugal) and *Lampetra planeri* Bloch (brook lamprey). *P. marinus* and *L. fluviatilis* are parasitic and anadromous, with a fresh water larval stage and a salt water post-metamorphic stage, whereas *L. planeri* is a non-parasitic, fresh water restricted lamprey. The species used in this study, *P. marinus,* occurs in the East coast of North America and from Finland to the Mediterranean Sea, reaching Greece, in Europe (Hubbs and Potter, 1971; Doadrio, 2002). In Spain, it is distributed mainly in rivers of the Cantabric coast (especially in Asturias and Galicia) and the Atlantic coast (many Galician rivers, Guadalquivir estuary, Guadalete, Barbate and Guadiana rivers) but also

in the Guadiaro estuary and the Ebro delta, in the Mediterranean coast (Doadrio, 2002).

The life cycle of the sea lamprey is exceptional among vertebrates (Fig. 4). During the breeding season, adult lampreys build their nests by removing stones with their sucking mouths. In the spawning act the male twists the posterior part of the body around the female to fertilize the eggs which are maintained adhered to the river sediment. The diameter of the eggs is around 1 mm and every female can lay about several hundreds of thousands. After spawning the progenitors die (Hardisty and Potter, 1971). The embryonic stage lasts for 10 - 13 days when the hatching takes place. The hatching leads to a prolarval stage which lasts over 20 - 27 days. Prolarvae feed on the yolk and acquire the necessary characteristics to the posterior larval life (Piavis, 1971). Larval lampreys are blind, filter-feeding and live buried in the mud. This period is very long, usually not less than 5 years and may be delayed until 18 years. As they are so different from adults, larval lampreys were initially considered a separate species (*Ammocoetes branchialis*). After this stage, larvae undergo a metamorphosis, in which drastic morphologic and physiologic changes occur, such as acquisition of functional eyes, transformation of the mouth into the sucking oral disc or changes in osmoregulation mechanisms (Hardisty and Potter, 1971; Nieuwenhuys and Nicholson, 1998). Following metamorphosis, the young lampreys migrate downstream to the sea where they remain from 2 – 4 years feeding as parasites on any class of fish. They attach to the bodies of fish with the sucking mouth, rasp the tissue with a rough, tongue-like structure and open a wound through which they suck the inner fluids of the prey (Nieuwenhuys and Nicholson, 1998). At the end of this stage, adult lampreys stop feeding and return to the river. They migrate upstream reaching the final sexual maturation and undergo a number of morphological and physiological changes, such as degeneration of the eyes or atrophy of the intestine (Hardisty and Potter, 1971). The cycle ends with the spawn and death of the adult lampreys.

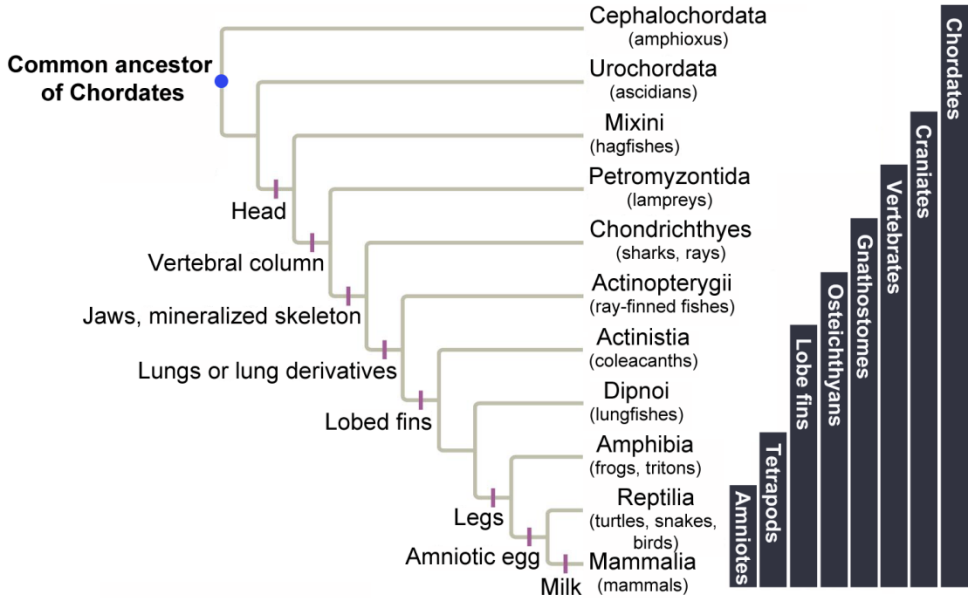

Fig. 3 Vertebrate phylogeny.

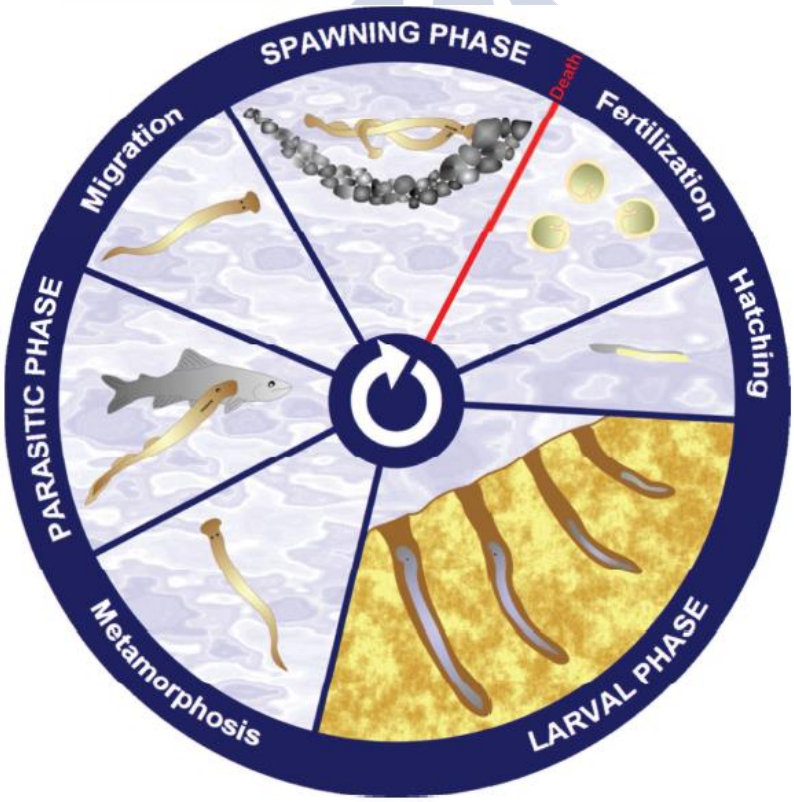

Fig. 4 Life cycle of the sea lamprey.

3. The lamprey as an animal model in Neurobiology and Neuroregeneration

Literature of the neurobiology of lampreys goes back to the middle 1800s when the gross anatomy of the nervous system and the axonal pathways were broadly studied (Reissner, 1860; Mayer, 1897; Johnston, 1902). Sigmund Freud, before he turned in his interests to the psychology, started to study the anatomy of the spinal cord of larval lampreys (Freud, 1877; Freud, 1878). Since then, the cytoarchitecture of the central nervous system (CNS) of lampreys has been well described by comparative neuroanatomists (see Nieuwenhuys and Nicholson, 1998).

The key phylogenetic position of lampreys between urochordates and gnathostomes (Delsuc et al., 2006; Osório and Rétaux, 2008; Shimeld and Donoghue, 2012) makes them strategic species to study the evolution of morphological as well as molecular and genomic characters at the base of the Craniates and throughout their evolution (Shimeld and Donoghue, 2012; for revision see Osório and Rétaux, 2008). Lampreys are, therefore, truly important in evolutionary and developmental studies (*evo-devo*). Specifically, they can help to clarify the origin and evolution of the brain (Murakami and Watanabe, 2009) as they share all the basic features of the CNS with the rest of vertebrates. During the last years, the whole genome of the sea lamprey has been sequenced and published (http://www.ensembl.org) so that the evolutionary studies are being conducted from a genomic point of view (Smith et al., 2013).

The isolated brainstem-spinal cord of lampreys can be maintained *in vitro* for several days. This, together with the fact that lampreys have much fewer neurons than other vertebrates, has allowed a detailed study and characterization of the circuits that underlie locomotion (for revision see Dubuc et al., 2008; see also Grillner et al., 2008). The motor pattern underlying locomotion can be elicited by electric stimulation of the diencephalic and mesencephalic locomotor regions (DLR and MLR,

respectively) or drug stimulation in the isolated spinal cord. The motor activity can be monitored by ventral root recordings. A number of studies have focused on the deciphering of the lamprey locomotor network (Rovainen, 1979; Buchanan et al., 1989; Dubuc and Grillner, 1989; Buchanan, 1999; Grillner, 2003). Briefly, the descending reticulospinal cells (RS), which receive inputs from the DLR and MLR, excite all classes of spinal interneurons and motor neurons. The excitatory intraspinal interneurons excite all types of spinal neurons, including the inhibitory interneurons that cross the midline to inhibit all interneuron types on the contralateral side and motor neurons, producing the alternating pattern (for revision see Grillner and Wallén, 2007). The activity of the RS descending system is modulated by feedback signals from the spinobulbar ascending system about the state of the locomotor networks and about sensory information produced during the movement, as well as by vestibular, optic, trigeminal, lateral line and olfactory nerves information (Rovainen, 1978; Vinay and Grillner, 1993; Buchanan and Einum, 2008).

In contrast to mammals, lampreys completely recover the locomotor function after a complete transection of the spinal cord (Rovainen, 1976; Selzer, 1979). From the beginning of the 1960s, the lamprey has been used as an animal model of spinal cord regeneration (Maron, 1959; Hibbard, 1963). In 1980, the National Institute of Health of the United States (NIH), proposed five criteria that should be achieved to demonstrate functional regeneration in the spinal cord (Guth et al., 1980). The lamprey is the unique vertebrate that meets all these five criteria, which are: (1) The experimental lesion must cause disconnection of nerve processes, (2) Processes of CNS neurons must bridge the level of injury, (3) The regenerated fibres must make junctional contacts, (4) The regenerated fibres must generate post-junctional responses and (5) Changes in function must derive from regenerated connections (for revision see Shifman et al., 2007).

Studies of the regeneration of the giant RS axons following a complete transection of the cord have suggested that the regenerating neurites suffer some changes, such as split into several branches or being located in abnormal positions in the cord. However, the larvae show normal swimming and crawling behaviour when the regenerating axons have not reach their normal length (Rovainen, 1976; Selzer, 1978). In fact, stimulation of the spinal cord rostral to the site of injury have led to polysynaptic but not monosynaptic, as it occurs in normal animals, activation of neurons caudal to the lesion (Selzer, 1978). Despite of this, true axonal pathway and synaptic specificity has been demonstrated (Yin and Selzer, 1983; Yin et al., 1984; Mackler and Selzer, 1987). However, only about the 50% of all the descending axons regenerate through the scar (Davis and McClellan, 1994; Jacobs et al., 1997). These findings indicate that plasticity mechanisms are involved in the restoration of the spinal circuits in lampreys. This supports the use of the lamprey as a suitable model for spinal cord regeneration, as the plasticity processes are very important in vertebrates with limited regeneration capacity, such as mammals. On the other hand, as not all the axons have the same capacity to regenerate (see above), the lamprey becomes a unique model to study the intrinsic molecular mechanisms that underlie the ability of the neurons to regenerate in the same permissive environment after SCI (Shifman and Selzer, 2000, 2007; Jin et al., 2009; Shifman et al., 2009).

Recently, lampreys have been proposed as a model to study the evolution from a regenerative point of view (*evo-rego*) (Barreiro-Iglesias, 2012). They are also used as a model to study the cytotoxic effects of proteins related to Alzheimer's disease on neurons (Hall, 1999; Hall et al., 2000; Honson et al., 2009; Lee et al., 2012).

4. Organization of the lamprey spinal cord

The spinal cord of the lamprey is flattened and transparent, lacks myelin and the blood vessels do not enter its parenchyma. The grey matter forms paired horns that extend laterally. The medial region corresponds to the dorsal horns of jawed vertebrates in terms of embryonic origin and the lateral region corresponds to the ventral horns (see Meléndez-Ferro et al., 2003). The white matter surrounds the grey matter and comprises a number of longitudinally running fibres. Most of the spinal neurons are situated in the grey matter although there are also some somas in the white matter (Fig. 5). There are dorsal and ventral roots which do not emerge at the same transverse levels and do not join to form mixed spinal nerves (Nieuwenhuys and Nicholson, 1998).

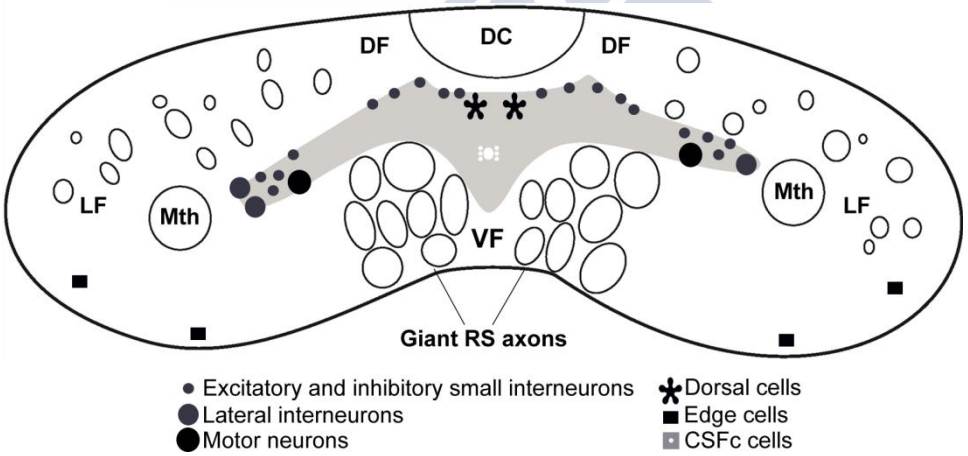

Fig. 5 Scheme of a transverse section of the rostral spinal cord of a lamprey. CSFc: Cerebrospinal fluid contacting cells; DC: Dorsal column; DF: Dorsal fascicle; LF: Lateral fascicle; Mth: Mauthner axon; RS: Reticulospinal; VF: ventral fascicle.

There are different neuron types within the spinal cord, such as motor neurons, dorsal cells and several kinds of intrinsic neurons (Fig. 5). The motor neurons constitute a ventrolaterally situated column on either side of the cord. Separate motor neurons innervate the slow, parietal muscle fibres, the twitch muscle fibres (Teräväinen and Rovainen, 1971) and the dorsal fins (Rovainen and Birnberger, 1971; for revision see Nieuwenhuys and Nicholson, 1998). Dorsal cells form two distinct longitudinal rows on either side of the dorsomedial part of the spinal grey. They are primary sensory bipolar cells with a long rostral projection and a short caudal projection which travel in the ipsilateral dorsal column (Rovainen, 1967a; Tang and Selzer, 1979) carrying information of touch and pressure (Christenson et al., 1988). In addition to these central processes, the dorsal cells have a peripheral process that leaves the spinal cord via a dorsal root, ending as a mechanoreceptor in the skin (Martin and Wickelgren, 1971). Intrinsic neurons are called in that way because their processes never leave the CNS. They are abundant in the grey matter of lampreys and heterogeneous in morphology and size. During the last decades, a number of studies have been focused on the characterization of these cells which have been classified by their morphology, projections and synaptic interactions in giant interneurons, lateral interneurons, edge cells, excitatory interneurons (EINs), crossed caudal interneurons (CCINs), small ipsilateral inhibitory interneurons (IINs) and cerebrospinal fluid-contacting cells (CSFc), among others (Rovainen, 1967a; Tang and Selzer, 1979; Buchanan, 1982; Buchanan and Cohen, 1982; Grillner et al, 1984; Buchanan and Grillner, 1987, 1988; Buchanan et al., 1989; Viana di Prisco et al., 1990; Ohta et al., 1991; Buchanan, 1999; Einum and Buchanan, 2006). Briefly, giant interneurons are second order sensory cells with contralateral rostral-projecting axons. They are only present in the caudal third of the cord. Lateral interneurons are large inhibitory neurons with ipsilateral descending axons. They inhibit the CCINs, allowing the opposite side of the cord to become active. The edge cells are situated in the lateral fascicle of the white matter. They are stretch

receptor neurons of two types: excitatory edge cells which excites ipsilateral neurons and inhibitory edge cells which cross the midline to inhibit contralateral neurons. EINs, CCINs and IINs are interneurons that participate in the lamprey locomotor network of the spinal cord. The EINs are related to the rhythm generation and the IINs to the left-right alternating locomotor pattern. During the excitatory phase in one side of the cord, the CCINs maintain inhibited all the neurons in the contralateral side. CSFc cells surround the central canal and have a lateral-extending process that reaches lateral edge forming a marginal plexus and making contacts with the dendrites of the edge cells (Christenson et al., 1991) and a process that protrude into the central canal. There are different types of spinal interneurons according to the neurotransmitter that they use: GABAergic (Meléndez-Ferro et al., 2003), dopaminergic (Abalo et al., 2005), cholinergic (Pombal et al., 2001) or serotonergic (Harris-Warrick et al., 1985).

The spinal white matter can be subdivided in dorsal, lateral and ventral fascicles, which are separated by the dorsal and ventral nerve roots. Ascending and descending fibre tracts run in these fascicles.

There are two descending systems to the spinal cord in lampreys, the reticulospinal (RS) and the vestibulospinal (VS). The RS neurons originate from the diencephalic nucleus of the longitudinal medial fascicle and the first and second pairs of the Müller cells, which are giant identifiable RS neurons, the mesencephalic reticular nucleus (MRN) and the three rhombencephalic reticular nuclei (anterior, middle and posterior rhombencephalic reticular nuclei or ARRN, MRRN and PRRN, respectively). In the reticular mesencephalic and rhombencephalic nuclei there are also a fewMüller cells.The Müller cells give rise to the giant RS axons which descend ipsilaterally in the ventral and also in the lateral fascicles (Fig. 5). The lateral fascicle also contains two RS axons from the identified Mauthner cells, which descend in the contralateral side of the cord (Fig. 5). The VS system is constituted by a number of medium-sized axons that

originate from the intermediate and posterior octavomotor nuclei and project only to the rostral spinal cord (revision in Nieuwenhuys and Nicholson, 1998). There are two large ascending systems, the dorsal column (DC) and the spinal lemniscus. The DC contains axons from primary afferents as well as from the dorsal cells. DC axons ascend in the dorsal fascicle carrying sensory information from the skin to the brainstem (Dubuc et al., 1993a). Spinal lemniscus fibres ascend in the lateral fascicle and convey tactile, pain, temperature stimuli to the brain and information about the motor pattern to the brainstem. Spinal lemniscus fibres innervate the octavolateral area, the optic tectum, and even the dorsal thalamus (Nieuwenhuys and Nicholson, 1998).

Regarding the glia two types of glial cells are present in the spinal cord of lampreys, astrocyte-like and ependymoglia (Fig. 6). Astrocyte-like cells are located in the narrow strip of spinal grey matter extending their processes radially to the dorsal and ventral surfaces of the cord. The ependymoglia is situated around the central canal and have a short central process and a long peripheral process (Nieuwenhuys and Nicholson, 1998).

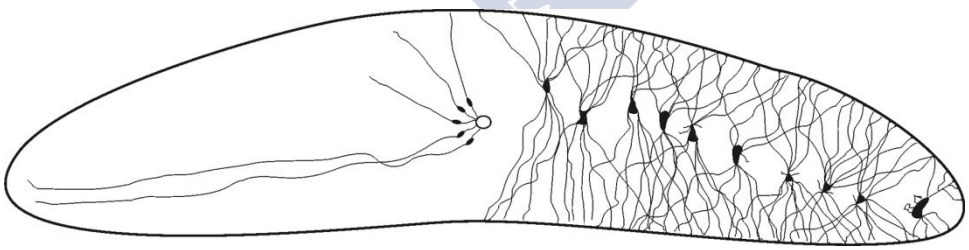

Fig. 6 Draw of the glial elements in the spinal cord of the lamprey showing astrocyte-like cells (right side) and ependymoglia (left side).

5. Glutamate in central nervous system health and disease

Glutamate (Fig. 7) is the major excitatory neurotransmitter in the vertebrate CNS. It has a role in most aspects of normal brain function including cognition and long-term neuronal potentiation, the molecular substrate for memory and learning (for revision see Fonnum, 1984; Ottersen and Storm-Mathisen, 1984a; Meldrum, 2000; Tapiero et al., 2002). Glutamate is also involved in the development of the CNS, participating in synapse formation and elimination, and in cell migration, differentiation and death (McDonald and Johnston, 1990; Rabacchi et al., 1992; Komuro and Rakic, 1993; Quinlan et al., 1999). It plays also a signalling role in peripheral organs and tissues and in endocrine cells (for revision see Moriyama et al., 2000).

Glutamate exerts its signalling role by acting on three different families of glutamate receptors, which are classified accordingly to their structure, pharmacology or intracellular activation cascade. Two of these families (NMDA- and AMPA/Kainate-receptors) constitute the ionotropic glutamate receptors, which are glutamate gated ion channels conducting Na^+ or Na^+ and Ca^{2+} ions. The third family comprises the metabotropic receptors (mGluR1-8), which are G-protein coupled receptors and are subdivided into group I (mGluR1 and 5), group II (mGluR2 and 3) and group III (mGluR4, 6, 7 and 8). Group I are coupled to phospholipase C, therefore, to diacylglycerol and inositol triphosphate whereas groups II and III are negatively coupled to adenylate cyclase (Danbolt, 2001). It is of vital importance that the extracellular glutamate concentration remains low. This is required for a good signal-to-noise ratio in synaptic transmission. Furthermore, a massive activation of glutamate receptors due to an excessive extracellular concentration is harmful for the CNS. Accordingly, glutamate removing mechanisms are truly important for the correct CNS function. Glutamate transporters carry out the glutamate uptake by using the electrochemical gradients across the plasma membranes as driving forces for uptake. Five different glutamate

transporters have been cloned so far (common for glutamate and aspartate): glutamate-aspartate transporter (GLAST) (Storck et al., 1992), glutamate transporter (GLT) (Pines et al., 1992), excitatory amino acid carrier (EAAC) (Kanai and Hediger, 1992), excitatory amino acid transporter (EAAT4) (Fairman et al., 1995) and EAAT5 (Arriza et al., 1997) (in humans these are referred to as EAAT1-5, respectively). Most of the transporters (GLAST, GLT and some EAAC) are located on astrocytes or on neuronal membranes facing astrocytes (EAAT4). Astrocytes play a major role in glutamate removal, as GLT and GLAST are responsible of most of the glutamate uptake and they are almost uniquely expressed by astrocytes (for revision see Danbolt, 2001). In the presynaptic terminals, the vesicular glutamate transporters (VGLUTs) take up glutamate into synaptic vesicles to use it as neurotransmitter. Three VGLUTs have been identified in mammals to date (VGLUT1, VGLUT2 and VGLUT3) (Herzog, et al., 2004).

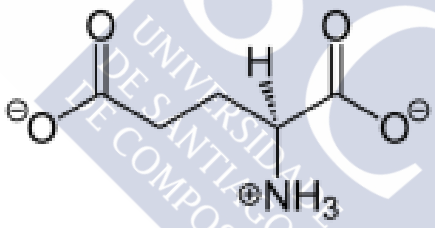

Fig. 7 L-Glutamate at physiological conditions

Once glutamate is released for neurotransmission, it is taken up by cells to reuse it. The major pathway that has been proposed to recycle glutamate is known as the glutamate-glutamine cycle (Fig. 8). In presynaptic terminals, glutamate is carried into synaptic vesicles by a VGLUT and is subsequently released by exocytosis to the synaptic cleft. Glutamate binds postsynaptic and presynaptic receptors and is taken up by glutamate transporters (EAATs) present postsynaptically, presynaptically, and extrasynaptically in astroglial cells. In astrocytes,

glutamate is transformed into glutamine, which is released to the extracellular fluid. Then, glutamine is taken up by neurons and reconverted to glutamate inside them. Apart from glutamine, glutamate can be synthetized from a-ketoglutarate through deamination by the glutamate dehydrogenase in astrocytes. Glutamate dehydrogenase has been also detected in presynaptic terminals (McKenna et al., 2000). Thus, glutamatergic neurons might not be as dependent on astrocytes for glutamate precursors supply as previously thought (revision in Danbolt, 2001).

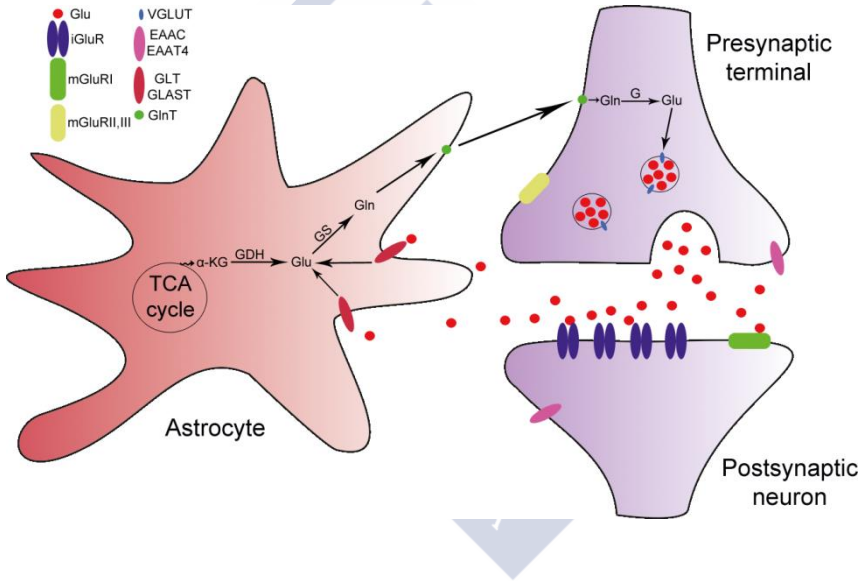

Fig. 8 Schematic drawing of a glutamatergic synapse showing the main structures involved in glutamate neurotransmission. α-KG: a-ketoglutarate; EAAC: excitatory amino acid carrier; EAAT: excitatory amino acid transporter; iGluR: ionotropic glutamate receptors; G: glutaminase; GDH: glutamate dehydrogenase; GLAST: glutamate-aspartate transporter; Gln: glutamine. GlnT: glutamine transporter; GLT: glutamate transporter; Glu: glutamate; GS: glutamine synthetase; mGluRI: group I metabotropic glutamate receptors; mGluRII, III: group II, III metabotropic glutamate receptors; TCA: tricarboxylic acid.

Glutamatergic systems, including metabolism, release mechanisms, receptors and transporters are implicated in nearly all aspects of normal brain function, development and ageing as well as in most neurological diseases. Because of its ubiquitous presence and the importance of its actions, it is necessary that glutamate is at the correct concentration, in the correct place and at the correct time. It has been observed that the excessive release of excitatory neurotransmitters results in neuronal damage. If the glutamate transporters do not work correctly for any reason (low expression, stop working, reverse releasing of glutamate from the cytoplasm) there is an increase in the extracellular glutamate concentration. This results in an over-stimulation of the glutamate receptors that leads to an increase in the Na^+ and Ca^{2+} influx, energy consumption, free radical production and, consequently, to neuronal death. A defect in the glutamate uptake has been suggested to participate in numerous neurological disorders (for revision see Meldrum, 2000; Danbolt, 2001; Platt, 2007), such as:

(1) Ischemia: massive release of glutamate with low expression of GLT has been observed after ischemia (Torp et al., 1995; Martin et al., 1997; Rao et al., 2000).

(2) Traumatic brain and spinal cord injury: glutamate release after CNS injury in mammals is mainly due to reversed uptake (Szatkowski et al., 1990; McAdoo et al., 2000; Rossi et al., 2000), although down-regulation of GLT1 expression has been also reported (Lepore et al., 2011).

(3) Amyotrophic lateral sclerosis (ALS): over-stimulation of the glutamate receptors might be important in the pathophysiology of motor neuron disease and ALS via: (a) increased glutamate levels (due to reduce uptake) (Rothstein et al., 1992, 1995), (b) increased sensitivity to glutamate (due to changed glutamate receptors, defective energy production or radical free defence) (Takuma et al., 1999) or (c) intake of excitotoxins (Spencer et al., 1986, 1987; Spencer, 1999).

(4) Alzheimer's disease: Free radicals and lipid peroxidation products generated by β-amyloid peptide inhibit glutamate transporters (Harris et al., 1995; Trotti et al., 1996; Blanc et al., 1998; Pedersen et al., 1999) which involve excessive glutamatergic transmission in the pathogeny of Alzheimer's disease.

(5) Epilepsy: Excessive activation of glutamate receptors plays a major role in the initiation and spread of seizure activity (Meldrum, 1994; Chapman, 2000) and changes in NMDA and AMPA receptors have been reported in human epilepsy (de Lanerolle et al., 1998; Musshoff et al., 2000).

(6) Hepatic encephalopathy: The ammonia toxicity could be responsible of the reduction in glutamate uptake (Blei and Larsen, 1999).

On the other hand, hypoactivation of the glutamate receptors is also harmful, especially during development, and it has been related to developmental abnormalities and to schizophrenic symptoms (Carlsson et al., 1997; Carpenter, 1999; Bressan and Pilowsky, 2000). Understanding the role of glutamate in these neurological diseases will lead to a better knowledge of clinical conditions and, likely, to improved treatments.

6. The glutamatergic system in lampreys

Despite the importance of glutamate in the CNS, the distribution of the glutamatergic populations in the brain and the spinal cord of most vertebrates is still unknown. The generation of antibodies raised against glutamate has allowed the study of the cellular distribution of glutamate in the rat brain by immunohistochemistry (Ottersen and Storm-Mathisen, 1984a, b). However, this method has been considered suspicious since glutamate is a metabolic molecule present in all the cells (Walberg et al.,

1990). Because of that, some authors have considered that there were no reliable markers for glutamatergic cells until recently, with the cloning of the VGLUTs. Recent studies in mammals and zebrafish have used *in situ* hybridization or antibodies against these specific markers increasing the knowledge of the anatomic distribution of the glutamatergic cells and fibres (rat: Oliveira et al., 2003; Landry et al., 2004; Persson et al., 2006; zebrafish: Higashijima et al., 2004; mouse: Brumovsky et al., 2007).

In the sea lamprey, immunohistochemical studies have described glutamate immunoreactive (-ir) neurons in the retina (Villar-Cerviño et al., 2006) and in the dorsal column nucleus (Rodicio et al., 2005). Villar-Cerviño et al., (2010), characterized the lamprey VGLUT providing a new tool to describe the glutamatergic neurons in this animal model. Thus, comprehensive studies of the glutamatergic populations in the forebrain and brainstem of the sea lamprey have been published recently (Villar-Cerviño et al., 2011, 2013). Moreover, these studies have revealed that glutamate immunohistochemistry constitutes a reliable method to study the glutamatergic neurons in the sea lamprey, as the results obtained with *in situ* hybridization for the VGLUT and glutamate immunohistochemistry have been almost the same (Villar-Cerviño et al., 2011, 2013). Glutamatergic neurons are widely distributed in the lamprey brain. In the forebrain of lampreys, VGLUT expression has been found in the olfactory bulb, pallium, septum, subhippocampal lobe, preoptic region, thalamic eminence, prethalamus, thalamus, epithalamus, pretectum, hypothalamus, posterior tubercle, and nucleus of the medial longitudinal fascicle (Villar –Cerviño et al., 2011). In the brainstem, glutamatergic neurons have been observed in the optic tectum, torus semicircularis, isthmus, dorsal and medial nuclei of the octavolateral area, dorsal column nucleus, solitary tract nucleus, motoneurons, and reticular formation, among other regions (Villar-Cerviño et al., 2013).

Before these anatomical studies, most of the works that characterized the excitatory (glutamatergic) transmission in some

identified cells of the CNS of lampreys were physiological (Rovainen, 1967b; Wickelgren, 1977; Matthews and Wickelgren, 1979; Homma, 1985; Buchanan and Grillner, 1987; Buchanan et al., 1987; Dryer, 1988; Brodin et al., 1989, 1994; Buchanan et al., 1989; Dubuc et al., 1993b) and showed the implication of glutamate in a number of brainstem and spinal cord circuits (see sections 3 and 4).

Regarding the spinal cord, apart from the physiological studies (see above), the distribution of the glutamatergic cells has been studied only with glutamate immunohistochemistry in synapses at ultrastructural level (Shupliakov et al., 1992) and in some commissural neurons (Mahmood et al., 2009). Therefore, in spite of the importance of glutamate in locomotor circuits and in the response to a CNS injury, together with the importance of the sea lamprey as an animal model for neuroregeneration studies, there is an almost complete lack of knowledge about the anatomical distribution of glutamatergic neurons in the spinal cord of lampreys, as well as of their changes after SCI and during the regeneration. Further, no report has been published in non-mammalian adult vertebrates of the expression of VGLUTs in the spinal cord.

7. Introduction to the methods used in this project

Animals

Large, developmentally stable larvae (80 – 160 mm in body length), downstream migrating young adult and upstream migrating adult sea lampreys, *Petromyzon marinus,* were used in the present study. Larvae and young adult lampreys were collected from the river Ulla (Galicia, NW, Spain) with permission from the Xunta of Galicia. Upstream migrating

adults were bought to a local supplier. Larvae were maintained in aerated fresh water aquaria whereas adults were used immediately after arrival to the laboratory.

All experiments were approved by the Bioethics Committee at the University of Santiago de Compostela and conformed to the European Union and the Spanish guidelines on animal care and experimentation to minimize the pain and suffering.

In situ hybridization

Digoxygenin-labelled riboprobes were synthesized using the amplified fragments of the cloned sequences (Villar-Cerviño et al., 2010) as templates, following standard protocols. *In situ* hybridizations on cryostat sections (14 µm thick) were conducted using standard protocols, with an RNAse A treatment (0.2 mg/ml, 37uC during 30 minutes) that was added to the post-hybridization washings. Staining was conducted in BM Purple (Roche, Mannheim, Germany) at 37°C until the signal was clearly visible. For more details see Material and Methods section of chapter 1.

Immunofluorescence

Immunofluorescence experiments were conducted to characterize glutamate, glycine, γ-aminobutyric acid (GABA), cytokeratins and/or Hu protein immunoreactive (-ir) cells and processes. Sections were pre-treated with 0.2% NaBH4 in deionized water for 45 minutes and incubated with a mixture of a rabbit polyclonal anti-glutamate antibody (Immunosolution, Jesmond, Australia; 1:4,500) and a mouse monoclonal anti-GABA antibody (Sigma, St. Louis, MO; 1:1,200) or a mouse monoclonal anti-cytokeratin antibody (supplied by Dr. Selzer, Philadelphia; 1:100) or a mouse monoclonal anti-HuC/HuD antibody

(Molecular Probes Europe, Leiden, The Netherlands; 1:100) or with a mixture of a rabbit polyclonal anti-glycine antibody (Immunosolution; 1:3,000) and a mouse monoclonal anti-glutamate antibody (Swant, Bellinzona, Switzerland; 1:1,000). For indirect immunofluorescence detection of each molecule, Cy3-conjugated goat anti-rabbit immunoglobulin (Chemicon, Temecula, CA; 1:100) and fluorescein-conjugated goat anti-mouse immunoglobulin (Millipore, Temecula, CA; 1:100) were used. All the antibodies were diluted in TBS containing 15% normal goat serum and 0.2% Triton X-100 as detergent. For more details see Material and Methods of each chapter.

Spinal cord injury surgery

After deeply anesthesia, larvae were placed dorsal side up in a Sylgard-lined dish filled with Ringer solution. The spinal cord was exposed by a transverse incision from the dorsal midline at the level of the fifth gill, a complete spinal cord transection was performed with a scalpel and the spinal cord cut ends were visualized under a microscope. After surgery, lampreys were maintained on ice for 1 h to allow the wound to air dry. After this time, the animals were allowed to recover in aerated freshwater tanks at 19.5 ºC. Each transected animal was examined 24 h after surgery to confirm that there was no movement caudal to site of injury. For more details see Material and Methods section of chapters 2, 3 and 4.

Treatment with glutamate transporter inhibitor

After deeply anesthesia, larvae were placed dorsal side up in a Sylgard-lined dish filled with Ringer solution. The skin, the muscles and the meninx of the region comprised between the third and the seventh gill were carefully removed exposing the spinal cord. The animals were

maintained in 30 μM DL-threo-b-Benzyloxyaspartic acid (DL-TBOA; Tocris Bioscience, UK) diluted in dimethyl sulfoxide (DMSO) in Ringer during 4 h. For more details see Material and Methods section of chapter 2.

Quantification of glutamate-ir profiles

The quantification of profiles was made using the plug-in Feature J Hessian for Fiji software (Image J, NIH). This computer software allows making a semi-automated quantification of the number of positive profiles in photomicrographs (Grider et al., 2006). For more details see Material and Methods section of chapter 3.

Statistical analysis

Statistical analysis was carried out by GraphPad Prism 5 (GraphPad software, La Jolla, CA). Variability of values was given by standard error of the mean (SEM). All the data passed the Kolmogorov-Smirnov normality test. Therefore, unpaired Student t test, one-way ANOVA with Bonferroni's multiple comparison tests were made to analyse the data. For more details see Material and Methods section of chapters 2, 3 and 4.

Image acquisition

Photomicrographs of *in situ* hybridization experiments were made with an Olympus photomicroscope (AX-70; Provis) equipped with a colour digital camera (Olympus DP70, Tokyo, Japan). Sections of immunofluorescence experiments were photographed with the spectral confocal microscopes TCS-SP2 and SP5 (Leica, Wetzlar, Germany). Stacks

of photographs were processed with LCS Lite and LAS software (Leica). For more details see Material and Methods section of each chapter.

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Abbreviations

ALS	Amyotrophic lateral sclerosis
ARRN	Anterior rhombencephalic reticular nucleus
CCINs	Crossed caudal interneurons
CNS	Central nervous system
CSFc	Cerebrospinal fluid-contacting
DC	Dorsal column
DLR	Diencephalic loco motor region
DL-TBOA	DL-threo-b-Benzyloxyaspartic acid
DMSO	Dimethyl sulfoxide
EAAC	Excitatory amino acid carrier
EAAT	Excitatory amino acid transporter
EINs	Excitatory interneurons
GABA	γ-aminobutyric acid
GLAST	Glutamate-aspartate transporter
GLT	Glutamate transporter
IINs	Ipsilateral inhibitory interneurons
ir	Immunoreactive
mGluR	Metabotropic glutamate receptor
MLR	Mesencephalic loco motor region
MRN	Mesencephalic reticular nucleus
MRRN	Middle rhombencephalic reticular nucleus
PRRN	Posterior rhombencephalic reticular nucleus
RS	Reticulospinal
SCI	Spinal cord injury
SEM	Standard error of the mean

VGLUT	Vesicular glutamate transporter
VS	Vestibulospinal

Justification and aims of the Thesis

JUSTIFICATION AND AIMS OF THE THESIS

Justification and aims of the thesis

The sea lamprey, *Petromyzon marinus*, occupies a key phylogenetic position in the base of the vertebrate evolutionary tree. Because of this, lampreys constitute an important animal model for studying the basic central nervous system structures and deciphering the circuits underlying the control of locomotion, situated at spinal levels (revision in Dubuc et al., 2008; Grillner et al., 2008). Further, lampreys are also a model in studies of spinal cord regeneration due to their ability of spontaneously recover the locomotor function following a complete transection of the cord.

Glutamate is the main excitatory neurotransmitter in the central nervous system of vertebrates (Fonnum, 1984; Persson et al., 2006; Brumovsky et al., 2007). Excitatory interneurons play an important role in the spinal locomotor circuits (Buchanan and Grillner, 1987; Buchanan, 1999). In spite of this fact, anatomical studies about this neurotransmitter system are really scarce in non-mammalian vertebrates. Recent studies in our group have studied the distribution of the glutamatergic populations in the brain of adult lamprey and during its development (Villar-Cerviño et al., 2010, 2011, 2013) but the distribution of the glutamatergic populations in the spinal cord of lampreys remained unknown. Furthermore, glutamate plays also an important role in the pathophysiology of the spinal cord injury (SCI) in mammals, including humans (revision in Lee-Liu et al., 2013). Understanding the differences between lampreys and mammals in their acute response to spinal injury, as well as studying the changes that take place during the regeneration in a regenerative-vertebrate may provide

valuable information about the necessary mechanisms to achieve a functional recovery after SCI.

The specific aims of the thesis are:

- To characterize the glutamatergic neuronal populations in the spinal cord of the sea lamprey and compare its distribution with that of GABA and glycine, the main inhibitory neurotransmitters in the central nervous system. This study will increase the knowledge about the glutamatergic spinal system, providing clues about the complexity of neurotransmission in the spinal cord of lampreys. Further, it provides the basis for the study of the changes in the glutamatergic spinal system during spinal regeneration in lampreys.

- To investigate the glutamate release and the subsequent response of astrocytes following SCI in lampreys. This will provide new clues about the impressive regenerative and survival abilities of the lamprey spinal cord.

- To describe a method to quantify the number of processes of the cells of each type of spinal neurotransmitter system. This will provide a tool to compare results when analyzing the changes in different neurotransmitter systems at different time points after injury.

- To analyse the changes in the spinal glutamatergic system during spinal cord regeneration in lampreys. This will provide new information about the regenerative and plasticity mechanisms underlying the recovery of the locomotor function after SCI in lampreys.

The thesis is organized in four chapters. The first two main objectives correspond to chapters 1 and 2 whereas the latter two correspond to chapters 3 and 4.

In the first chapter, the pattern of expression of the vesicular glutamate transporter (VGLUT) in the spinal cord of lampreys by means of *in situ* hybridization is reported. Glutamate distribution in cells and fibres and colocalization between glutamate and GABA or glycine is also studied with immunofluorescence techniques. VGLUT expression was observed in dorsal cells, caudal giant cells, two grey matter populations, cerebrospinal fluid-contacting cells, edge cells and in some other white matter cells. Colocalization of glutamate and GABA or glycine was found in some spinal cells. The use of anti-glutamate antibodies to study the spinal glutamatergic populations is also validated in this study.

In the second chapter, the glutamate release and the subsequent astrocyte response during the first week following a complete transection of the cord is studied. Glutamate immunoreactivity in neurons was lost, close to the site of injury, immediately after the lesion. Strikingly, astrocytes showed glutamate immunoreactivity after SCI, and a treatment with an inhibitor of the glutamate transporters indicated that astrocytes actively uptake the glutamate released following SCI. These data suggest that, in contrast to mammals, astrocytes of lampreys have a great capacity to actively uptake glutamate after SCI.

In the third chapter, an immunofluorescence method to detect aminoacidergic neurotransmitters as well as a semiautomatic quantification method to analyse the changes in the number of glutamate immunoreactive (-ir) fibres during spinal cord regeneration are described.

In the fourth chapter, by means of the method described in chapter 3, glutamate-ir processes are quantified at different time points during the first six months following a complete transection of the cord.

Additionally, glutamate-ir cells are manually quantified in normal animals and in animals which have been recovering for 24 weeks post lesion (wpl). A significant decrease in the number of fibres was observed 2 wpl, whereas at later time points the expression returned mostly to normal levels. Significant changes were observed in the number of cells at 24 wpl in most of the populations, indicating that plasticity mechanisms are involved in the recovery of function in the lamprey.

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Chapter 1

The glutamatergic neurons in the spinal cord of the sea lamprey: an *in situ* hybridization and immunohistochemical study

THE GLUTAMATERGIC NEURONS IN THE SPINAL CORD OF THE SEA LAMPREY: AN *IN SITU* HYBRIDIZATION AND IMMUNOHISTOCHEMICAL STUDY

Introduction

Since the 1970s, glutamate has been recognized as the major excitatory neurotransmitter in the central nervous system of vertebrates (Fonnum, 1984; Persson et al., 2006; Brumovsky et al., 2007). It acts on several types of glutamate receptors: three groups of ionotropic receptors and three groups of metabotropic receptors (mGluR) (Meldrum, 2000; Platt, 2007). Glutamate is also involved in important processes in the developing brain such as neuronal differentiation and migration (Hack and Balzás, 1994; Yano et al., 1998; Meldrum, 2000). Glutamate probably exerts influence on neuronal responses to some basic guidance molecules (Kreibich et al., 2004). As a neurotransmitter, glutamate plays a major role in the processing and transmission of sensory information in the spinal cord (Todd et al., 2003; Todd, 2010) and in the spinal circuits involved in locomotion (Roberts et al., 1998; Higashijima et al., 2004). In mammals, all primary afferents to the spinal cord use glutamate as their major fast transmitter (Landry et al., 2004; Todd, 2010). Glutamate is introduced from the extracellular medium to the neuron cytoplasm by an excitatory amino acid transporter (EAAT; common for glutamate and aspartate) and then it is transported into the synaptic vesicles by vesicular glutamate transporters (VGLUTs) specific for this amino acid. Three VGLUTs have been identified in mammals to date (VGLUT1, VGLUT2 and VGLUT3) (Herzog et al., 2004). Early anatomical studies of the glutamatergic system in mammals depended on the use of antibodies raised against glutamate-

protein conjugates (Ottersen and Storm-Mathisen, 1984a, b). The use of these antibodies to characterize glutamatergic neurons was often considered problematic, because glutamate is a metabolic molecule found in all cells and because staining of perikarya was somewhat inconsistent (Walberg et al., 1990). Accordingly, some authors have considered that there were no reliable immunocytochemical markers for the cell bodies of glutamatergic neurons in mammals. In this way, glutamatergic cells were often defined by negative criteria (those cells that were not immunoreactive for GABA and glycine) (Todd, 2010). The new studies with in situ hybridization for vesicular glutamate transporters allowed identifying unequivocally glutamatergic neuronal perikarya, whereas immunohistochemistry with antibodies raised against these transporters have been useful to identify axons of glutamatergic cells although they fail to stain the perikaryon (Landry et al., 2004; Todd, 2010). Studies of VGLUT distribution in the rat lumbar spinal cord indicate that the proportion of glutamatergic neurons clearly exceeds the half of the total number of neurons in all laminae (Landry et al., 2004). The morphology of glutamatergic neurons of lamina I and II of the rat dorsal horn has been investigated in detail using combined physiological and immunohistochemical methods to characterize vesicular glutamate transporters in axons of neurobiotin-injected single cells (Maxwell et al., 2007; Todd, 2010; Yasaka et al., 2010).

The spinal cord has an intrinsic circuitry that controls locomotion generating a coordinated rhythmic output; this circuitry is known as the spinal cord central pattern generator (CPG) (Grillner et al., 2008). The excitatory interneurons play an important role in the rhythm generation (reviewed by Buchanan and Grillner, 1987; Grillner et al., 2008; Kiehn et al., 2008). Lampreys have been used for many years as a model to identify the neuronal circuits involved in the control of locomotion (see Dubuc et al., 2008; Grillner et al., 2008). Actually, lampreys are the vertebrates for which more detailed knowledge about the spinal neuronal network is

available, mostly based on electrophysiological and anatomical studies (see Buchanan, 2001; Grillner, 2003). Distribution of glutamatergic neurons in the spinal cord has been studied in a few animal species by immunohistochemistry with antibodies raised against glutamate-protein conjugates or antibodies raised against vesicular glutamate transporters (VGLUTs), and by *in situ* hybridization with probes for VGLUTs. The majority of these later studies were made in adult mammals (rat: Oliveira et al., 2003; Landry et al., 2004; Persson et al., 2006; mouse: Brumovsky et al., 2007) and in developing zebrafish (Higashijima et al., 2004). The CPG is composed by several types of interneurons that control the discharge of motor neurons (reviewed by (Grillner and Wallén, 2007; Grillner et al., 2008). Excitatory interneurons excite motor neurons and other interneurons, inhibitory glycinergic interneurons ensure alternate left-right side segmental activation by inhibiting all neuronal types on the contralateral side In addition to interneurons, lamprey edge cells (a class of intraspinal stretch receptor neurons) provide sensory feedback to the rhythm-generating network. A part of edge cells excite ipsilateral neurons, whereas the other edge cells inhibit contralateral neurons (Viana di Prisco et al., 1990).

Lampreys belong to the Agnathans, the oldest group of extant vertebrates, and thus have a great value for deciphering the early evolution of neurochemically-defined systems. Several studies have revealed the organization of glycinergic (Shupliakov et al., 1996; Gustafsson et al., 2002; Villar-Cerviño et al., 2008a) and GABAergic (Batueva et al., 1990; Brodin et al., 1990; Meléndez-Ferro et al., 2003; Ruiz et al., 2004; Robertson et al., 2007) cells in the lamprey spinal cord. However, the distribution of glutamatergic cells in the spinal cord has been studied only with glutamate immunohistochemistry either in synapses at ultrastructural level (Shupliakov et al., 1992) or in some cell perikarya of commissural neurons (Mahmood et al., 2009). At the best of our knowledge, there has been no report in non-mammalian adult

vertebrates of the expression of VGLUTs in the spinal cord, or comprehensive studies of spinal glutamatergic populations using glutamate immunohistochemistry. The recent cloning of a cDNA coding for a lamprey VGLUT (Villar-Cerviño et al., 2010a) affords an alternative tool to assess the glutamatergic character of lamprey neurons, as reported in the brain (Villar-Cerviño et al., 2011, 2013).

Over the past two decades, evidence indicating that transmission by multiple messengers released by single neurons was the norm rather than the exception has been accumulated (Seal and Edwards, 2006; Somogyi, 2006; Trudeau and Gutiérrez, 2007; Barreiro-Iglesias et al., 2009a, b). The functional implications of neurotransmitter co-release are not clear but it likely plays an important role in the maturation and refinement of synapses, in precision of motor activity, in the homeostatic opposition to hyperexcitability during seizures (Seal and Edwards, 2006) or possibly in reducing the metabolic cost and errors of signaling (Somogyi, 2006). Glutamate has been shown to colocalize with other neurotransmitters in some neurons. Colocalization of glutamate and GABA immunoreactivity has been reported in several regions of the central nervous system of mammals (for review see Gutiérrez, 2003, 2005). Colocalization of glutamate and glycine immunoreactivity has also been observed in some central neurons of mammals and amphibians (Reichenberger et al., 1997; Somogyi and Llewellyn-Smith, 2001; Noh et al., 2010). With regard to lampreys, several reports indicate that they are good to study distribution and colocalization of amino acid neurotransmitters at cellular level (Rodicio et al., 2008; Villar-Cerviño et al., 2008a, b, 2009, 2010b, 2011; Barreiro-Iglesias et al., 2009a, b, 2010). Knowledge of the colocalization of glutamate and GABA or glycine in cells of the lamprey spinal cord may contribute to a better understanding of their roles in the locomotor circuits.

The main aim of this study was to characterize the glutamatergic neuronal populations of the spinal cord of the sea lamprey, *Petromyzon marinus*. For this goal, we investigated the expression of VGLUT in neuronal perikarya to assess the glutamatergic character of cells. We also compared, by means of double immunofluorescence and confocal microscopy, the distribution of glutamate with that of GABA and glycine, the major inhibitory neurotransmitters in the central nervous system. This study determined the distribution of several populations of glutamatergic neurons along the spinal cord. Moreover, the results indicate that some glutamate-ir cells also show GABA or glycine immunoreactivity. These results were discussed in a comparative and functional context.

Material and methods

Ethical statement

All experiments were approved by the Bioethics Committee at the University of Santiago de Compostela and conformed to the European Union (86/609/EEC) and Spanish (Royal Decree 223/1998) regulations for the care and handling of animals in research.

Subjects

Large larvae (n = 19: 3 larvae for *in situ* hybridization, 16 for immunohistochemistry; body length between 130-160 mm), downstream migrating young adults (n = 4: 2 for *in situ* hybridization, 2 for immunohistochemistry; body length between 150-170 mm) and upstream migrating adults (n = 6: all of them for immunohistochemistry; length more than 650 mm) of the sea lamprey (*Petromyzon marinus* L.) were used.

Larval and young lampreys were collected from the River Ulla (Galicia, NW, Spain), with permission from the Xunta of Galicia. Upstream migrating adults were obtained from a commercial supplier. Larval lampreys were maintained in aerated fresh water aquaria with a bed of river sediment, while adults were processed immediately after arrival to the laboratory.

Tissue collection and processing

Animals were deeply anesthetized with 0.05% benzocaine (Sigma, St. Louis, MO) in fresh water and killed by decapitation. Three spinal cord regions were selected for study. The region between the fourth and the seventh gill is referred to as rostral spinal region, the long region between the end of the gill region and the dorsal fin is referred to as the middle region, and that corresponding to the levels where the dorsal and caudal fins are located is referred to as the caudal region. Pieces of spinal cord for each of the regions (5 mm in length, which corresponds to about 5-6 muscle segments in large larvae and 1 segment in adult lampreys) were fixed by immersion in 5% glutaraldehyde and 1% sodium metabisulfite (MB) in 0.05M Tris buffered saline (TBS; pH 7.4) for 20 h for immunohistochemistry or with paraformaldehyde 4% in phosphate buffered saline (pH 7.4) for 24 hours for *in situ* hybridization. The fixed samples were embedded in Tissue Tek (Sakura, Torrance, CA), frozen in liquid nitrogen-cooled isopentane, sectioned on a cryostat in the transverse plane (14 µm thick) and mounted on Superfrost ® Plus glass slides (Menzel, Braunschweig, Germany).

In situ hybridization

For VGLUT *in situ* hybridization, we performed the same protocol used previously in studies about glutamatergic cells in the brain of

lamprey (Villar-Cerviño et al., 2011, 2013). The probes used correspond to two sequence fragments (of 584 and 3,483 bp) of a lamprey VGLUT recently cloned in Sylvie Mazan's laboratory from a lamprey EST database, as reported elsewhere (Villar-Cerviño et al., 2010a). Plasmid DNA was purified from the selected clones, and the corresponding inserted fragments were excised as control. Templates for in vitro transcription were prepared by PCR amplification from plasmid DNA. A 584-bp 5' probe was obtained from clone NY0AAA51YH17RM1 using 5'-TTACTGCCGCTGCCAAATC-3' and the T7 promoter containing sequence 5'-AAGCTCTAATACGACTCACTATAGGGGTAACGCTTGGGCATTCCG-3' as forward and reverse primers, respectively. A second 3' 483-bp probe, spanning the region coding for transmembrane domains 9-12, was synthesized using 5'-TGCCCATCGGAGGACAAC-3' and 5'-AAGCTCTAATACGACTCACTATAGGGGCTCGTCCTCGTTGATGAAG-3' as forward and reverse primers, respectively. Digoxygenin-labelled riboprobes were synthesized by using the amplified fragments as templates, following standard protocols. In situ hybridizations on cryostat sections (14 µm thick) were conducted using standard protocols with an RNAse A treatment (0.2 µg/ml, 37ºC during 30 minutes) that was added to the post-hybridization washings. Staining was conducted in BM Purple (Roche, Mannheim, Germany) at 37ºC until the signal was clearly visible. The sections were mounted with Mowiol and photographed with a colour digital camera in an Olympus photomicroscope.

Immunofluorescence

For immunofluorescence, sections were pretreated with 0.2% $NaBH_4$ in deionized water for 45 minutes at room temperature to quench autofluorescence. Then, sections were incubated with a mixture of a rabbit polyclonal anti-glutamate antibody (Immunosolution, Jesmond, Australia; 1:4,500) and a mouse monoclonal anti-GABA antibody (Sigma,

St. Louis, MO; 1:1,200) or with a mixture of a rabbit polyclonal anti-glycine antibody (Immunosolution; 1:3,000) and a mouse monoclonal anti-glutamate antibody (Swant, Bellinzona, Switzerland; 1:1,000) in TBS with 1% sodium metabisulfite during 3 days at 4ºC or overnight at room temperature. After rinsing in TBS, sections were incubated for 1 hour at room temperature with Cy3-conjugated goat anti-rabbit immunoglobulin (Chemicon, Temecula, CA; 1:200) and fluorescein-conjugated goat anti-mouse immunoglobulin (Chemicon; 1:100), rinsed in TBS and mounted with Vectashield (Vector, Burlingame, CA).

Antibodies

The polyclonal anti-glutamate antibody was raised in rabbit against a glutamate-glutaraldehyde-porcine thyroglobulin conjugate. The antibody has been tested by the supplier in sections of retina and cerebellum from various mammals and other vertebrates, as well as in dot blot immunoassays with a variety of amino acid-protein conjugates. These include the standard 20 amino acids found in proteins, the non-protein amino acids D-serine, D-alanine and D-aspartate, GABA and the glycine-containing tripeptide glutathione, which did not yield significant cross reactivity. This antibody has been developed by Dr David V. Pow (University of Newcastle, New South Wales, Australia) and used in previous studies of the lamprey brain (Villar-Cerviño et al., 2011, 2013). In addition, the antibody was tested by Western blotting of lamprey brain/spinal cord protein extracts. This antibody did not stain any sea lamprey brain native protein band in these extracts (Villar-Cerviño et al., 2011).

The mouse monoclonal anti-glutamate antibody was raised against glutaraldehyde-linked L-glutamate-bovine serum albumin (BSA) conjugate by P. Streit (Liu et al., 1989), and this clone was made commercially available through Swant. This antibody has been

characterized with respect to cross reactivity by antibody dilution experiments as well as by absorption experiments (Adám and Csillag, 2006). In addition, the staining pattern obtained with both monoclonal and polyclonal anti-glutamate antibodies in this study and in sections of the brain and retina (unpublished observations) were the same.

The rabbit polyclonal anti-glycine antibody was raised against a glycine-glutaraldehyde-porcine thyroglobulin conjugate. It was tested in sections of retina and cerebellum from various mammals and other vertebrates as well as in dot blot immunoassays against a variety of amino acid-carrier protein conjugates, including the standard 20 amino acids found in proteins; the non-protein amino acids D-serine, D-alanine and D-aspartate; GABA; and the glycine containing peptide glutathione, which did not yield significant reactivity. This antibody has been used in a number of studies of glycinergic neurons of the retina, brain and spinal cord of the sea lamprey (Villar-Cerviño et al., 2008a, b) and other vertebrates (*Xenopus laevis*: (Dullin et al., 2007); sturgeon: (Adrio et al., 2011); zebrafish: (Moly and Hatta, 2011); bat: (Yavuzoglu et al., 2011).

The mouse monoclonal anti-GABA antibody was raised against GABA conjugated to BSA with glutaraldehyde and was evaluated by the supplier for activity and specificity by dot blot immunoassay. No cross-reaction was observed with BSA, L-a-aminobutyric acid, L-glutamic acid, L-aspartic acid, glycine, δ-aminovaleric acid, L-threonine, L-glutamine, taurine, putrescine, L-alanine, or carnosine. This antibody showed weak cross-reaction with β-alanine. This antibody has been used in previous studies of the sea lamprey (Villar-Cerviño et al., 2008a, b), and the pattern of immunostaining reported was the same as in studies with other anti-GABA antibodies (Meléndez-Ferro et al., 2001, 2002, 2003; Rodicio et al., 2005; Villar-Cerviño et al., 2006; Robertson et al., 2007). Moreover, the anti-glycine and the monoclonal anti-GABA antibodies were tested by Western blotting of lamprey brain protein extracts and they did not

recognize any brain native protein in blots (Villar-Cerviño et al., 2006, 2008a).

Image acquisition and measurements

Immunocytochemically stained sections were photographed and analysed with a spectral confocal microscope TCS-SP2 (Leica, Wetzlar, Germany). Confocal image stacks were processed with LITE software (Leica). Photographs were adjusted in brightness and contrast with Adobe Photoshop 7 software. To compare the number of cells of each population that showed VGLUT expression or glutamate immunoreactivity, at least thirty sections of two different larvae were counted. In each section, all the cells of each population were counted. Values are expressed as mean ± standard deviation. To measure the cell diameters of glutamate-ir cells, the LITE software was also used. For each neuronal population, at least fifteen cells of two different individuals from each group of animals (larvae and adults) were measured. Values are expressed as mean ± standard deviation. To establish the percentage of colocalization, cells in one out of each four sections were counted in two different individuals from each stage (larvae and adults). Since the small size of cells showing colocalization and due to our aim was not to compare absolute cell numbers or densities in larvae and adults, but the percentage of cells showing neurotransmitter colocalization, no correction factor was used. This need to be bore in mind when comparing percentages in larvae and adults, because the neurons are very different in size.

Results

VGLUT expression and glutamate immunoreactivity in neuronal populations in the lamprey spinal cord

In lampreys, the spinal cord is flattened and the grey matter forms paired "wings" or "horns" that extend laterally. The medial region corresponds to the dorsal horn of the spinal cord in jawed vertebrates in terms of embryonic origin and the lateral region corresponds to the ventral horns (see Meléndez-Ferro et al., 2003). VGLUT-expressing and glutamate-immunoreactive neurons were distributed in the grey and white matter of the spinal cord. Interestingly, similar neuronal populations were observed with both VGLUT *in situ* hybridization and glutamate immunohistochemistry. The number of labelled cells per section with both methods is shown in Table 1. The means and standard deviations founded suggest that the two methods are revealing the same neuronal populations. Important differences among individuals in the number of neurons in the lamprey spinal cord has been previously reported (Selzer, 1979). Since the possibility that some glutamate-ir neurons do not actually correspond with VGLUT positive cells cannot be ruled out, in the following we refer to glutamate-ir neurons as putative glutamatergic cells. Accordingly, the description of the populations is based on *in situ* hybridization experiments whereas a more detailed description of the size, morphology and processes is based on immunohistochemistry studies.

The size of larval cells is much different from the size of cells in adults. For descriptive purposes, larval neurons with mean minor cell diameter less than 10 µm are described as small, cells comprised between 10 and 25 µm as large, and those larger than 25 µm as giant. In adult animals, cells less than 25 µm in diameter are referred as small, cells between 25 and 50 µm as large, and those clearly exceeding 50 µm in diameter as giant.

The patterns of VGLUT expression and glutamate immunoreactivity in the spinal cord of larval and adult sea lamprey are quite similar (Fig.1, 2, 3 and 4). A notable difference was the size of glutamatergic cells, which were two or three times larger in adults than in larvae (as example, Table 2 shows the sizes of grey matter glutamatergic interneurons). In our slices of the most caudal spinal cord (caudal fin region) of upstream migrating adults, the levels of glutamate immunoreactivity were low so it was not possible to measure the diameters of cells. However, VGLUT expression and glutamate immunoreactivity were clearly observed at this level of the spinal cord in downstream migrating adults (Fig. 3B). Moreover, a decrease in the cellular density occurred in the adult spinal cord, and the glutamate-ir cells appear more scattered than in larval lampreys, as observed with immunohistochemistry. It was easier to distinguish the cell morphology in larvae than in adults: accordingly, the descriptions were based in larval observations. As is stated in Material and Methods, the VGLUT expression in upstream migrating adults was not investigated.

In general, the distribution of VGLUT expression and glutamate-ir were similar at all the levels of the spinal cord: rostral (Fig. 1A), middle (Fig. 1B), dorsal fin (Fig. 1C) and caudal fin (Fig. 1D), excepting for the presence of giant glutamatergic cells at the dorsal fin level, the absence of cells associated to medium-sized dorsolateral axons at the dorsal and caudal fin levels and the absence of cells associated to Müller axons at the caudal fin level. In addition, at the caudal fin level, the transverse area of the spinal cord was reduced with respect to the other regions, and also were the size of the cells (Table 2) and the diameter of the giant and medium-sized axons. (Fig 1D).

VGLUT expression was observed in cells of both the grey matter and the white matter (Fig. 1A-D). The reaction signal was located in the cytoplasm around the cell nucleus and occasionally in proximal dendrites, and showed a grainy appearance. There were large differences in

intensity of signal (number of positive granules) among neurons. In the grey matter, prominent VGLUT expression was observed in primary sensory neurons (dorsal cells) situated in the mediodorsal region of the grey matter just adjacent to the central canal and below the dorsal column. In transverse sections the dorsal cells showed a characteristic rounded profile (Fig. 2A, B; 3A). In larvae, dorsal cells were faintly glutamate-ir (Fig. 4A), but they were strongly glutamate-ir in adults (Fig. 4J). Hybridization signal was observed in a population of small interneurons located in the dorsomedial grey and in a heterogeneous cell population with regard cell size and intensity of reaction situated in the lateral grey matter (Fig. 2C, D; B, C). The putative glutamatergic interneurons of the dorsal population showed strong glutamate immunostaining. According to their position, two types of cells were distinguished: dorsomedial and dorsolateral. Dorsomedial cells were located in the medial region of the dorsal population and under the fibres of the dorsal column. These putative glutamatergic cells had one or more processes of dendritic appearance coursing dorsally or dorsolaterally to the ipsilateral dorsal column or to the dorsolateral region near the dorsal column (Fig. 4B). Some of these cells had a dendritic process crossing the midline dorsal to the central canal, under the dorsal column (Fig. 4F). Glutamate-ir dorsolateral cells were observed only in the rostral spinal cord and in the rostral part of the middle spinal region. These glutamate-ir cells had a process coursing ventromedially, surrounding the medial longitudinal fascicle and another process directed to the ventrolateral area (Fig. 4C). In some of these cells, a dorsal branch arising from the ventrolateral process coursed to the dorsal column (not shown). The glutamate-ir interneurons of the lateral population also showed strong immunostaining. According to their size (Table 2), two VGLUT-positive cell subpopulations were distinguished: large and small (Fig. 2D; 3C). Large lateral cells were situated in the most lateral region of the grey matter. They were multipolar neurons, irregular in shape and with processes directed to the medial, ventral and lateral funiculi (Fig.

4D). Some of the processes coursing laterally reached the area adjacent to the Mauthner axon (Fig. 4E). Small lateral cells were located medially to the large lateral cells. They were multipolar cells, with irregular morphology. Lateral, ventral and medial processes were distinguished in some of these cells (Fig. 4D). In the caudal fin region the density of cells of the lateral population was higher than the observed in the other regions of the cord and both, large and small cells occupied both lateral and ventrolateral positions (not shown). Bipolar ventrolateral glutamate-ir cells with processes directed laterally and ventromedially (Fig. 4G) were observed in the caudal spinal cord.

Surrounding the central canal of the spinal cord, there were small VGLUT-positive and glutamate-ir bipolar neurons of cerebrospinal fluid-contacting (CSFc) type with perikarya located in the ependymal walls. The number of positive granules per cell was usually scant (Fig. 2E; 3D). In larvae, most of these cells were faintly glutamate-ir, although some of them presented strong glutamate immunoreactivity (Fig. 4H). In adults, they showed different degrees of glutamate immunoreactivity, although most of them were faint or moderately glutamate-ir (Fig. 4I, R). A short dendrite of these cells coursed to the central canal, ending as a club. Occasionally, a thin axonal process was observed arising from the opposite pole of the cell.

At dorsal fin levels of the spinal cord, giant VGLUT-positive (Fig. 2F, 3E) and glutamate-ir (Fig. 4K) cells [32.7 µm × 19.4 µm (larvae), 56.1 µm in width × 109.1 µm (adult) in major and minor diameters, respectively] were situated in a ventrolateral position of the grey matter. These multipolar neurons showed an oval perikaryon and processes directed medially, laterally and ventrally (Fig. 2F, 3E 4K).

In the white matter, there were VGLUT signal (Fig. 2G-K; 3F, G) and glutamate immunoreactivity (Fig. 4L-R) in numerous cells of the lateral, ventromedial and dorsolateral regions. In lateral and ventrolateral spinal

regions, different white-matter VGLUT positive and glutamate-ir cells were distinguished according to their location and morphology. VGLUT-positive and glutamate-ir edge cells were situated in the ventrolateral (Figs. 2G, H; 3F, 4L) and lateral marginal (Fig. 4L, M) zones of the spinal cord. Some of them were bipolar cells and showed a long dendritic process that coursed laterally to the marginal neuropil and a process that coursed medially (not shown). Other edge cells were tripolar in appearance, with ventrolateral and lateral processes directed to the edge of the spinal cord, and a medial process (Fig. 4M). Some VGLUT positive and glutamate-ir cells were observed closely associated with the Mauthner axon (Figs. 2I, 4N), except at the caudal fin level. These cells were bipolar with processes surrounding it (Fig. 4N).

In the dorsolateral funiculus, occasional glutamate-ir and VGLUT expressing cells were located among the medium-sized axons of this column (Figs. 2J, 4O) at rostral and middle spinal cord levels. Some of these cells were bipolar neurons with processes coursing among dorsomedial axons (Fig. 4O). In the ventromedial funiculus, some glutamatergic cells were observed among the giant Müller axons (Fig. 3G). Some perikarya of similar glutamate-ir cells were almost vertical and spindle-shaped, with dorsal and ventral processes (Fig. 4P). Some other glutamatergic cells were situated under the giant axons (Fig. 2K) and similar glutamate-ir cells showed their processes coursing laterally and medially (Fig. 4Q, R).

Colocalization of glutamate and GABA or glycine immunoreactivities

Previous studies of the lamprey spinal cord reported the presence of glycine and/or GABA in some small-celled spinal populations, whereas immunoreactivity to these substances was absent in dorsal cells, motor neurons and large lateral cells, among others (Ruiz et al., 2004; Villar-

Cerviño et al., 2008a; Mahmood et al., 2009), which is confirmed with present observations. Here, colocalization with glutamate was only investigated in those populations exhibiting GABA and glycine.

In the spinal cord, colocalization of glutamate and GABA immunoreactivities was observed in some medial glutamate-ir interneurons of the dorsal population (Fig. 5A-A'', 6A-A''), in small lateral cells (Fig. 5B-B'', 6B-B'') and in most of the CSFc cells (Fig. 5C-C''). Whereas these populations were observed in both larval and adult lampreys, the percentage of glutamate-ir cells showing colocalization with GABA varied among stages and spinal regions. Table 3 shows the percentages of double-labelled interneurons. They correspond to arbitrary lengths of spinal cord in which both dorsal and lateral glutamate-ir cells were counted. No colocalization with GABA immunoreactivity was observed in the glutamate-ir cells situated in the white matter or in other glutamate-ir cellular types of the grey matter. In the CSFc cells, the cellular distribution of glutamate and GABA immunoreactivities was not homogeneous along the cell. Thus, glutamate and GABA were colocalized in the apical dendrite including the terminal club and in the central region of the soma but only some of the CSFc cells showed glutamate immunoreactivity in the exit of the basal process. The periphery of the soma and the basal process in their whole length were only GABA-ir. Colocalization of glutamate and glycine was observed in in cells located in both the grey and the white matter. In the grey matter, some interneurons of the dorsal population (Fig. 7A-A''), some small lateral cells (Fig. 7B-B'', 8A-A'') and some CSFc cells (Fig. 7C-C'') showed glutamate and glycine colocalization. In these CSFc cells, the cellular distribution of immunolabelling was similar for both neurotransmitters, which is in contrast to that reported for glutamate and GABA (see above). As reported above for GABA, regional differences in the degree of colocalization of glycine in glutamate-ir interneurons were also observed (Table 4), and the

colocalization percentages obtained correspond to arbitrary lengths of spinal cord.

In the white matter, some ventral edge cells (Fig. 7D-D'', 8B-B'') and, occasionally, lateral edge cells (not shown) showed glutamate and glycine immunoreactivity in both larval and adult lampreys. All edge cells that express glycine also express glutamate immunoreactivity.

Fibres

The spinal cord of adult and larval lampreys showed a heterogeneous distribution of glutamate-ir fibres. The highest density of glutamate-ir fibres was observed in the dorsal column (Fig. 4A), except for the caudal spinal region where these fibres were faintly glutamate-ir. The density of glutamate-ir fibres is higher in the dorsal funiculus next to the dorsal column than in the ventral funiculus, although the glutamate-ir fibres were thicker in the ventral funiculus. The glutamate-ir fibres were thicker in adults than in larvae, as reported with respect to the size of the cells. A very rich region of glutamate-ir processes were also observed in the edge cell neuropil. Most of the processes in the lateral neuropil appear to correspond to dense terminal dendritic branches of glutamate-ir edge cells (Fig. 6C-C'). In general, the density of GABA-ir fibres is lower than that of glutamate-ir fibres and the highest density was observed on both sides of the dorsal column and in the margin around the lateral processes of edge cells (as shown with glutamate immunoreactivity), forming a rich GABA-ir marginal neuropil (Fig 6C-C''). On the other hand, the number of glycine-ir fibres was lower than that of glutamate-ir fibres and they were concentrated in the ventral, ventrolateral and dorsolateral funiculi. However, glycine-ir fibres were thicker than glutamate-ir ones. With respect to the giant axonal system of the spinal cord, the axoplasm of Mauthner and Müller axons did not show glutamate immunoreactivity

above background either in larvae or in adults, despite the fact that their perikarya express clearly VGLUT.

Discussion

Our results demonstrate for the first time the presence of VGLUT mRNA, a highly reliable glutamatergic marker, in numerous neurons of the lamprey spinal cord, thus revealing actual glutamatergic cells. In addition, this is the first comprehensive study reporting the distribution of glutamate immunoreactivity in neurons in the spinal cord of the sea lamprey and the colocalization of glutamate and GABA or glycine immunoreactivities in some spinal cord neurons.

Glutamate in spinal cord circuits

Excitatory (glutamatergic) cells play an essential role in the CPG networks of the spinal cord. Electrophysiological studies have demonstrated that excitatory spinal interneurons excite both motor neurons and inhibitory premotor interneurons (Buchanan and Grillner, 1987). Present results indicate that these excitatory neurons most probably express a vesicular glutamate transporter, giving support to the opinion that they are actually glutamatergic.

Our results revealed morphological variability among cells of the same type. Although some differences in size can be appreciated among the glutamate-ir cell photographed in a recent study of lamprey (Mahmood et al., 2009), their authors focused only in the neurotransmitter phenotypes of commissural cells. A great morphological variability within

each type of spinal neurons, including motor neurons, has also been observed in *Xenopus* embryos (Roberts and Clarke, 1982).

Our results indicated that the types of glutamatergic cells were similar in larvae and adults, although their cell sizes were quite different. Another difference between larval and adult lampreys was that the cells were more scattered in the sections in adults than in larvae, as a probable result of the growth of spinal cord volume in adults without accompanying neuronal proliferation. Accommodation of existing neuronal populations to the increasing length and width of the spinal cord between larvae and the adulthood is partially compensated by the great increase in size that takes place in perikarya and their processes.

Our results showed some differences in the glutamatergic populations along the different regions of the spinal cord: rostral, middle and caudal. Specifically, the glutamate-ir dorsolateral cells of the grey matter were observed in the rostral and middle regions but not in the caudal spinal cord. Moreover, the giant glutamatergic cells were only observed in the caudal spinal cord, which is in agreement with anatomical observations on giant interneurons of Rovainen, (1967) and Selzer, (1979). In the following, the main glutamatergic cell populations of the lamprey are discussed and compared with those observed in other species.

Primary sensory neurons (dorsal cells)

Our results showed VGLUT expression in the primary sensory dorsal cells, which is in agreement with electrophysiological studies that indicated that these cells make excitatory monosynaptic and/or polysynaptic contacts with motor neurons (el Manira et al., 1996) and giant relay interneurons (Brodin et al., 1987). The absence of GABA and

glycine immunoreactivity in these cells is also consistent with their excitatory role. It has been proposed that lamprey dorsal cells are homologous to Rohon-Beard cells, which are observed transiently in other fishes and in amphibians during development (Nieuwenhuys, 1964; Rovainen, 1967). Electrophysiological studies have demonstrated that Rohon-Beard cells are glutamatergic in frog tadpoles (Li et al., 2003), and they express VGLUTs in larval zebrafish (Higashijima et al., 2004).

Interneurons of the dorsal population

A population of glutamatergic cells was observed in the dorsal region of the spinal cord of the sea lamprey. A dorsal domain of cells that express VGLUTs has also been observed in the spinal cord of zebrafish embryos (Higashijima et al., 2004). In lamprey, two types of interneurons can be distinguished in this glutamatergic population: dorsomedial and dorsolateral cells. Dorsomedial cells showed processes directed to the dorsal column region. The dorsal column is constituted by the axons of intraspinal primary sensory neurons (dorsal cells) and spinal ganglion cells that course in the dorsal funiculus to the dorsal column nucleus in the caudal rhombencephalon (Ronan and Northcutt, 1990). Because some cells of the lateral population are excited polysynaptically by dorsal cells, these findings suggest that dorsomedial cells are involved in sensory processing by relaying sensory information (Rovainen, 1974a). Some cells of this population had a long process that crosses the midline dorsally to the central canal. Cells with similar morphology have been reported in *Lampetra planeri* with classical staining methods (Whiting, 1948) and in the sea lamprey using calretinin immunohistochemistry (Megías et al., 2003). Cells near the dorsal funiculus that show a dendrite crossing dorsally the spinal midline have also been observed in newt embryos prior to hatching (Harper and Roberts, 1993). These cells showed an axon crossing

ventrally, which could not be observed in the dorsomedial glutamate-ir cells of the sea lamprey.

The dorsolateral glutamatergic cells were bipolar neurons with a process coursing ventromedially and the other coursing ventrolaterally, but some of these cells showed a branch of the ventrolateral process directed to the dorsal column. This suggests that these cells are also involved in processing sensory information. The ventromedial process of these cells appear to cross the midline ventrally to the central canal in a different transversal level than that occupied by the soma, so they could not be followed to the contralateral side (see Fig. 2C). Their position and cell morphology indicated that these cells are similar to the excitatory dorsolateral commissural cells (dlc) described in *Xenopus* embryos. In *Xenopus*, electrophysiological studies have demonstrated that the dlc cells were excited by the axons of Rohon-Beard cells and in turn, they excite contralateral neurons (Roberts and Sillar, 1990).

Cells of the lateral population

In the sea lamprey spinal cord, a population of glutamatergic cells was observed in the lateral region of the grey matter. During development, the spinal cord of lamprey prolarvae becomes flattened and the cells of the intermediate column migrate away from the midline, giving rise to the lateral grey matter of larvae (Meléndez-Ferro et al., 2003). Accordingly, this lateral glutamatergic population probably corresponds to the ventral VGLUT2-expressing cell domain of the spinal cord of the embryonic zebrafish (Higashijima et al., 2004) and to the VGLUT-expressing neurons of the intermediate region of the rodent spinal cord (Oliveira et al., 2003; Brumovsky et al, 2007).

Two types of glutamatergic lateral neurons, small and large, were observed in the lamprey spinal cord. Small lateral cells may correspond to interneurons involved in the CPG that excite all types of spinal neurons, including motor neurons, commissural interneurons, inhibitory lateral interneurons and other excitatory interneurons of the ipsilateral side (Buchanan and Grillner, 1987; Grillner et al., 2008). These lamprey interneurons were considered involved in CPG rhythm generation (Grillner, 2003; Kiehn, 2006). With regard to the large glutamatergic neurons observed here, they are probably interneurons since (Mahmood et al. 2009) did not observe labelled glutamate-ir cells after tracer application to the motor roots of the river lamprey spinal cord. Large lateral cells of lampreys were described as multipolar neurons with dendritic processes directed to the medial, ventral and lateral tracts of fibres, including the Müller and Mauthner axons, which made contacts with them (Martin and Ringham, 1975; Ringham, 1975). However, the large glutamatergic interneurons observed here probably do not correspond with these large lateral interneurons studied in lamprey (Rovainen, 1974a; Selzer, 1979; Buchanan, 1982), because electrophysiological studies have demonstrated that most of them were inhibitory and only an excitatory lateral cell could be recorded by Rovainen, (1974a). Our results suggest that large glutamatergic (excitatory) lateral interneurons may be more numerous than expected. Excitatory ipsilateral cells with processes directed to the Mauthner axon and making excitatory synapses on motor neurons have been observed in goldfish (Fetcho and Faber, 1988).

Giant cells

We observed big glutamatergic cells in the caudal spinal cord at the level of the dorsal fin. By their location, size and morphological features, these cells correspond to the giant cells described by Rovainen, (1967) and Selzer, (1979). Electrophysiological studies have demonstrated

that these cells are excitatory (Rovainen, 1967), which is in agreement with the VGLUT expression observed in these cells (present results).

Cerebrospinal fluid-contacting cells

These cells belong to the CSFc rhombencephalic-spinal system observed in all vertebrate species, although they are more numerous in fishes and amphibians (revision in Vigh et al., 2004). About the functions of these cells there have been several hypotheses, including among others mechanosensory, chemosensory or secretory functions (see Vigh et al., 2004). The recent finding of expression of mRNA of a candidate sour taste sensor (PKD2L1) in spinal CSFc cells of mice (Huang et al., 2006) supports the hypothesis that they are chemosensory although does not preclude additional functions. In the lamprey spinal cord, some CSFc cells showed VGLUT expression and many were glutamate-ir. The levels of glutamate immunoreactivity varied from faint to strong. All the glutamate-ir CSFc cells were also GABA-ir, which raises the question about the functional significance of this colocalization. GABA-ir CSFc cells have been previously reported in lamprey (Batueva et al., 1990; Brodin et al., 1990; Christenson et al., 1991; Shupliakov et al., 1996; Meléndez-Ferro et al., 2003; Ruiz et al., 2004). It is worth pointing out that it is the apical dendrite and soma of CSFc cells which primarily showed glutamate and GABA colocalization, whereas only in a few CSFc cells colocalization was observed in the exit of the basal process. The basal (axonal) process of GABA-ir CSFc cells course to the lateral margin of the spinal cord forming a rich marginal plexus (Grillner et al., 2008; Villar-Cerviño et al., 2008a), making contacts with the dendrites of edge cells (Christenson et al., 1991). The GABAergic terminals that form the marginal neuropil were glutamate-ir negative, suggesting differential distribution and use by the cell of these neurotransmitters. CSFc cells exert a tonic inhibition on the excitatory edge cells (Vinay et al., 1996).

Differences in the cellular distribution of two neurotransmitters have been observed in other neurons. For instance, rat motor neurons express glutamate in addition to acetylcholine; whereas the endplate at the neuromuscular junction releases only acetylcholine, glutamate is released in the collateral axonal terminals over the Renshaw cells and acts as a cotransmitter (Herzog et al., 2004; Nishimaru et al., 2005). The functions of glutamate in CSFc cells need to be further investigated. In some lamprey CSFc cells, GABA is also colocalized with other neurotransmitters as glycine (Villar-Cerviño et al., 2008a, b), dopamine (Rodicio et al., 2008) and somatostatin (Christenson et al., 1991), suggesting the existence of neurochemically specialized CSFc cell subpopulations.

Edge cells

Although lamprey edge cells were first described by Reissner, (1860), only more than one century later they were characterized physiologically as intraspinal mechanoreceptors (Grillner et al., 1984). These white matter cells are situated near the lateral edge of the spinal cord and their axons are mainly directed rostrally (Rovainen, 1974a; Buchanan, 2001). Previous electrophysiological results have indicated that edge cells projecting ipsilaterally were excitatory on target cells and presumably glutamatergic, whereas those projecting contralaterally where inhibitory and presumably glycinergic (Rovainen, 1974a; Viana di Prisco et al., 1990). The existence of glutamate-ir or glycine-ir edge cells has not been mentioned by Mahmood et al. (2009), who studied these immunoreactivities in combination with tract-tracing from the contralateral spinal cord. The presence of VGLUT expression observed here in some edge cells is consistent with the physiological characterization of some edge cells as glutamatergic (Viana di prisco et al., 1990). The observation of glycine immunoreactivity in some edge cells is also in agreement with the characterization of other edge cells as

glycinergic inhibitory. Concerning the glycine-ir edge cells, however, a striking result of present experiments is the presence of glutamate immunoreactivity in all these cells, which was observed in both larvae and adults. This fact poses the question about the functional significance of this excitatory amino acid in these edge cells (see below).

Other glutamatergic cells situated in the white matter

Numerous small glutamatergic cells situated in the white matter of the spinal cord of the lamprey are associated with the giant Mauthner and Müller axons, and with axons of other reticulospinal cells. These small cells are in a key position to receive descending information from the brain and to participate in locomotion networks. The cells situated in the ventromedial region of the white matter may receive inputs from Müller axons and other reticulospinal axons and may be involved in the locomotion circuits. The association of white matter glutamatergic small cells with giant axons or specific tracts may be complementary to the specific contacts between reticulospinal cells and identified spinal neurons studied by Rovainen, (1974b). The existence of small glycinergic cells in the white matter receiving contacts from large descending fibres has been also reported previously (Gustafsson et al., 2002; Villar-Cerviño et al., 2008a).

Glutamate-ir commissural cells

It seems that there are commissural cells in both dorsal cell populations, but in this study only the cells of which axons decussate at the level of the soma were demonstrated. Excitatory glutamate-ir commissural neurons have been previously described in lamprey using combined immunohistochemical and tract-tracing methods (Mahmood

et al., 2009), but neither their position in the cord nor their morphology have been studied in detail. Electrophysiological studies have also reported that excitatory contralateral interneurons with descending axons situated in the rostral spinal region excite fin motor neurons in alternating activity with regard to myotomal motor neurons when the lamprey is swimming forwards (Mentel et al., 2008). However, these authors did no report morphological details that would allow us to compare these cells with the dorsal commissural cell populations described in this study.

Significance of colocalization of glutamate and GABA or glycine

Double immunofluorescence methods allowed directly comparing the distribution of glutamate immunoreactivity with those of glycine or GABA in spinal neurons. In several populations of the larval and adult spinal cord, some neurons showed colocalization of glutamate with GABA or glycine immunoreactivities.

As regards colocalization of GABA and glutamate, present results reveal that the cell types that show colocalization in larvae also show colocalization of these neurotransmitters in adults, although some differences were observed in the percentage of glutamate-ir cells showing colocalization. Colocalization of GABA and glutamate in lamprey has been previously reported in some cells of the adult retina (Villar-Cerviño et al., 2006) and in a few populations of the larval and adult brain (Villar-Cerviño et al., 2011, 2013). In other vertebrates, colocalization of glutamate and GABA, or of glutamate vesicular transporters and/or GABA synthesizing enzymes or GABA vesicular transporters, has been described in mossy fibres of the hippocampus (Sandler and Smith, 1991; Gutiérrez and Heinemann, 2006), in retinal cells (Yang et al., 1994; Sun and Crossland, 2000; Kao et al., 2004) and in cerebellar mossy fibre terminals (Zander et al., 2010). A striking observation

was the colocalization of glutamate and GABA in the body and apical dendrite of some of the CSFc cells, but not in their basal axons, which are only GABA-ir. Release of glutamate and dopamine from different terminals of the retinal bipolar cells has been suggested (Kao et al., 2004) and release of glutamate and dopamine from different sites of the same cell has been reported in mesoaccumbens projections (Joyce and Rayport, 2000). Therefore, a similar situation could occur in lamprey CSFc cells containing glutamate and GABA.

The functional implications of glutamate and GABA colocalization are not known yet. It has been suggested that co-release of transmitters could play an important role in improving the precision of the locomotor activity in the spinal cord of mammals (Ornung et al., 1998; Seal and Edwards, 2006). Other functions that have been suggested for this colocalization were its participation in maturing and refinement of synapses (Gutiérrez, 2003, 2005; Seal and Edwards, 2006) and in adaptive processes in the adult brain (Zander et al., 2010). However, the functional significance of glutamate and GABA colocalization needs further investigation.

Studies of colocalization of glutamate and glycine are very scarce. In the lamprey spinal cord, colocalization of glutamate and glycine has been previously reported in giant fibre synapses using immunoelectron microscopy (Vesselkin et al., 1995), although we could not confirm these results. Instead, our study reveals colocalization of glutamate and glycine immunoreactivity in some types of cells of the spinal cord, both in larvae and adults. In other vertebrates, colocalization of glutamate and glycine immunoreactivity has been reported in cells of the retina (human: Davanger et al., 1991; chick: Sun and Crossland, 2000) and in vestibular neurons of frog (Reichenberger et al., 1997). Colocalization of glutamate and glycine was also observed in vestibular afferents of frog (Reichenberger and Dieringer, 1994), in nerve terminals in

the rat *locus coeruleus* (Somogyi and Llewellyn-Smith, 2001) and in axons from the medial nucleus of the trapezoid body in the lateral superior olive (Gillespie et al., 2005; Noh et al., 2010).

In relation to the functional significance of the glutamate and glycine colocalization, co-release of both neurotransmitters from some central synapses might modulate responses of target neurons. It is known that glycine contributes to excitatory neurotransmission acting as an allosteric modulator for the NMDA receptor (Johnson and Ascher, 1987; Dingledine et al., 1990; Liu et al., 2010). Moreover, glutamate exerts an allosteric potentiation of the glycine receptor chloride currents. This reciprocal modulation could act as a rapid homeostatic control mechanism for neuronal excitability (Liu et al., 2010). A recent study has shown that glutamate co-release during development is crucial for the synaptic reorganization and topographic specification of an inhibitory pathway in the auditory system (Noh et al., 2010). Our results showed that glutamate is also present in the glycine-ir (inhibitory) edge cells during both the larval and adult periods. This fact suggests that glutamate and glycine are co-released by some edge cells, but if this is involved in functional modulation of edge cells synapses on target cells needs to be investigated.

In conclusion, this neurochemical study reports for the first time the presence of a variety of glutamatergic cell populations distributed along the spinal cord and raises the number of known glutamatergic cell types by two: CSFc cells and white matter interneurons. The pattern of VGLUT expression and glutamate immunoreactivity observed in larvae and adults was similar, indicating that no major changes occurred in this system during metamorphosis. Colocalization of glutamate and GABA or glycine has been frequently observed in both larval and adult sea lampreys, indicating that the glutamatergic cells of the lamprey spinal

cord are neurochemically more complex than previously thought. This work provides a base to study the function of white matter glutamatergic interneurons, the role that glutamate plays in the CSFc cells and the putative changes in spinal glutamate distribution during spinal cord regeneration in lampreys.

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Abbreviations

CPG	Central pattern generator
CSFc	Cerebrospinal fluid-contacting
dlc	Dorsolateral commissural cells
EAAT	Excitatory aminoacid transporter
ir	Immunoreactive
mGluR	Metabotropic glutamate receptor
VGLUT	Vesicular glutamate transporter

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Tables and Figures

Table 1. Comparison between numbers of glutamate and VGLUT positive cells per section.

	GREY MATTER						WHITE MATTER			
	DORSAL CELLS	DORSAL POPULATION		LATERAL POPULATION		CSFc CELLS	EDGE CELLS	MAC	MTAC	MDLAC
		DM	DL	LARGE	SMALL					
Glutamate	0.05 ± 0.22	0.92 ± 1.05	0.63 ± 0.82	0.88 ± 0.84	7.28 ± 2.97	1.32 ± 1.18	0.22 ± 0.41	0.28 ± 0.49	0.05 ± 0.22	0.07 ± 0.25
VGLUT	0.25 ± 0.44	0.60 ± 0.67	0.88 ± 0.60	0.92 ± 0.79	9.07 ± 2.35	1.68 ± 0.75	0.48 ± 0.51	0.30 ± 0.46	0.12 ± 0.33	0.20 ± 0.41

DM: Dorsomedial; DL: Dorsolateral; MAC: Müller axons associated cells; MDLAC: Medium-sized dorsolateral axons associated cells; MTAC: Mauthner axons associated cells.

Table 2. Cell sizes of main glutamatergic populations.

	SIZES OF CELLS (µm)					
	LARVA			ADULT		
	DI	SLP	LLP	DI	SLP	LLP
ROSTRAL	9.2 ± 1.4	9.5 ± 1.0	14.4 ± 2.3	16.8 ± 2.4	21.1 ± 3.1	45.6 ± 6.5
MEDIAL	9.7 ± 1.7	9.6 ± 1.6	16.1 ± 3.0	15.2 ± 1.8	16.4 ± 2.2	26.9 ± 5.2
CAUDAL	6.2 ± 0.8	6.9 ± 0.9	11.1 ± 1.5	NOT DONE	NOT DONE	NOT DONE

DI: dorsal interneurons; LLP: large lateral population; SLP: small lateral population.

Table 3. Percentages of glutamate-ir cells showing GABA colocalization.

	LARVA		ADULT	
Spinal level	DI	SLP	DI	SLP
ROSTRAL	38% (n=138)	27% (n=322)	22% (n=268)	17% (n=420)
MEDIAL	28% (n=144)	21% (n=371)	18% (n=117)	12% (n=276)
CAUDAL	18% (n=243)	19% (n=637)	NOT DONE	NOT DONE

DI: Dorsal interneurons; SLP: Small lateral population.

Table 4. Percentages of glutamate-ir cells showing glycine colocalization.

	LARVA		ADULT	
Spinal level	DI	SLP	DI	SLP
ROSTRAL	19% (n=172)	24% (n=335)	20% (n=184)	19% (n=297)
MEDIAL	23% (n=65)	17% (n=193)	14% (n=210)	16% (n=335)
CAUDAL	25% (n=221)	22% (n=441)	NOT DONE	NOT DONE

DI: Dorsal interneurons; SLP: Small lateral population.

Figure 1. Schematic draws showing the distribution of glutamatergic cell types in rostral (A), middle (B), dorsal fin (C) and caudal fin (D) spinal levels. A: Major VGLUT-positive cell types in rostral levels of the larval spinal cord. Primary sensory neurons or dorsal cells (black asterisks); dorsal interneurons (black squares); cells of the lateral population (gray circles); cerebrospinal fluid-contacting cells (CSFc) cells (black circles); edge cells (gray squares); cells associated to reticulospinal and Mauthner axons (black stars). **B:** Schematic draw showing the distribution of the major VGLUT-positive cell types in middle levels of the larval spinal cord. Cell type symbols as in Figure 1A. **C:** VGLUT-positive neuronal populations in the dorsal fin region; same symbols as in Figure 1A. **D:** Schematic draw showing the VGLUT-positive neuronal populations in the caudal fin region; same symbols as in Figure 1A. Scale bars = 100 µm (A); 50 µm (B, C, D).

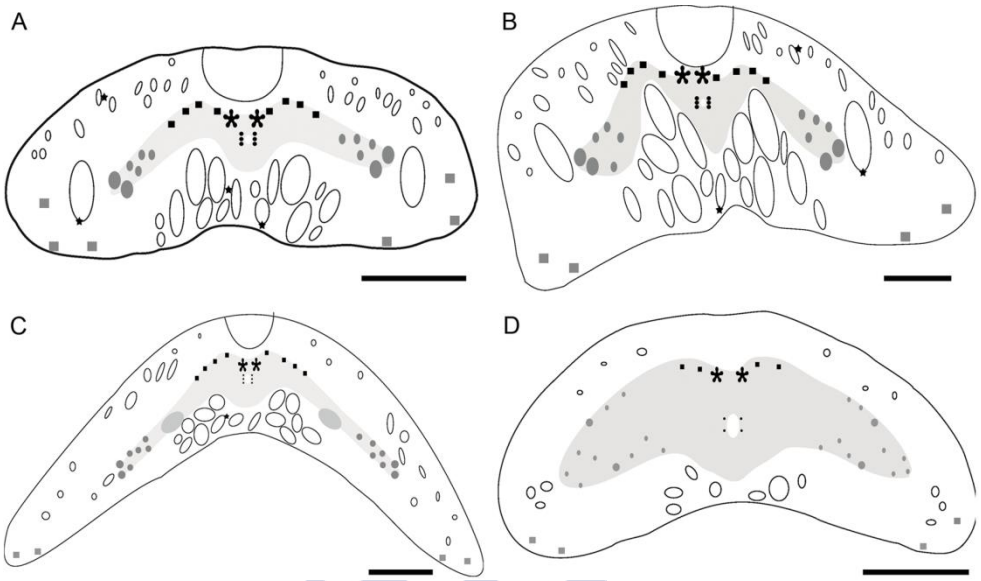


Figure 1

Figure 2. Lamprey vesicular glutamate transporter (VGLUT) expression in transverse sections of spinal cord of larvae. High magnification photomicrographs of larva showing details of VGLUT-positive cells (arrows) in the spinal gray (A-F) and white matter (G-K). **A, B:** Dorsal cells in the rostral (A) and caudal (B) level of the cord. Star indicates the central canal. **C:** Dorsal interneurons. **D:** Small and large lateral interneurons. **E:** CSFc cells, star indicates the central canal. **F:** VGLUT-positive giant cell. **G:** VGLUT-positive edge cell situated laterally. **H:** VGLUT-positive edge cells. **I:** Glutamatergic neuron situated ventrally to the Mauthner axon (star). **J:** Glutamatergic cell associated to medium-sized axons of the lateral column. **K:** VGLUT-positive cells situated ventrally to Müller axons. Dorsal is at the top. Lateral is on the left except in D, G, H and K, in which lateral is on the right. A, D, G, I, J and K correspond to the rostral spinal cord; C, E, F and H correspond to the dorsal fin level; B corresponds to the caudal fin level. Scale bars = 10 µm (A, B, C, D, E, F, I, J, K); 5 µm (G, H).

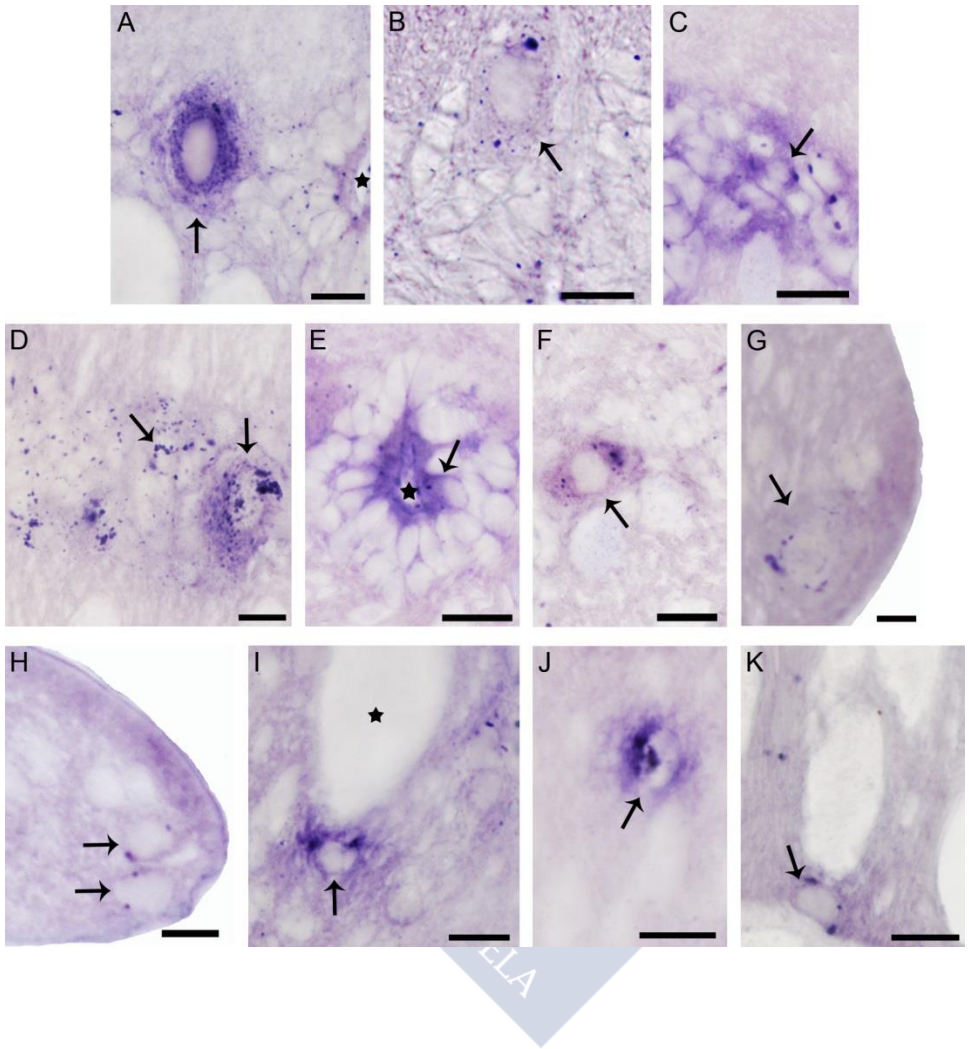


Figure 2

Figure 3. Lamprey vesicular glutamate transporter (VGLUT) expression in transverse sections of spinal cord of adults. High magnification photomicrographs of adult showing details of VGLUT-positive cells (arrows) in the spinal gray (A-E) and white matter (F, G). **A:** Dorsal cell. **B:** Dorsal interneurons. **C:** Small and large lateral neurons. **D:** CSFc cells, the star indicates the central canal. **E:** VGLUT-positive giant cell. F: VGLUT-positive edge cell. **G:** VGLUT-positive neurons situated among the Müller axons. Dorsal is at the top. Lateral is on the left except in A and C in which lateral is on the right. A, C, D, F and G correspond to the rostral spinal cord; E corresponds to the dorsal fin level; B corresponds to the caudal fin level. Scale bars = 10 µm.

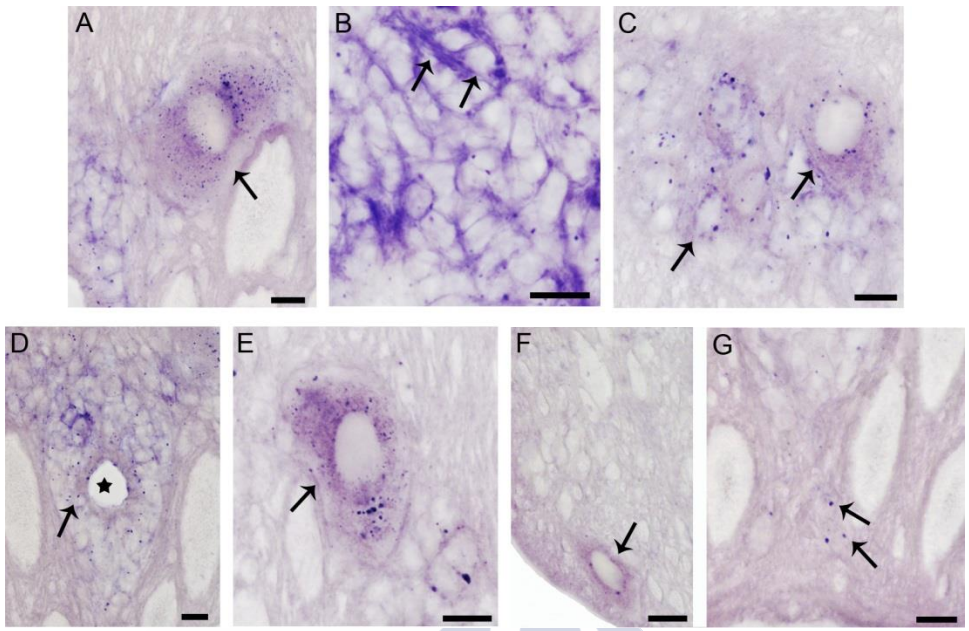


Figure 3

Figure 4. Glutamate immunoreactivity in transverse sections of the sea lamprey spinal cord. High magnification photomicrographs of larvae and adults showing details of glutamate-ir cells and processes in the spinal cord. **A:** Dorsal cells (arrowhead), DC indicate the dorsal column. **B, C:** Dorsal interneurons. **B:** Glutamate-ir cell with thick (dendrite) and thin (axon) processes directed to the dorsal column. **C:** Glutamate-ir bipolar cell showing ventrolateral (barbed arrowhead) and ventromedial (pointed arrowheads) processes. **D:** Glutamate-ir small (arrow) and large (arrowhead) neurons of the lateral gray. **E:** Lateral cell with a process surrounding the Mauthner axon (star). **F:** Glutamate-ir cells (arrows) of the dorsal population showing processes directed to the dorsal column (DC) and a dendrite (arrowheads) that crosses the midline (dashed line) dorsally. **G:** Detail of a glutamate-ir bipolar ventrolateral cell (arrow) of the caudal region of the cord. **H:** Cerebrospinal fluid-contacting (CSFc) cells (arrowhead). The star indicates the central canal. **I:** Glutamate-ir cerebrospinal fluid-contacting (CSFc) cells of an adult. **J:** Strong glutamate-ir primary sensory dorsal cells. **K:** Giant glutamate-ir cell. **L:** Glutamate-ir edge cells (arrows) and edge neuropil (asterisk). **M:** Glutamate-ir edge cell (arrow) laterally. The white star indicates the Mauthner axon. **N:** Positive neuron (arrow) situated ventrally to the Mauthner axon (white star). **O:** Glutamate-ir cell (arrow) associated to medium-sized axons of the lateral column (White star: Mauthner axon). **P, Q:** Glutamate-ir cells (arrows) situated among (P) and ventrally (Q) to Müller axons. **R:** Glutamate-ir cells situated in the ventromedial region of the white matter. Note also CSFc cells around the central canal. Note that the giant Müller axons (in C, R) are apparently glutamate-negative. In all figures dorsal is at the top. Lateral is on the left except in B, C, D, G and K, in which lateral is on the right. I, J, K and R correspond to adult individuals. A, B, C, E, H, J, M, N, O, P correspond to the rostral spinal cord; D, I, L, Q, R correspond to the middle spinal cord; K corresponds to the dorsal fin level; F, G correspond to the caudal fin level. Scale bars = 100 µm (I, J, R); 50 µm (K); 20 µm (A, B, C, D, E, L, M, N, O, P Q); 10 µm (F, G, H).

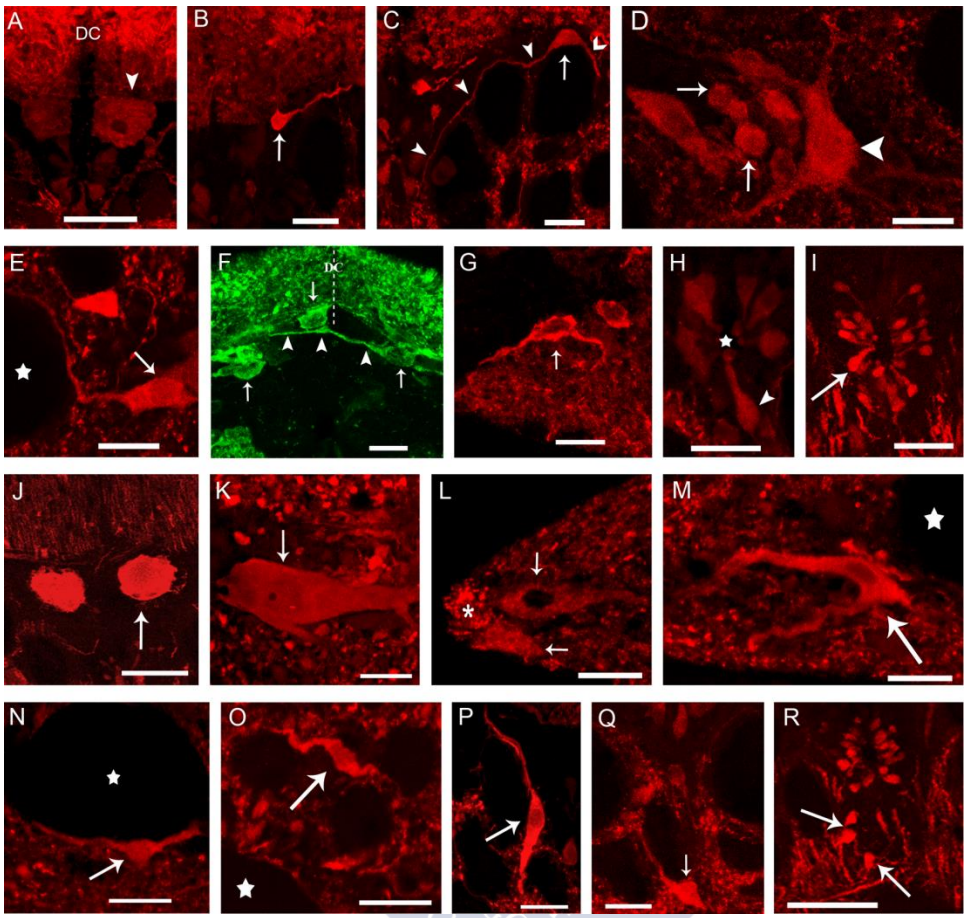
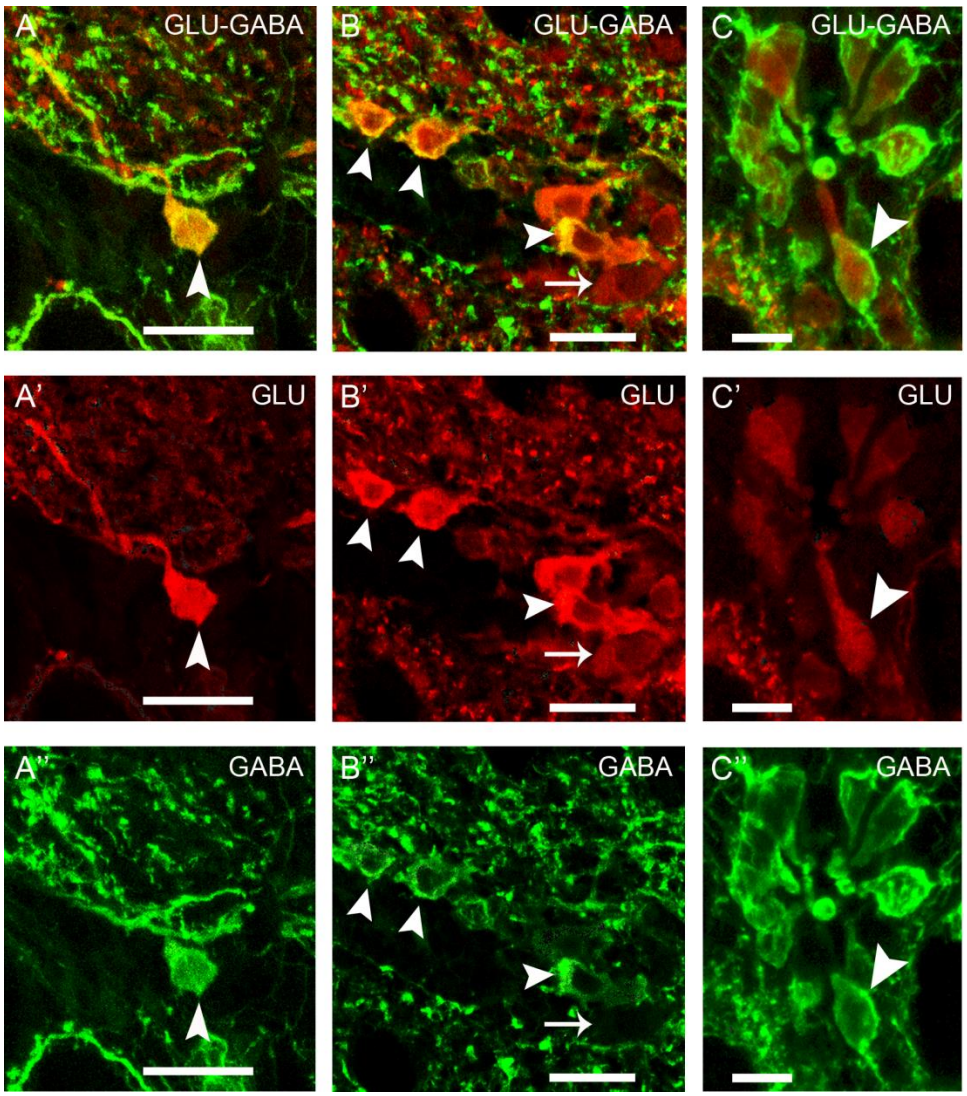


Figure 4

Figure 5. Glutamate and GABA colocalization in the lamprey spinal cord. Confocal photomicrographs of transversal sections through the rostral spinal cord of larvae showing double immunolabelled cells (arrowheads) for glutamate and GABA. Arrows point to single immunolabelled cells. **A-A'':** Dorsal glutamate-ir population. **B-B'':** Lateral glutamate-ir population. **C-C'':** Cerebrospinal fluid contacting cells. Dorsal is at the top. Lateral is on the right except for A-A'', in which lateral is on the left. A, B, C: Overlay; A', B', C': Glutamate; A'', B'', C'': GABA. Scale bars = 20 µm (A-B''); 10 µm (C-C'').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GABA

Figure 5

Figure 6. Glutamate and GABA colocalization in the lamprey spinal cord. **A-B'':** Confocal photomicrographs of transversal sections through the medial and caudal spinal cord of larvae showing double immunolabelled cells (arrowheads) for glutamate and GABA. Arrows point to single immunolabelled cells. A-A'': Medial spinal cord. B-B'': Caudal spinal cord. **A-A'':** Dorsal glutamate-ir population. **B-B'':** Lateral glutamate-ir population. **C-C'':** Confocal photomicrograph of a transverse section through the rostral spinal cord of a larva showing the glutamate-ir processes (curved arrow) surrounded by the GABA-ir fibres constituting the marginal neuropil (arrowhead). Dorsal is at the top. Lateral is on the right. A, B, C: Overlay; A', B', C': Glutamate; A'', B'', C'': GABA. Scale bars = 20 µm (C-C''); 10 µm (A-B'').

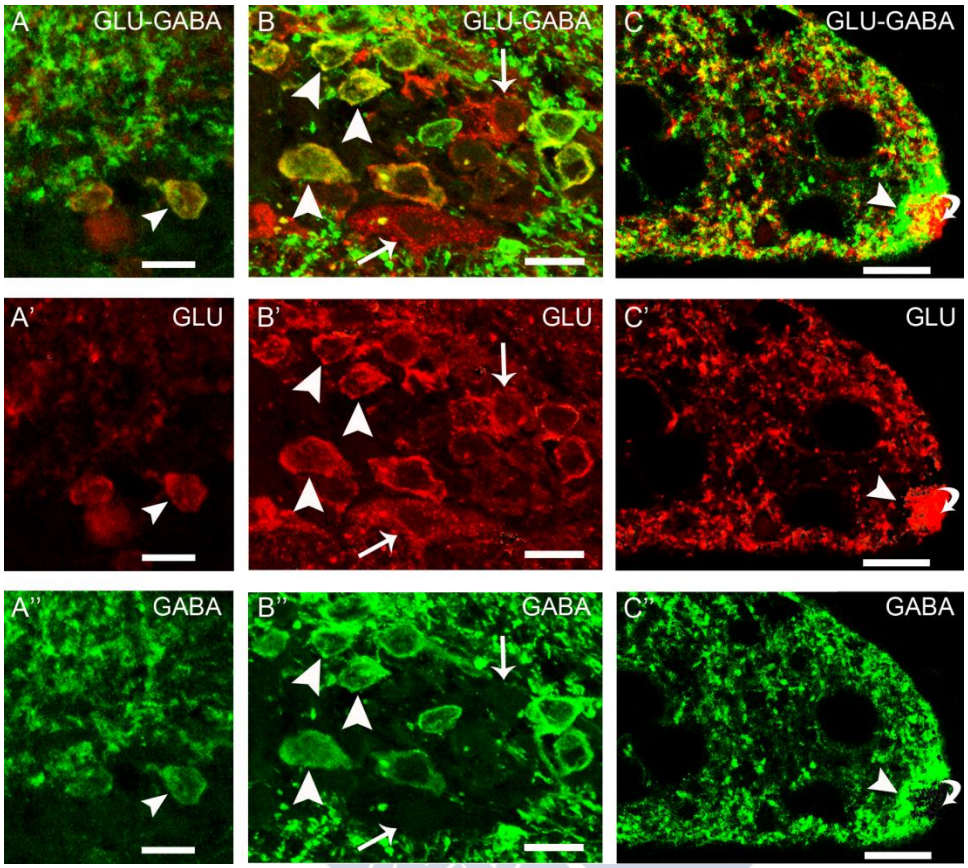
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Figure 6

Figure 7. Glutamate and glycine colocalization in the lamprey spinal cord. Confocal photomicrographs of transversal sections through the rostral spinal cord of larvae showing double immunolabelled cells (arrowheads) for glutamate and glycine. **A-A'':** Dorsal glutamate-ir interneurons. **B-B'':** Lateral glutamate-ir population. **C-C'':** Cerebrospinal fluid contacting cells. **D-D'':** Edge cell and marginal neuropil. Arrowheads point to double immunolabelled cells and arrows point to single glutamate-ir cells. Dorsal is at the top. Lateral is on the left except for A-A'', in which lateral is on the right. Scale bars = 10 µm (A-A', C-D''), 20 µm (B-B'').

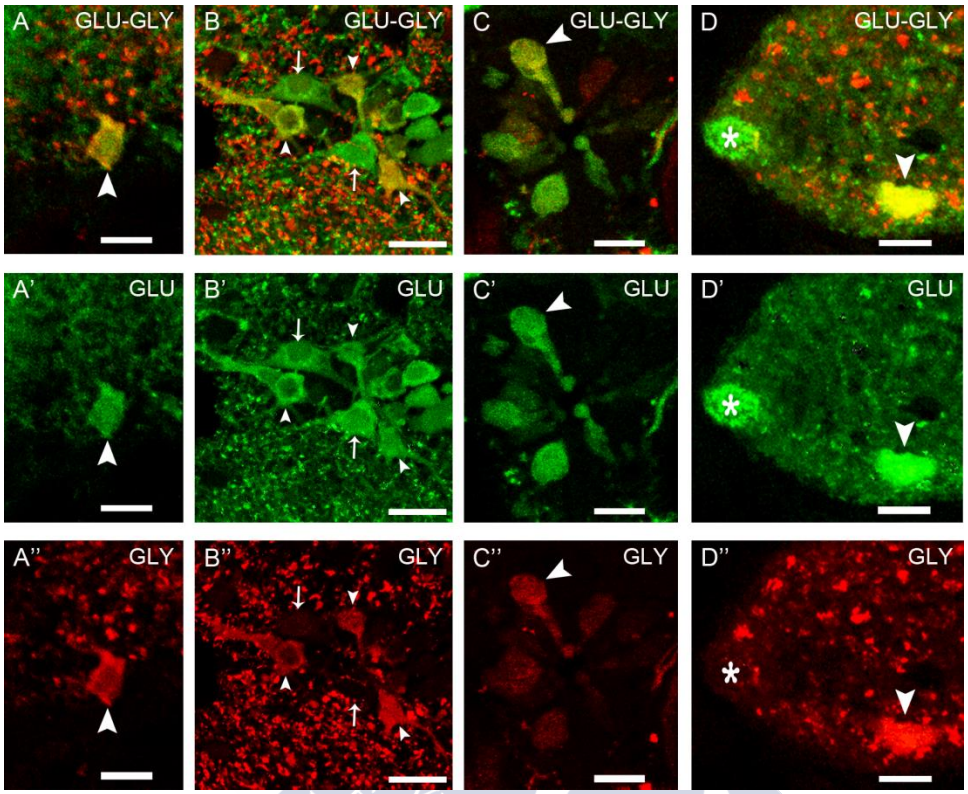

Figure 7

Figure 8. Glutamate and glycine colocalization in the lamprey spinal cord. Confocal photomicrographs of transversal sections through the medial (A-A'') and caudal (B-B'') spinal cord of larvae showing double immunolabelled cells (arrowheads) for glutamate and glycine. **A-A'':** Lateral glutamate-ir population. **B-B'':** Edge cell and marginal neuropil. Note that the glutamate-ir marginal neuropil mostly lacks glycine immunoreactivity (asterisk). Arrowheads point to double immunollabeled cells and arrows point to single glutamate-ir cells. Dorsal is at the top. Lateral is on the left in A-A'', and on the right in B-B''. Scale bars = 10 µm.

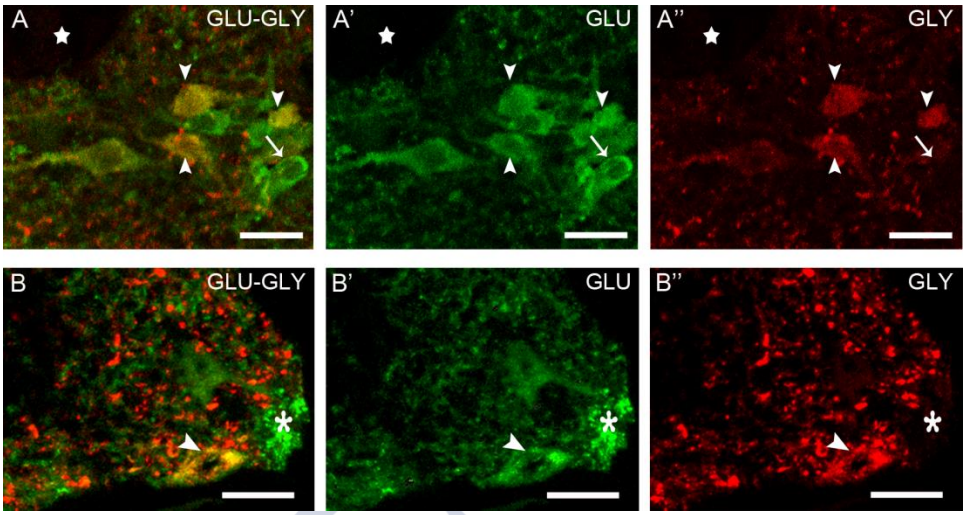

Figure 8

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Chapter 2

Neuronal release and successful astrocyte uptake of glutamate following complete spinal cord transection in lampreys

NEURONAL RELEASE AND SUCCESSFUL ASTROCYTE UPTAKE OF GLUTAMATE FOLLOWING COMPLETE SPINAL CORD TRANSECTION IN LAMPREYS

Introduction

Spinal cord injury (SCI) leads to loss of function and sensitivity below the lesion site in non-regenerating vertebrates (e.g. mammals) and, so far, an appropriate therapy does not exist. In other vertebrates (regenerating vertebrates), such as lampreys (Wood and Cohen, 1979; Yin and Selzer, 1983; Cohen et al., 1986), fishes (Bernstein and Gelderd, 1970; Coggeshall et al., 1982; Bunt and Fill-Moebs, 1984; Becker et al., 1997) and amphibians (Lee, 1982; Davis et al., 1990) there is a process of spontaneous axonal regeneration after SCI leading to functional recovery. Lampreys have been used as a model of spontaneous spinal regeneration since the late 1950s (Maron, 1959). They are the only vertebrates that have been shown to satisfy the 5 NIH criteria to demonstrate functional regeneration after SCI (Guth et al., 1980; Rodicio and Barreiro-Iglesias, 2012). The high regenerative ability of identifiable descending (reticulospinal) neurons confers them several advantages respect to other models for studying the factors that impede/facilitate spinal regeneration. In lampreys, functional recovery after SCI is achieved not only due to regeneration processes, but also to plastic and neuronal reorganization events (Rodicio and Barreiro-Iglesias, 2012). This is very important because recovery after SCI in humans will also rely on neuronal circuit reorganization (Esclarín-De Ruz et al., 2009). Interestingly, a recent study using TUNEL and Fluoro Jade staining has shown that there is an almost complete lack of cell death in the sea lamprey spinal cord during

the first month after a complete spinal cord transection (Shifman et al., 2012).

In mammals, SCI causes a secondary response in the spinal cord that involves the release of glutamate and a subsequent astrocyte reaction. Reactive astrocytosis after central nervous system (CNS) injury appears to be a conserved phenomenon throughout vertebrate evolution (Larner et al., 1995). Because of that, it has been suggested that this process could have selective survival advantages in mammals (Faulkner et al., 2004; Karimi-Abdolrezaee and Billakanti, 2012). However, in spite of this, it also leads to some detrimental effects like being an obstacle for axonal regeneration (Karimi-Abdolrezaee and Billakanti, 2012). Beneficial activities of astrocytes include clearance of glutamate and other neurotransmitters (Seifert et al., 2006), which could protect neurons from excitotoxicity and cell death (Eulenburg and Gomeza, 2010). The astrocytes of lamprey express cytokeratins (Merrick et al., 1995), whereas, glial fibrillary acid protein (GFAP) is only expressed by ependymal cells of the central canal (Wasowicz et al., 1994). Even though a glial scar is present in lampreys after SCI, it creates a permissive environment for axon regrowth (Selzer, 1978). A system of inactivation of synaptically released glutamate is also present in lampreys (Brodin and Grillner, 1985; Gundersen et al., 1995) and the astrocytes appear to be involved in neurotransmitter clearance, like in mammals (Baudoux and Parker, 2008). Special properties of the astrocytes of lampreys could also be one of the reasons that lead to a lack of cell death after SCI in the lamprey spinal cord (Shifman et al., 2012).

Glutamate release after SCI causes glutamate excitotoxicity (Liu et al., 1991), which can lead to neuronal and oligodendrocyte death (Liu et al., 1999; Xu et al., 2004). Glutamate uptake by astrocytes could protect neurons and oligodendrocytes from excitotoxicity. However, in mammals, a decrease in GLT1 (EAAT2) (the main glutamate transporter of astrocytes) expression has been observed for at least 2 weeks following SCI (Lepore et

al., 2011), while the extracellular levels of glutamate rise to toxic levels in minutes (Liu et al., 1991).

The process of glutamate release after SCI has not been studied in any regenerating species and a direct histological observation of this phenomenon has not been reported in any vertebrate either. Knowing how regenerating vertebrates deal with this process could clearly help to propose new lines of research or therapies to protect the injured spinal cord from secondary damage. We aimed to investigate the release of glutamate in response to SCI and the subsequent astrocytic response in the sea lamprey. Specifically, we have studied: (1) the short-term changes in cytokeratins expression (a marker of astrocytes in lampreys) in response to a complete spinal cord transection, (2) the changes in glutamate immunoreactivity that take place in the first week after SCI, (3) the colocalization of glutamate with cytokeratins to investigate whether glutamate is taken up by the astrocytes of lampreys after SCI and (4) the effect of DL-TBOA (an inhibitor of glutamate transporters) on the active uptake of glutamate by astrocytes after SCI. We present evidence showing that the astrocytes of lampreys have a better response against glutamate release than those of mammals.

Material and methods

Ethical statement

All experiments were approved by the Bioethics Committee at the University of Santiago de Compostela and conformed to the European Union and the Spanish guidelines on animal care and experimentation.

Subjects

Mature and developmentally stable larval sea lampreys, *Petromyzon marinus L*. (n= 45; between 80 and 156 mm in body length, 5 to 7 years of age), were used in the present study. Animals were collected from the river Ulla (Galicia, north-western Spain) with permission from the Xunta de Galicia and maintained in aerated fresh water aquaria at 15 ºC with a bed of river sediment until their use for experimental procedures.

Spinal cord transection

Animals were deeply anaesthetized by immersion in 0.1% tricaine methanesulphonate (MS-222 ; Sigma, St. Louis, MO) in Ringer solution (pH 7.4) of the following composition: 137 mM NaCl, 2.9 mM KCl, 2.1 mM $CaCl_2$, 2 mM HEPES. Larvae were placed with their dorsal side up in a Sylgard-lined dish filled with Ringer solution. The spinal cord was exposed by a transverse incision made on the body wall from the dorsal midline at the level of the 5th gill. A complete spinal cord transection was performed with a scalpel at this spinal level, and the spinal cord cut ends were visualized under a microscope. After surgery, lampreys were maintained on ice for 1 hour to allow the wound to air dry (except for those processed immediately after the transection). During this hour, the animals were maintained in a paper towel soaked with Ringer solution not in direct contact with the ice. After this time, the animals were allowed to recover in aerated freshwater tanks at 19.5 ºC. Lampreys are known to be able to recover normal appearing locomotion at this temperature (Cohen et al., 1999). Each transected animal was examined 24 hours after surgery to confirm that there was no movement caudal to the site of injury. A spinal transection was considered complete if on the stimulation of the head, the animal could move only its body rostral to the lesion site.

Tissue collection and processing

After the different recovery periods [0 (n=8), 1 (n=8), 2 (n=6), 3 (n=6), 4 (n=5) and 7 days after injury (n=5)], animals were deeply anaesthetized with 0.1% MS-222 in Ringer solution and killed by decapitation. The body of the animal was fixed by immersion in 5% glutaraldehyde and 1% sodium metabisulfite (MB) in 0.05 M Tris-buffered saline (TBS; pH 7.4) for 20 hours. After fixation, the body region comprising the spinal cord between the 4th and the 6th gills (i.e. 3.5 mm rostral and caudal to the site of injury) was cut and embedded in Neg 50™ (Microm International GmbH, Walldorf, Germany), frozen in liquid nitrogen-cooled isopentane, sectioned on a cryostat in the transverse plane (14 µm thick) and mounted on Superfrost ® Plus glass slides (Menzel, Braunschweig, Germany).

Immunofluorescence

For immunofluorescence, sections were pretreated with 0.2% $NaBH_4$ in deionized water for 45 minutes to quench autofluorescence. Sections were incubated with a cocktail of a rabbit polyclonal anti-glutamate antibody (Immunosolution; 1:4.500) and a mouse monoclonal anti-cytokeratin (LCM29) antibody (supplied by Dr. Selzer, Philadelphia; 1:100) or a mouse anti-HuC/HuD antibody (Molecular Probes Europe, Leiden, The Netherlands; 1:100) in 0.05 M TBS with 1% MB during 3 days at 4ºC. After rinsing in TBS with 1% MB, sections were incubated for 1 hour at room temperature with Cy3- conjugated goat anti-rabbit immunoglobulin (Chemicon, Temecula, CA; 1:100) and fluorescein-conjugated goat anti-mouse immunoglobulin (Millipore, Temecula, CA; 1:100) rinsed in TBS and mounted with Mowiol. All antibodies were diluted in TBS (pH 7.4) containing 0.2% Triton X-100 and 15% normal goat serum.

Antibodies

The polyclonal anti-glutamate antibody was raised in rabbit against a glutamate- glutaraldehyde-porcine thyroglobulin conjugate. The antibody has been tested by the supplier in sections of retina and cerebellum from various vertebrates, as well as in dot blot immunoassays with a variety of amino acid-protein conjugates. These include the standard 20 amino acids found in proteins, the non-protein amino acids D-serine, D-alanine and D-aspartate, GABA and the glycine containing tripeptide glutathione, which did not yield significant cross reactivity. This antibody has been developed by Dr David V. Pow (University of Newcastle, New South Wales, Australia) and used in previous studies of the lamprey brain and spinal cord (Villar-Cerviño et al., 2011, 2013; see chapter 1). This antibody did not stain any sea lamprey brain native protein band (Villar-Cerviño et al., 2011).

The mouse monoclonal anti-cytokeratin antibody (LCM 29) is a lamprey specific antibody that has been previously characterized (Merrick et al., 1995) and used in studies about the spinal cord of lampreys (Lurie et al., 1994; Uematsu et al., 2004; Vidal Pizarro et al., 2004).

The mouse monoclonal anti-HuC/HuD antibody was raised against the human neuronal proteins HuC and HuD and, as the supplier stated, it binds specifically to antigens present exclusively in neuronal cells and are thus useful as marker of neuronal cells in tissue. It has been also used in lampreys as neuronal marker (Zhang et al., 2014).

Treatment with an inhibitor of glutamate transporters

Animals were deeply anesthetized by immersion in 0.1% MS-222 (Sigma, St. Louis, MO) in Ringer solution and were placed with their dorsal side up in a Sylgard-lined dish filled with Ringer. The skin, the muscles and

the meninx of the region comprised between the 3rd and the 7th gill were carefully removed from the top of the spinal cord; therefore, the spinal cord was completely exposed. Then, the animals were maintained in Ringer solution with DMSO (n=3) or in 30 μM DL-*threo*-β-Benzyloxyaspartic acid (DL-TBOA; Tocris Bioscience, UK) diluted in DMSO in Ringer during 4 hours (n=4). The inhibitor DL-TBOA is a highly selective blocker of excitatory aminoacid transporters, especially for EAAT1, EAAT2 and EAAT3 (Shimamoto et al., 1998). After this incubation period, a complete spinal cord transection was performed and the animals were processed immediately after the transection as explained above.

Image acquisition

After immunofluorescence the sections were photographed and analysed with the spectral confocal microscopes TCS-SP2 and SP5 (Leica, Wetzlar, Germany). Stacks of photographs were processed with LITE and LAS software (Leica). Photographs were minimally adjusted for brightness and contrast with Adobe Photoshop CS4 software.

Results

In control larval sea lampreys, with no SCI, the spinal cord at the level of the 5th gill is flattened and the central canal has a regular rounded profile. From 4 days post-lesion (dpl) some morphological changes were observed in the central canal. In the first 400 to 600 μm rostral and caudal from the injury site, the central canal was enlarged and acquired an irregular shape, which is in agreement with previous reports (Selzer, 1978).

The expression of glutamate and cytokeratins in cells of the spinal cord of lampreys has been already described (glutamate: Shupliakov et al., 1992; Mahmood et al., 2009; see chapter 1; cytokeratins: Lurie et al., 1994, Merrick et al., 1995) and can be observed in Figure 1. Briefly, in control larval sea lampreys glutamate (Fig.1A, C-E) immunoreactivity was observed in cells of the dorsal (Fig. 1A) and lateral (Fig. 1C, D) grey populations and in cerebrospinal fluid contacting (CSFc) cells (Fig. 1E). Dorsal primary sensory cells (not shown) and individual cells located in the white matter (Fig. 1D), including edge cells, were glutamate inmunoreactive (-ir). The anti-cytokeratin antibody labelled the cell bodies of astrocytes located in the grey matter (Fig. 1G), the lateral funiculus (Fig. 1F), the perimeter of the giant axons and also the astrocytic processes that course radially (Fig. 1B, F). No cells around the central canal showed cytokeratin expression (Fig.1B). Figure 2 shows colocalization of glutamate with Hu (neuronal marker) (Fig. 2A-A'') and the absence of colocalization of this neurotransmitter with cytokeratins (Fig. 2B-B'') in control animals. This indicates that glutamate is probably present in astrocytes at very low levels, which precludes the observation of immunolabelling in control animals.

Spinal cord injury led to a clear decrease in the expression of glutamate in neurons and to its accumulation in astrocytes in the regions adjacent to the lesion site. Glutamate immunoreactivity was progressively maintained in neurons in more distal regions from the site of injury. The changes observed during the first week after the complete spinal cord transection are shown in Figures 3, 4 and 5.

Neuronal and astrocytic acute response on the day of injury

At 0 dpl (immediately after the injury), glutamate was released from neurons and their processes and was accumulated in astrocytes in the rostral and caudal regions close to the injury site. Neuronal cell bodies

and fibres were not glutamate-ir in the first 500 µm rostrally (Fig. 3A) and caudally (Fig. 3B) from the lesion site, whereas astrocytes and glial processes showed now glutamate immunoreactivity (Fig. 4A-B''). In more distal regions (from 500 µm to 3 mm from the injury site), presence of glutamate immunoreactivity in neuronal somata and processes increased progressively from the lesion site, whereas astrocytes were glutamate-negative (Fig. 3C (rostral); 3D (caudal)).

Based on this observations and to investigate whether the astrocytes actively uptake neuronal glutamate in response to SCI, complete spinal cord transections were carried out in lampreys that were previously treated with DL-TBOA for 4 hours. Absence of glutamate immunoreactivity in neurons was also observed in the first 500 µm rostrally and caudally to the site of injury in control and treated animals. However, absence of glutamate immunoreactivity in the astrocytes was observed (Fig. 3F-F'') in DL-TBOA treated animals in contrast to controls, which showed glutamate immunoreactivity in the astrocytes (Fig. 3G-G''). This indicated that glutamate is actively accumulated in astrocytes by the action of glutamate transporters after SCI.

The usual pattern of expression of glutamate immunoreactivity observed in the spinal cord of control samples was observed from 3 mm from the transection site (data no shown). Glutamate immunoreactivity was observed in at least some cells of all the populations (dorsal, lateral and CSFc) from 500 µm, both rostral and caudal to the lesion site. Glutamate-ir primary sensory dorsal cells were observed from 600-900 µm from the injury site. Glutamate-ir edge cells were observed for the first time at 600 µm from the lesion site. These edge cells appear to maintain better their glutamate expression rostrally to the lesion site, where they were observed as often as in control samples, while caudally to the injury, they were only occasionally observed. The rest of white matter cells were observed occasionally as in control larvae from 1 mm from the lesion site.

At 0 dpl, the complete spinal cord transection also led to changes in cytokeratin expression that extended for about 500 µm rostral and caudal to the lesion site. The astrocytes became reactive acquiring a more rounded profile with wider processes and showing intense immunoreactivity (Fig. 4C). Some processes of astrocytes showed a beaded appearance (Fig. 4D, E). From the first 500 µm from the site of injury onwards, the cytokeratin expression was rather similar to that observed in control animals [Fig. 4F (rostral) and 4G (caudal)], although, processes with beaded appearance were occasionally observed (Fig. 4H).

Changes from 1 to 7 dpl.

The presence of glutamate immunoreactivity in astrocytes was observed during the first six days following the complete SCI (Fig. 5A) in the regions close to the site of injury. However, the portion of the spinal cord in which astrocytes showed glutamate immunoreactivity became progressively shorter over the week reaching only 40-80 µm rostrally and caudally from the lesion site at 7 dpl. At 7 dpl glutamate-ir astrocytes cell bodies were not observed, but glial processes and end feet still were glutamate-ir in the first 40-80 µm from the site of injury (Fig. 5B). A pattern of glutamate immunoreactivity similar to that of control larvae was observed from 3 mm from the transection site in 1-2 dpl animals, from 1.5 mm in 3-4 dpl animals and from 200 µm in 7 dpl animals.

Since 2 dpl, astrocytes began to down-regulate cytokeratin expression leading to an appearance similar to that of the control samples. However, some reactive strongly cytokeratin-ir astrocytes were still observed (Fig. 5C). From 1dpl, at the border of the lesion, processes of astrocytes transversally cut could be observed (Fig. 5D). These processes were more clearly observed at 2 dpl (Fig. 5E) and 7 dpl (Fig. 5F). At 7 dpl, the astrocytes looked like in control larvae, although some kind of

reorganization of the astrocyte processes or of the cytokeratin expression on them was observed; the perimeningeal ends were faintly cytokeratin-ir, whereas perineuronal and periaxonal processes were observed better than in control samples (Fig. 5G).

Discussion

The short-term response in terms of glutamate expression following a complete SCI in the sea lamprey has been studied for the first time. The substances studied, glutamate and cytokeratins (a lamprey glial marker), experienced alterations during the first week following SCI. The main change was observed in the regions adjacent to the lesion site and it mainly consisted of a massive release of this excitatory neurotransmitter by neurons and their capture by astrocytes. In animals without injury, immunoreactivity for glutamate was exclusively observed in neurons, as it is shown by colocalization with Hu immunoreactivity. No colocalization with cytokeratins was observed in control animals, probably reflecting the very low levels of glutamate in astrocytes under normal conditions.

Regarding the expression of cytokeratins, some changes were also observed. Lurie et al., (1994) showed that from 7 dpl, there were no differences in cytokeratins immunoreactivity, which is in agreement with our observations. This was interpreted as a lack of glial reaction after SCI in the sea lamprey. However, we have observed that during the first 2-3 dpl, cytokeratins immunoreactivity increases and the astrocytes appeared enlarged. Similar features together with others, such as molecular and functional changes in astrocytes, have been observed in animals that suffer reactive astrogliosis and glial scar formation (Sofroniew and Vinters, 2010). Present observations in the sea lamprey suggest the occurrence of an astrocytic reaction, which occurs in the first days after SCI.

Glutamate release by neurons and their capture by astrocytes

Cytokeratins-expressing astrocytes have been observed in CNS locations which regenerate well after a lesion (Lurie et al., 1994). Under normal physiological conditions, the concentrations of glutamate in the extracellular space are tightly controlled by some neuronal, EAAC1 (EAAT3) and glial, GLT1 (EAAT2) and GLAST (EAAT1) transporters (for a review see Danbolt, 2001), being GLT1 the physiologically dominant (Rothstein et al., 2005). Glutamate uptake is very important because high extracellular glutamate levels result in excessive activation of glutamate receptors, triggering massive Ca^{2+} influx into cells and leading to neuronal death (Choi, 1988). Following SCI in mammals, a massive release of glutamate that raises it to excitotoxic levels within minutes has been reported (Panter et al., 1990; Liu et al., 1999; McAdoo et al., 1999; Xu et al., 2004). The increased levels of glutamate in the extracellular space has been observed for periods of 3 to 4 days following traumatic injury in humans (Baker et al., 1993; Bullock et al., 1995) and in other mammals it has been shown that it can persist for 2 to 4 weeks after SCI (Olsen et al., 2010; Lepore et al., 2011). It has been shown that glutamate release after CNS injury in mammals is mainly produced by means of reversed uptake (Szatkowski et al., 1990; McAdoo et al., 2000; Rossi et al., 2000), contributing to increase glutamate excitotoxicity. Lepore and co-workers (2011) have also reported the existence of apoptosis of GLT1-expressing astrocytes, down-regulation of GLT1 expression in surviving astrocytes and lack of GLT1 expression in reactive/proliferating astrocytes after SCI in mammals.

In this work, we present clear evidence suggesting a massive release of glutamate from neurons after SCI in lampreys. Moreover, glutamate immunoreactivity was observed in astrocytes during the first week after a complete transection of the cord indicating that glutamate uptake by astrocytes works properly after SCI. Treatment with the glutamate transporter inhibitor DL-TBOA showed that astrocytes of

lampreys actively uptake glutamate after SCI. This shows that in contrast to mammals (Lepore et al., 2011), glutamate transporters of lampreys continue working properly after SCI. A recent study has shown that in lampreys there is an amazing lack of death of intrinsic spinal cord cells in the first days after a complete SCI (Shifman et al., 2012). So, we suggest that the successful and active astrocytic uptake of glutamate could be one of the main reasons to explain the absence of cell death in lampreys after SCI. Further investigation to elucidate why in contrast to mammals the glutamate transporters of astrocytes of lampreys maintain their functionality after SCI is needed. This could provide clues to propose new therapies to prevent secondary damage after SCI.

In summary, the results of our study demonstrate that the astrocytes of lamprey have a high capacity of glutamate uptake after SCI, which is maintained during the first days after a complete transection of the cord. This feature appears to contribute to the impressive regenerative and survival capacity of the spinal cord of lampreys.

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Abbreviations

CNS	Central nervous system
DL-TBOA	DL-*threo*-β-Benzyloxyaspartic acid

dpl	Days post-lesion
EAAC	Excitatory aminoacid carrier
EAAT	Excitatory aminoacid transporter
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate/aspartate transporter
GLT	Glutamate transporter
ir	Immunoreactive
SCI	Spinal cord injury

Figures

Figure 1. Confocal photomicrographs of transverse sections of the spinal cord of the sea lamprey showing glutamate and cytokeratin (CK) immunoreactivities in control animals. **A:** Glutamate immunoreactivity. Outlined arrowheads point to neurons of the dorsal population. Arrows point to neurons of the lateral population. Outlined arrows point to CSFc cells and arrowheads point to white matter neurons. DC indicates the dorsal column. **B:** CK immunoreactivity. Curved arrows point to astrocytes. Note the absence of CK immunoreactivity in cells around the central canal. **C-E:** Details of glutamate-ir neurons (**C:** Lateral population cells. **D:** Lateral cell and cells located in the white matter. **E:** CSF-c cells. Star indicates the central canal). **F, G:** Details of CK immunoreactivity. **F:** Astrocyte in the lateral funiculus. Note the astrocytic radial processes. Outlined curved arrow points to an astrocyte end-foot. **G:** Astrocytes located in the grey matter. **H:** Schematic drawing showing the different neuronal populations observed in the lamprey spinal cord. Orange medium sized circles indicate cells of the dorsal population. Green squares indicate cells of the lateral population. White small circles indicate the CSFc cells. Purple big circles indicate edge cells. Blue stars indicate white matter cells. In all figures dorsal is at the top. Lateral is on the left. Scale bars = 75 μm (A, B, H); 15 μm (C, D, F, G); 10 μm (E).

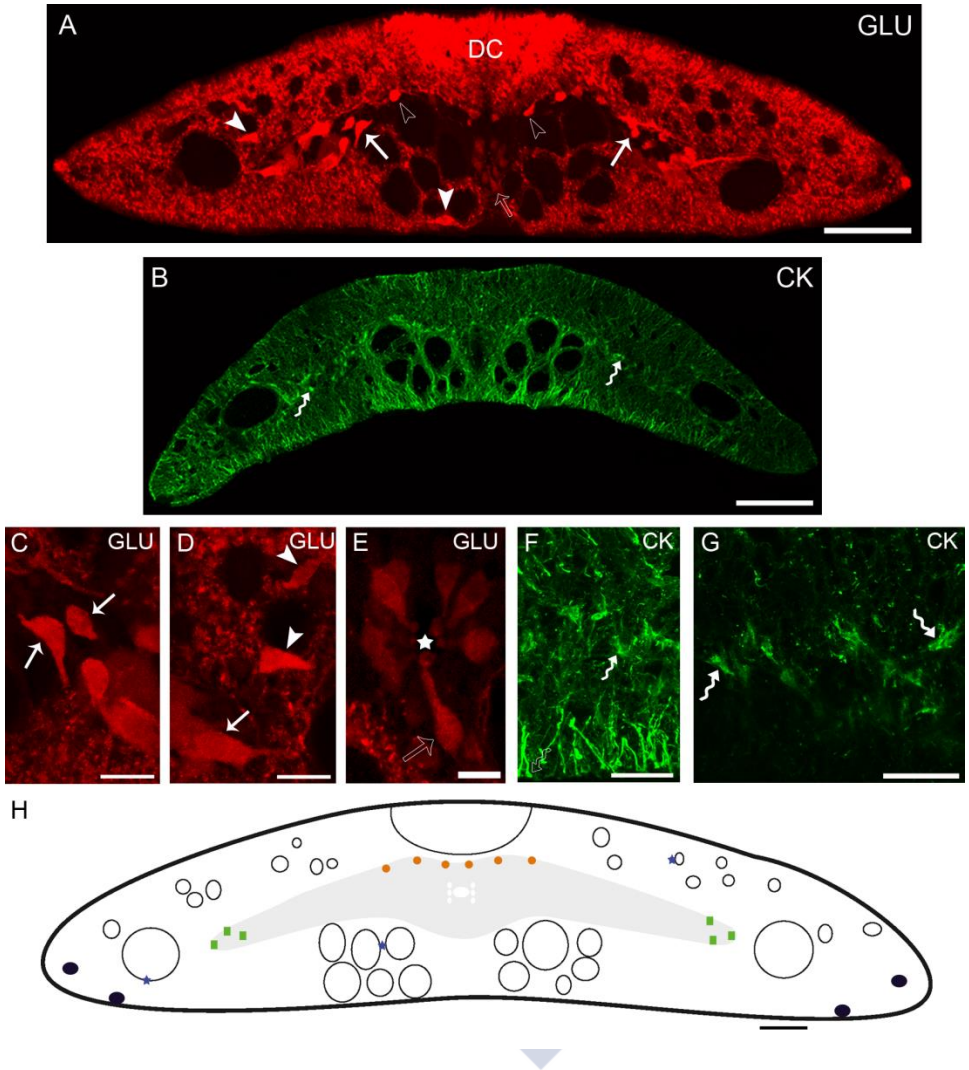

Figure 1

Figure 2. High magnification photomicrographs showing details of double labelled neurons (arrowheads) for glutamate and Hu and absence of colocalization between glutamate and CK in the spinal cord of control lampreys. **A-A'':** Glutamate-ir neurons. Arrows point to Hu single labelled neurons. **B-B'':** Glutamate-ir single labelled neurons (arrows) and singled labelled astrocytes immunoreactive to cytokeratins (arrowheads). In all figures dorsal is at the top. Lateral is on the right in A-A'' and on the left in B-B''. A, B: Overlay; A', B': Glutamate; A'': Hu; B'': CK. Scale bars = 20 µm.

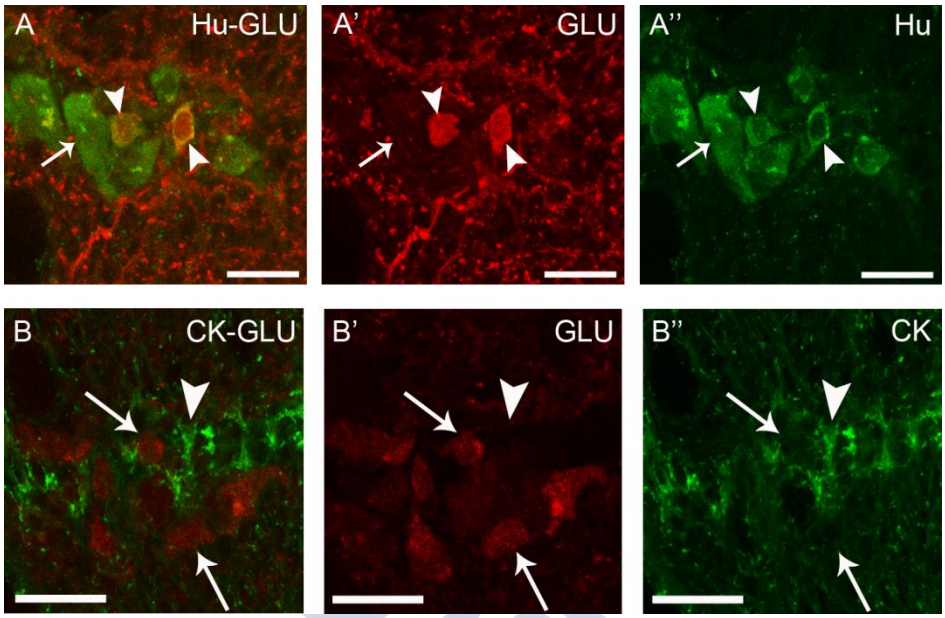

Figure 2

Figure 3. Confocal photomicrographs of transverse sections of spinal cord showing details of glutamate immunoreactivity at 0 dpl in transected animals (A-D), in DL-TBOA treated samples (F-F'') and in not treated samples (G-G''). **A, B:** Neurons and fibres glutamate-negative in the adjacent region to the lesion site, rostral (A) and caudal (B). Note the presence of glutamate-ir astrocytes (arrows). **C, D:** Glutamate-ir neurons (arrows) and fibres in the distal region from the injury site, rostral (C) and caudal (D). Note the absence of glutamate immunoreactivity in astrocytes. DC indicates the dorsal column. **E:** Schematic drawing of a transverse section of the spinal cord showing the locations of the photomicrographs in Figures 3, 4 and 5. **F-F'':** Glutamate negative astrocytes in DL-TBOA treated larval lampreys. **G-G'':** Glutamate-ir astrocytes (arrowheads) in non-DL-TBOA treated larval lamprey. In all photographs, the star indicates the central canal and the asterisk indicates the Mauthner axon. In all figures dorsal is at the top and lateral is on the right. F, G: Overlay; F', G': Glutamate; F'', G'': CK. Scale bars = 75 µm (E); 20 µm (A-D, F-F'', G-G'').

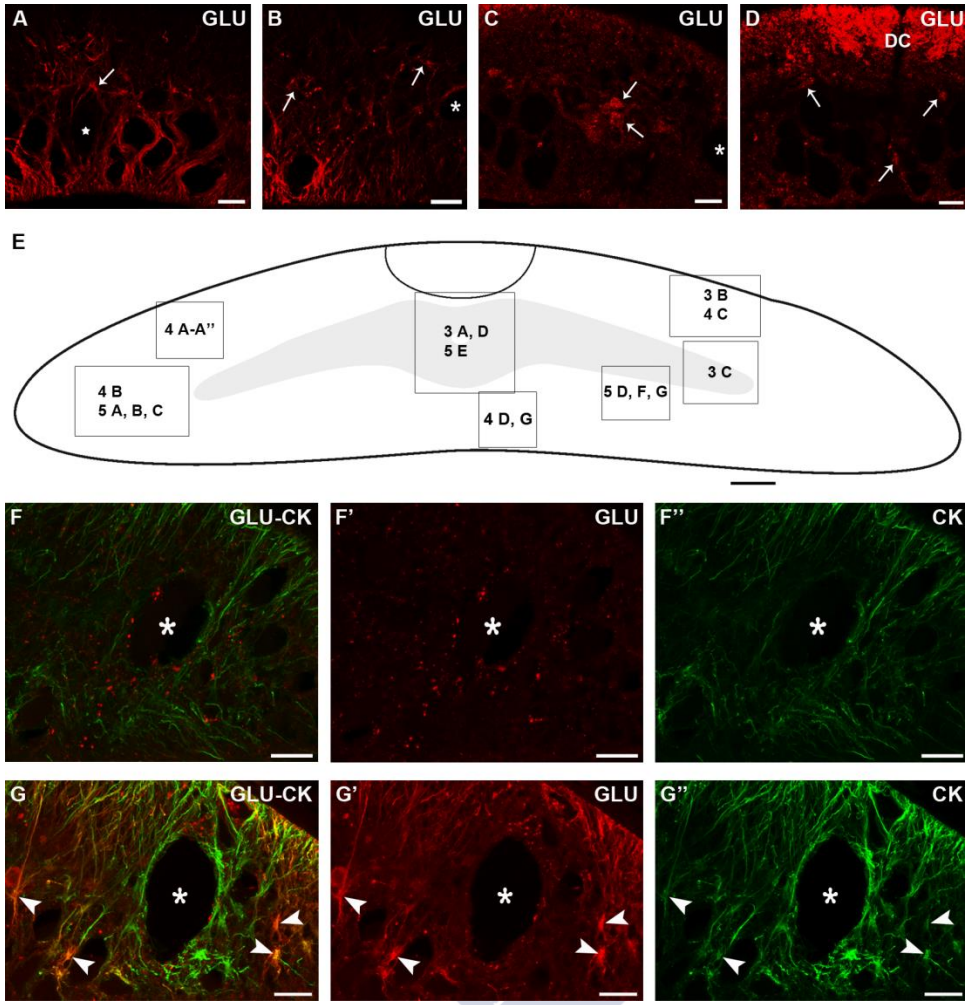
A
GLU
B
GLU
C
GLU
D
GLU
DC
E
4 A-A''
4 B
5 A, B, C
3 A, D
5 E
4 D, G
5 D, F, G
3 B
4 C
3 C
F
GLU-CK
F'
GLU
F''
CK
G
GLU-CK
G'
GLU
G''
CK

Figure 3

Figure 4. High magnification photomicrographs of transverse sections of spinal cord showing details of double immunolabelled astrocytes (arrowheads) for glutamate and CK and details of CK immunoreactivity in larvae processed immediately after the injury. **A-B'':** Glutamate-ir astrocytes. **C:** CK-ir reactive astrocyte (arrow). **D:** CK-ir astrocyte processes with beaded appearance (arrowheads). The star indicates the Mauthner axon. **E:** CK immunoreactivity with a dotted (arrowheads) appearance. Star indicates the central canal. **F, G:** CK immunoreactivity beyond the adjacent region to the injury site, rostral (F) and caudal (G). **H:** CK-ir processes with beaded appearance (arrowheads). The star indicates the central canal. In all figures dorsal is at the top. Lateral is on the right except for A-A'', B-B'', F and H, in which lateral is on the left. A, B: Overlay; A', B': Glutamate; A'', B'': CK. Scale bars = 40 µm (F, G); 20 µm (A-A'',B-B'', C, D, E); 10 µm (H).

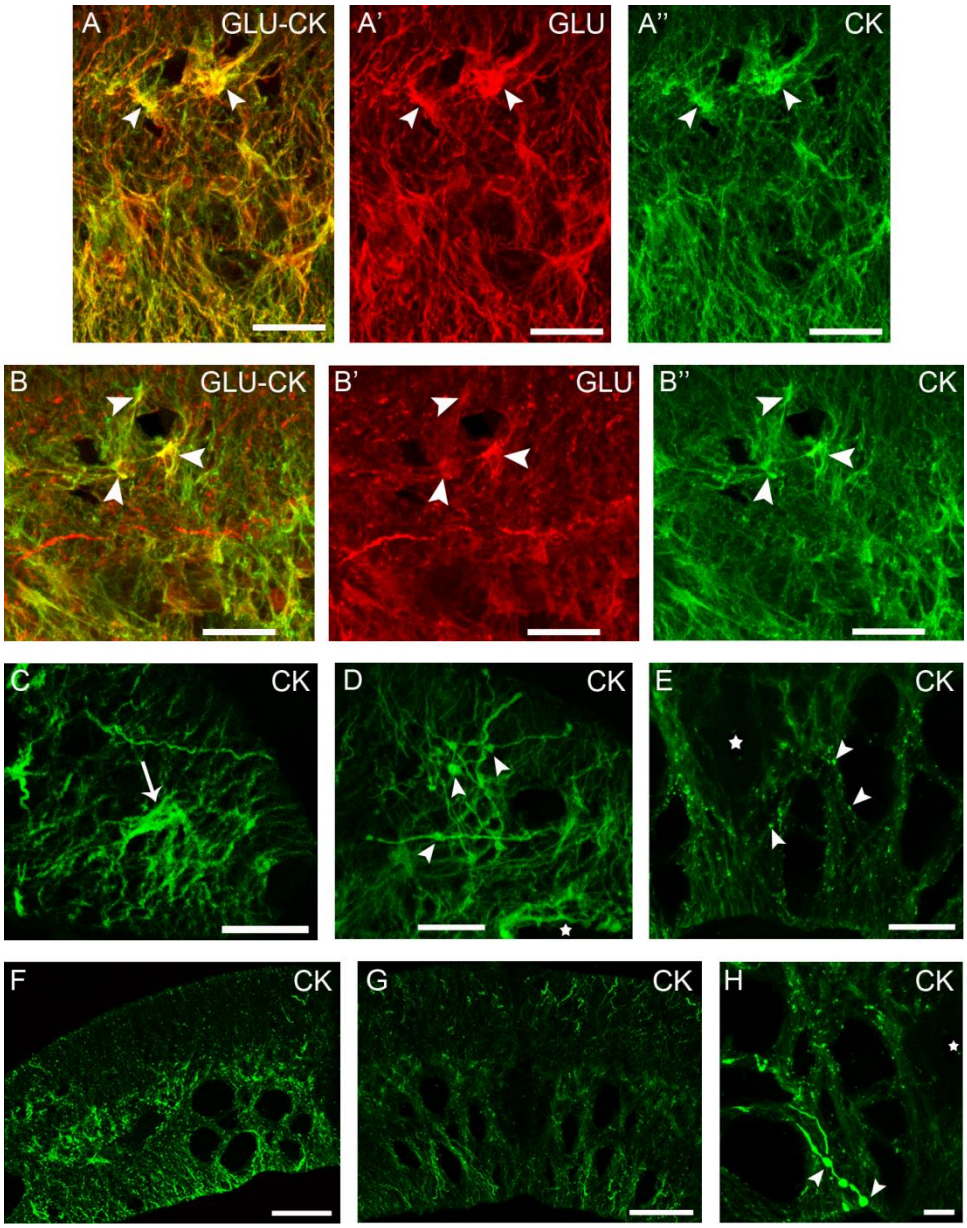

Figure 4

Figure 5. High magnification photomicrographs of transverse sections of spinal cord showing details of glutamate and CK immunoreactivities in larvae processed 1-7dpl. **A:** Glutamate-ir astrocyte (arrow) 3 dpl. The asterisk indicates the Mauthner axon. **B:** Glutamate-ir glial processes and end-feet (arrowheads) 7 dpl. **C:** CK-ir reactive astrocyte (arrow) 2 dpl. **D:** CK-ir processes of astrocytes (arrowheads) transversally cut, 1dpl. **E:** CK-ir astrocyte 3 dpl. Note the faint CK immunoreactivity in the perimeningeal ends. **F:** CK-ir processes of astrocytes (arrowheads) transversally cut, 7dpl. **G:** CK immunoreactivity appearance at 7 dpl. Dorsal is at the top. Lateral is on the left except for D, F and G, in which lateral is on the right. Scale bars = 20 µm (A, B, C, D, F); 10 µm (E, G).

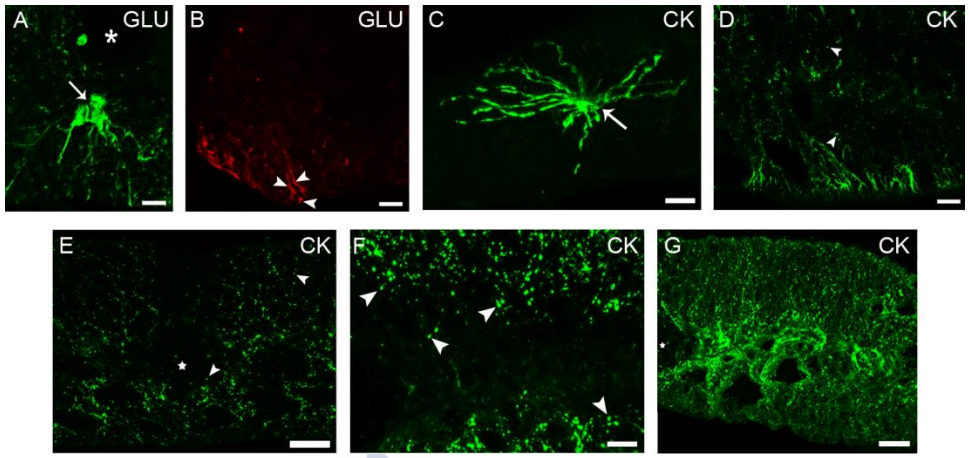

Figure 5

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Chapter 3

Confocal microscopy used for the semiautomatic quantification of the changes in aminoacidergic fibres during spinal cord regeneration

CONFOCAL MICROSCOPY USED FOR THE SEMIAUTOMATIC QUANTIFICATION OF THE CHANGES IN AMINOACIDERGIC FIBRES DURING SPINAL CORD REGENERATION

Introduction

In mammals, including humans, spinal cord injury (SCI) leads to permanent disability and to an irreversible loss of function. SCI has become an important public health and economical problem because so far, an appropriate therapy does not exist. In contrast to mammals, it is long known that most of anamniote vertebrate species are capable of impressive spinal cord regeneration (Tanaka and Ferretti, 2009). Lampreys have been used as a model of successful spinal regeneration since the late 1950s (Maron, 1959). Two characteristics of lampreys, the absence of myelin (Bullock et al., 1984) and the high regenerative ability of their descending neurons (Rodicio and Barreiro-Iglesias, 2012), confer them several advantages with respect to other vertebrates for studying the factors that control spinal cord regeneration.

SCI leads to changes in the spinal neurotransmitters systems, including aminoacidergic neurotransmitters. Glutamate is the main excitatory neurotransmitter, whereas glycine is one of the main inhibitory neurotransmitters in the central nervous system of vertebrates (Curtis et al., 1967; Fonnum, 1984). In lampreys, these neurotransmitters are involved in the spinal networks that control the locomotion (Valle-Maroto et al., 2011; see chapter 1). Spinal excitatory glutamatergic interneurons play an important role in the rhythm generation (Buchanan and Grillner, 1987; Cangiano and Grillner, 2003), whereas the inhibitory glycinergic interneurons are involved in the left–right alternating pattern (Grillner et al.,

1987; Alford and Williams, 1989). So, it is important to understand how these neurotransmitter systems adapt and change after a SCI. Confocal microscopy together with the use of appropriate computer software could be a suitable method to quantify the changes in the aminoacidergic neurotransmitter systems during the regeneration of the spinal cord of lampreys. Getting knowledge about how the spinal cord of lampreys reacts to injury to achieve functional recovery can provide new clues to propose new lines of research or therapies for SCI.

Confocal microscopy permits to obtain high-resolution images, allowing differentiating between two immunofluorescence profiles that are very close to each other. We have combined high- resolution images of confocal microscopy with the use of available free software (Fiji and the software's plug-in Feature J). This computer software allows making a semiautomated quantification of the number of positive profiles in photomicrographs (Grider et al., 2006), which permits to avoid the hard task of manual counting or to spend considerable amounts of money in expensive commercially programs. The use of this software to quantify fibre densities has been previously validated in studies for the quantification of serotonin positive profiles in the rat cortex after corticospinal tract lesions (Grider et al., 2006) or in the zebrafish spinal cord after SCI (Kuscha et al., 2012). Our group has developed immunofluorescence methods to detect the aminoacidergic neurotransmitters in the spinal cord of the sea lamprey by using glutaraldehyde tissue fixation and for the subsequent quantification of the neuronal fibres during spinal cord regeneration, although it can be applied to other animal models. The establishment of a standard protocol to carry out this type of studies can provide a meaningful way to compare results when looking at the changes in different neurotransmitters systems and different time points after injury. In addition, this method can also be used with other types of fixation (e.g., paraformaldehyde fixation) or even with bright-field photomicrographs.

Moreover, the protocol can be adapted to study changes under other experimental conditions and systems. The main steps of the protocol are the spinal cord surgery, tissue collection and processing, immunofluorescence procedure, image acquisition, quantification, and statistical analysis (Fig. 1).

Materials

Reagents

Tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO). Sodium chloride (NaCl; Panreac, Castellar del Vallés, Barcelona, Spain).

Potassium chloride (KCl; Panreac).

Calcium chloride-2 Hydrate cryst. ($CaCl_2$; Merck, Darmstadt, Germany).

Hydrochloric acid (HCl; Sigma). Caution: Can cause skin burns, eye damage, and respiratory irritation. Avoid breathing dust/ fume/gas/mist/vapors/spray. Use protective gloves, safety glasses, and mask.

HEPES (Sigma). Caution: Irritating to eyes, respiratory system, and skin. Use gloves, safety glasses, and mask.

Sylgard 184 (Dow Corning, Midland, Michigan).

Glutaraldehyde (Panreac). Store at 4°C. Caution: Toxic by inhalation and if swallowed. Use gloves, safety glasses, and mask.

Tris(hydroxymethyl)aminomethane (Panreac). Caution: Irritating to eyes and skin. Use gloves and safety glasses.

Sodium metabisulfite (MB; Panreac). Caution: Harmful if swallowed. Risk of damage to eyes. Use safety glasses.

Sucrose (Sigma).

Neg 50™ (Microm International GmbH, Walldorf, Germany).

Isopentane (Sigma). Store at 4°C. Caution: Maybe fatal if swallowed and enters airways. Avoid breathing dust/ fume/gas/mist/vapors/spray. Use mask.

Liquid nitrogen. Caution: It can cause terrible "burns." (Death of living tissue caused by the extreme cold.) Hand protection and goggles (not safety glasses) are to be worn when dispensing and handling liquid nitrogen.

Sodium borohydride ($NaBH_4$; Sigma). Caution: Toxic in contact with skin and if swallowed. Do not breathe dust. Use gloves, safety glasses, and mask.

Triton X-100 (Sigma). Caution: Harmful if swallowed. Eye irritation. Use gloves and safety glasses.

Normal serums of the host species of the secondary antibodies (e.g., normal goat serum; Millipore, Temecula, CA). Store in small aliquots at −20°C.

Distilled water (dH_2O).

Primary antibodies (see Table 1). Store following the supplier indications.

Secondary antibodies coupled to a fluorochrome (see Table 1). Store in the dark and following the supplier indications.

Mowiol 4–88 (Calbiochem, La Jolla, CA) (see *Reagent setup* section).

Glycerol (Panreac).

Equipment

Superfrost ® Plus glass slides (Menzel, Braunschweig, Germany). Coverglass 24 x 60 mm (Menzel, Braunschweig, Germany).

Incubation chamber for slide incubation (custom made) (see *Equipment setup* section).

Scalpel #3 and blades #11.

Stereomicroscope (e.g., Nikon SMZ-U; Nikon, Tokyo, Japan).

Spectral confocal microscope (e.g., TCS-SP2 and SP5; Leica, Wetzlar, Germany) equipped with suitable lasers.

Image acquisition and processing softwares (e.g., Photoshop).

Fiji software with the plug-in Feature J.

Software for statistical analysis (e.g., GraphPad Prism 5; GraphPad software, La Jolla, CA).

Reagent setup

Tris buffer 0.4 M: To prepare 1 l, add 48.4 of Tris(hydroxymethyl) aminomethane to 1 l of distilled water (dH_2O). Store at room temperature (RT).

Tris buffer saline (TBS) 0.05 M, pH 7.4: To prepare 1 l of buffer, add 125 ml of Tris buffer 0.4 M, 42 ml of HCl 1 N, and 29.2 g of NaCl. Adjust pH to 7.4 and add dH_2O to reach the final volume. Store at RT.

Lamprey ringer solution, pH 7.4: To prepare 1 l add 8 g of NaCl (137 mM), 0.22 g of KCl (2.9 mM), 0.308 g of $CaCl_2$ (2.1 mM), and 0.48 g of HEPES buffer (2 mM) to 600 ml of dH_2O. Adjust pH to 7.4 and add dH_2O to reach the final volume. Store at 4°C.

Glutaraldehyde solution (5% glutaraldehyde and 1% MB in 0.05 M TBS): To prepare 100 ml, add 20 ml of glutaraldehyde and 1 g of MB to 80 ml of TBS. Prepare just before use.

1% MB in 0.05 M TBS (TBS-MB): To prepare 500 ml, add 5 g of MB to 400 ml of TBS. Adjust pH to 7.4 and add TBS to reach the final volume. Prepare just before use.

0.1% MS-222 in Ringer solution: To prepare 100 ml, add 0.1 g of MS-222 to 100 ml of Ringer solution. Prepare just before use.

30% sucrose in TBS-MB: To prepare 100 ml, add 30 g of sucrose to 100 ml of TBS-MB. Prepare just before use.

1:1 solution of 30% sucrose in TBS-MB and Neg50™: To prepare 100 ml, add 50 ml of 30% sucrose in TBS-MB and 50 ml of Neg50™.

0.2% sodium borohydride ($NaBH_4$) in dH_2O: To prepare 100 ml, add 0.2 g of $NaBH_4$ to 100 ml of dH_2O.

Primary antibody(ies) solution: Dilute the antibodies in TBS-MB containing 0.2% Triton X-100 and 15% normal goat serum (for antibody dilutions, see Table 1).

Secondary antibody(ies) solution: Dilute the antibodies in TBS containing 0.2% Triton X-100 and 15% normal goat serum (for antibody dilutions, see Table 1).

Tris 1 M, pH 9.5: To prepare 1 l, add 121.1 g of Tris (hydroxymethyl) aminomethane to 800 ml of dH_2O, adjust pH to 9.5, and add dH_2O to reach the final volume. Store at RT.

Tris 0.2 M, pH 8.5: To prepare 100 ml, add 20 ml of Tris 1 M, pH 9.5 to 70 ml of dH_2O, adjust pH to 8.5, and add dH_2O to reach the final volume. Store at RT.

Mowiol: Add 9.6 g of Mowiol to 19.08 ml of glycerol and shake for 1 h. Add 24 ml of dH_2O and shake for 2 h. Add 48 ml of Tris 0.2 M, pH 8.5, put the solution in a water bath at 60 °C, and shake until it is dissolved. Clarify by centrifuging at 5,000x *g*, 15 min. Make small aliquots and store at −20°C. All the steps need to be done in the dark.

Equipment setup

Humid chamber for slides incubation: A simple homemade humid chamber can be made as follows: Place filter paper embedded in water at the bottom of an opaque plastic box. Place the slides inside the box. Cover the box and place it in an incubator at the necessary temperature.

Methods

Animals

For this study, larval sea lampreys, *Petromyzon marinus* L. (body length 80 to 150 mm, 5–7 years of age), were used (n = 25). They were maintained in aerated freshwater aquaria at 15°C with a bed of river sediment until their use for experimental procedures. All experiments were approved by the Ethics Committee of the University of Santiago de Compostela and were done following the European Community and Spanish guidelines on animal care and experimentation.

Spinal cord surgery

Animals were deeply anesthetized by immersion in 0.1% MS-222 in Ringer solution. Then, the larvae were placed with their dorsal side up in a Sylgard-lined dish filled with Ringer solution. The spinal cord was exposed by a transverse incision made on the body wall from the dorsal midline at the level of the fifth gill. A complete spinal cord transection was performed with a scalpel at this spinal level and the spinal cord cut ends were visualized under a stereomicroscope. After surgery, larval lampreys were maintained on ice for 1 h to allow the wound to air dry. During this hour, the animals were maintained on a paper towel soaked with Ringer solution not in direct contact with the ice. After this time, the animals were allowed to recover in aerated freshwater tanks at 19.5°C. Each transected animal was examined 24 h after surgery to confirm that there was no movement caudal to the site of injury. A spinal transection was considered complete if on the stimulation of the head, the animal could move only its body rostral to the lesion site.

Tissue collection and processing

The animals were allowed to recover in the water tanks for different periods after the complete SCI [2: n = 5; 4: n = 5, 10: n = 5, and 24: n = 5; weeks post-lesion (wpl)]. Larvae were fed with yeast during the period of recovery. Control non-injured animals (n = 5) and animals from the different experimental groups after the different recovery periods were deeply anesthetized with 0.1% MS-222 in Ringer solution and killed by decapitation. The portion of the body comprised between the fourth and the sixth gills was cut and processed as follows:

1. Fixation: It was done by immersion of the sample in glutaraldehyde solution (see *Reagent setup* section) for 20 h at 4°C.
2. Washes: TBS-MB 4 x 2 h. at 4°C.

3. Cryoprotection: 30% Sucrose in TBS-MB until the sample is completely sunken. 4°C.
4. The sample is placed in 1:1 solution of 30% sucrose in TBS-MB and Neg50™, 20 min. at 4°C.
5. Inclusion: The sample is embedded in Neg 50™ and frozen in liquid nitrogen-cooled isopentane.
6. Sectioning: The sample is sectioned on a cryostat in the transverse plane (14 µm thick), and the sections are mounted on glass slides (see *Equipment* section).
7. Tissue drying: Leave the slides at RT overnight and then place the slides in the oven for at least 45 min. at 37°C.

Immunofluorescence on the cryostat sections

1. Washes: TBS-MB 2 x 15 min. at RT, shaking.
2. Pretreatment with 0.2% $NaBH_4$ in dH_2O: 45 min. at RT.
3. Washes: TBS-MB 2 x 15 min. at RT, shaking.
4. Incubation of slides in the primary antibody solution (300 µl/slide): overnight, in the humid chamber (see *Equipment setup* section) at RT.
5. Washes: TBS 2 x 30 min. at RT, shaking.
6. Incubation of slides in the secondary antibody solution (300 µl/slide): 1 h. at RT in humid chamber and in the dark.
7. Washes: TBS 2 x 10 min. at RT, shaking in the dark.
8. Washes: TBS 1 x 5 min. at RT, shaking in the dark.
9. Washes: dH_2O 2 x 10 min. at RT, shaking in the dark.
10. Place the slides in the oven for 25 min. at 37°C.
11. Mount the coverslip with Mowiol.
12. Place the slides in the oven for at least 30 min. at 37°C.
13. Store the slides at −20°C until the day of scanning.

Image acquisition

The sections were photographed and analysed with the spectral confocal microscopes TCS-SP2 and SP5 (Leica, Wetzlar, Germany). Ten spinal cord hemisections (non-consecutive; 1 out of 3 sections) were photographed between 400 - 450 µm rostrally and caudally from the site of injury. In control animals, 20 sections at the level of the fifth gill were photographed. Photographs were made at 20x magnification with 1.5 zoom, to visualize the hemisection of the spinal cord. Stacks of photographs were processed with LITE and LAS softwares (Leica) to make a Z projection of the stack to generate a single tiff file of the photomicrograph.

Quantification

For the quantification of positive profiles in the spinal cord hemisections, three different regions were independently analysed (dorsomedial, ventromedial, and lateral) (Fig. 2A).

1. Open the confocal photomicrograph file (Fig. 2B) with the Fiji software.
2. Convert this fi le to an 8-bit image (Fig. 2C).
3. Click in *Plugins,* select *Feature Extraction*, then *Feature J* and click in *Feature J Hessian*. A window opens, select *Smallest eigenvalue of Hessian tensor* and click *OK*.
4. Click in *Image*, select *Adjust*, and click in *Threshold* (Fig. 2D). It is necessary to determine a threshold that gives the most accurate image to decrease background and increase edge and shape detection of the axons. The same threshold should be used for all the images (see Discussion). Click in *Apply* and *OK*.
5. Click in *Process*, select *Binary*, and click in *Make Binary* to convert the image to a binary B & W image (Fig. 2E).

6. Select the area in which you want to make the quantification. In our work, the chosen regions were the dorsomedial, ventromedial, and lateral areas (Fig. 2F).
7. Click in *Analyze* and select *Analyze particles*. Use the settings particle size from 1 to infinity and circularity from 0.00 to 1.00. Select *Display results* and *Summarize*. In the *Summary* window, the number that appears below *Count* is the number of profiles of the selected area.
8. Repeat steps 6 and 7 for every region of the hemisection you want to quantify.
9. Repeat steps 1–7 for every stack of confocal photomicrographs.
10. Calculate the means of the ten hemisections rostral and caudal to the site of injury and for every region of interest (e.g., dorsomedial, ventromedial, and lateral). The mean values for the rostral and caudal spinal cord of each animal are used for the statistical analysis.

Statistical analysis

For statistical analysis, the program GraphPad Prism was used. Variability of values was given as statistical error of mean (SEM). To test the normality of the data, the Kolmogorov–Smirnov test was used. Data sets were normally distributed and statistically analysed using a one-way ANOVA with Bonferroni's multiple comparison tests.

Results

In this chapter we described a protocol to quantify the changes in the aminoacidergic neurotransmitter systems during the regeneration of the spinal cord of lampreys. We took advantage of the developed immunofluorescence methods to detect aminoacidergic neurotransmitters in the central nervous system (CNS) of the lamprey by glutaraldehyde tissue fixation and combined this with confocal imaging and appropriate software.

This method was used to quantify the changes in the number of glutamate-immunoreactive (-ir) spinal fibres in control animals and at different time points (2, 4, 10 and 24 wpl) after a complete spinal cord transection in the sea lamprey (see chapter 4).

In control animals and after the different recovery periods of the injured animals, we performed glutamate immunofluorescence. For this, the tissue was fixated in glutaraldehyde, pretreated with sodium borohydride 0.2 % and incubated in polyclonal anti-glutamate antibody, which was detected with Cy3-conjugated goat anti-rabbit immunoglobulin (Table 1). Fig. 2A, B shows glutamate immunoreactivity in the spinal cord of a normal lamprey larva. Photomicrographs were made in a confocal spectral microscope and Fiji software was used for the quantification of the fibres. Finally, the data were statistically analysed using GraphPad Prism software.

As an example of the results that can be obtained following this protocol, the analysis of the three regions studied in the photomicrograph of Fig. 2, which shows glutamate immunoreactivity in a normal larva, gave the following results: dorsomedial region, 854 positive profiles; ventromedial region, 398 positive profiles; and lateral region, 883 positive profiles. Table 2 shows the results obtained after the quantification of the profiles in the three regions studied in ten sections of the rostral stump of

one larva from each group (control, 2, 4, 10 and 24 wpl). The same is observed in Table 3 for profiles of the caudal stump of the cord.

This method constitutes a very useful tool that facilitates the quantification of the changes in spinal cord innervation following injury.

Discussion

A useful tool to quantify the aminoacidergic neurotransmitter fibres in the spinal cord of the sea lamprey after SCI has been described in this work. Establishing standard protocols to make this kind of studies is really important to be able to compare results between different works. The software used here has been validated in previous studies in which serotonin positive profiles were quantified after corticospinal tract lesions in rats (Grider et al., 2006) or after SCI in zebrafish (Kuscha et al., 2012). Further, this method can be adapted to quantify fibres from other neurotransmitter systems, with different types of fixation or even with bright-field microscopy (Cornide-Petronio et al., 2014).

To carry out this protocol successfully, some things should be taken into consideration. Glutaraldehyde molecule has two aldehyde groups. The aldehyde groups interact with the proteins of the tissue to fix it. However, glutaraldehyde fixation introduces many free aldehyde groups, which leads to autofluorescence problems. Sodium metabisulfite (MB) is used as an antioxidant to preserve the neurotransmitters epitopes that can be damaged by glutaraldehyde fixation (Kiernan, 2000). In addition, MB can adduct to aldehyde to give sulfonates helping to quench the glutaraldehyde autofluorescence (Hopwood, 1967). Sodium borohydride ($NaBH_4$) is a good reducing agent that can reduce aldehyde groups to alcohol so it is also used to quench the glutaraldehyde autofluorescence.

The use of the MB and the treatment with $NaBH_4$ are not necessary when the fixation is made with paraformaldehyde (PFA). When using glutaraldehyde fixation, steps 1-13 of *Immunofluorescence on the cryostat sections* part must be done immediately after cutting the tissue. Slides cannot be conserved in the freezer after the cryostat sectioning because the immunofluorescence does not work. With PFA fixation, slides can be stored after sectioning in the freezer until the day of use. Once mounted, the slides can be stored at -20 °C for months and even years without losing the fluorescence. The thickness of the sections could be larger (18 – 20 µm) with PFA fixation because the antibody penetration is better with PFA than glutaraldehyde. With the confocal microscope, the images should be taken trying not to vary the amplifier gain neither the offset. It is desirable not to vary these parameters much to avoid the introduction of experimental variability between samples. However, fluorescence intensity, even from one section to another of the same slide, can vary significantly. With this in mind, images should be taken in the narrowest margin of values of amplifier gain and offset to reduce as much as possible the introduction of variability in the quantifications. Further, it is also necessary to establish a threshold in the Fiji software to consider the profiles as positive. To establish it, some images with different profile densities should be opened, the most suitable threshold value should be selected and a medium value should be chosen to use it for all the photomicrographs.

Any model of confocal microscope can be used in these experiments as long as it has the suitable lasers and any other software for statistical analysis can also be used. Here, the Kolmogorov–Smirnov normality test have been used because the sample size is small (n = 5). Other normality tests such as the D'Agostino or Shapiro–Wilk tests are recommended when the sample size is higher.

Therefore, this method pretends to standardize this kind of studies, avoiding the hard task of manual counting or to spend considerable

amounts of money in expensive commercially programs, since Fiji is a free software that can be downloaded by all users and for different operating systems.

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Abbreviations

CNS	Central nervous system
ir	Immunoreactive
PFA	Paraformaldehyde
RT	Room temperature
SCI	Spinal cord injury
wpl	Weeks post-lesion

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Tables and Figures

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Table 1. Antibodies used in our studies.

Antibody	Species	Supplier	Code/Clone, Lot/Batch	Dilution	Immunogen	Characterization
Anti-Glutamate	Rabbit polyclonal	Immunosolution, Jesmond, Australia	IG1007	1:4500	GLU-porcine thyroglobulin conjugate	Dot blot, Western blot
Anti-Glycine	Rabbit polyclonal	Immunosolution, Jesmond, Australia	IG1003	1:3000	GLY-porcine thyroglobulin conjugate	Dot blot, Western blot
Anti-Glutamate	Mouse monoclonal	Swant, Switzerland	mAB 2D7	1:1000	Glutaraldehyde-linked L-glutamate-BSA conjugate	
Anti-GABA	Mouse monoclonal	Sigma, San Louis, MO	GB 69	1:1200	Purified GABA conjugated to BSA	Dot blot, Westem blot
Anti-rabbit IgG	Cy3-labelled Goat polyclonal	Chemicon, Temecula, CA	GAR-Cy3, AP132C	1:100		
Anti-mouse IgG	FITC-labelled Mouse monoclonal	Chemicon, Temecula, CA	GAM-F, AQ303F	1:100		

Table 2. Results obtained after the quantification of the profiles in the three regions studied in ten sections of the rostral stump of one larva from each group.

CONTROL			2 wpl			4 wpl			10 wpl			24 wpl		
DM	VM	LAT	DM	VM	LAT	DM	VM	LAT	DM	VM	LAT	DM	VM	LAT
765	383	968	176	58	88	119	82	74	593	346	732	893	388	740
673	369	1024	121	63	47	116	118	124	736	412	778	616	537	741
650	356	502	214	163	347	96	98	60	911	455	726	765	518	612
760	310	706	176	183	232	158	99	125	533	475	700	798	631	782
664	358	487	304	279	381	156	67	81	1094	520	676	928	643	891
720	323	646	149	63	80	508	303	396	929	338	798	992	678	965
527	256	415	260	188	122	382	327	432	1091	566	1078	1067	737	1044
759	301	798	407	248	400	396	265	297	1049	610	673	1081	771	974
656	369	444	386	302	617	331	201	334	1159	698	981	944	736	965
808	369	662	315	226	244	280	173	150	784	340	629	917	748	980

DM: Dorsomedial; LAT: Lateral; VM: Ventromedial; wpl: Weeks post-lesion

Table 3. Results obtained after the quantification of the profiles in the three regions studied in ten sections of the caudal stump of one larva from each group.

CONTROL			2 wpl			4 wpl			10 wpl			24 wpl		
DM	VM	LAT	DM	VM	LAT	DM	VM	LAT	DM	VM	LAT	DM	VM	LAT
659	307	684	98	108	301	109	29	94	864	567	1231	799	618	947
778	456	690	319	104	457	75	48	116	635	399	966	1067	681	944
688	378	638	168	181	247	80	28	85	670	522	1188	671	693	738
636	345	753	246	225	272	132	71	117	568	345	708	749	551	777
854	398	883	151	144	163	349	206	355	510	415	520	753	609	871
827	441	955	167	238	354	189	97	130	986	462	830	913	744	752
852	402	757	302	143	249	491	191	419	952	442	949	848	438	1207
840	411	746	140	155	280	442	148	381	800	461	894	765	636	772
882	468	880	58	82	128	607	237	644	866	382	630	1078	881	1291
812	275	1065	276	239	324	700	362	627	884	406	556	1065	933	1068

DM: Dorsomedial; LAT: Lateral; VM: Ventromedial; wpl: Weeks post-lesion

Figure 1. Flow diagram showing an overview of the protocol.

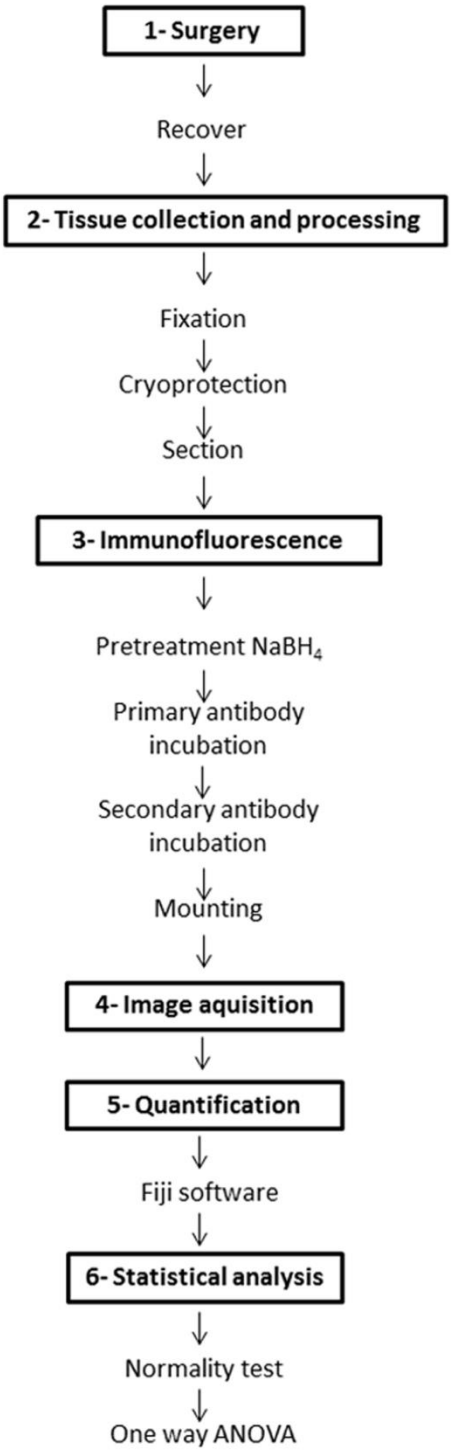
1- Surgery
Recover
2- Tissue collection and processing
Fixation
Cryoprotection
Section
3- Immunofluorescence
Pretreatment $NaBH_4$
Primary antibody incubation
Secondary antibody incubation
Mounting
4- Image aquisition
5- Quantification
Fiji software
6- Statistical analysis
Normality test
One way ANOVA

Figure 1

Figure 2. Photomicrographs showing the steps taken for the quantification of the number of positive profiles. **A:** Glutamate immunoreactivity in a transversal hemisection of a normal lamprey spinal cord showing the 3 regions that were independently quantified: dorsomedial (DM), ventromedial (VM) and lateral (LAT). **B:** Confocal photomicrograph opened by Fiji software; yellow lines mark the selected area that was copied and pasted in a new file. **C:** Appearance of the photomicrograph after converting the file to an 8-bit image. **D:** Appearance of the image after Feature J Hessian application and clicking in *Threshold*. E: Appearance of the image after the binary conversion. F: Yellow lines delimiting the DM area to be quantified.

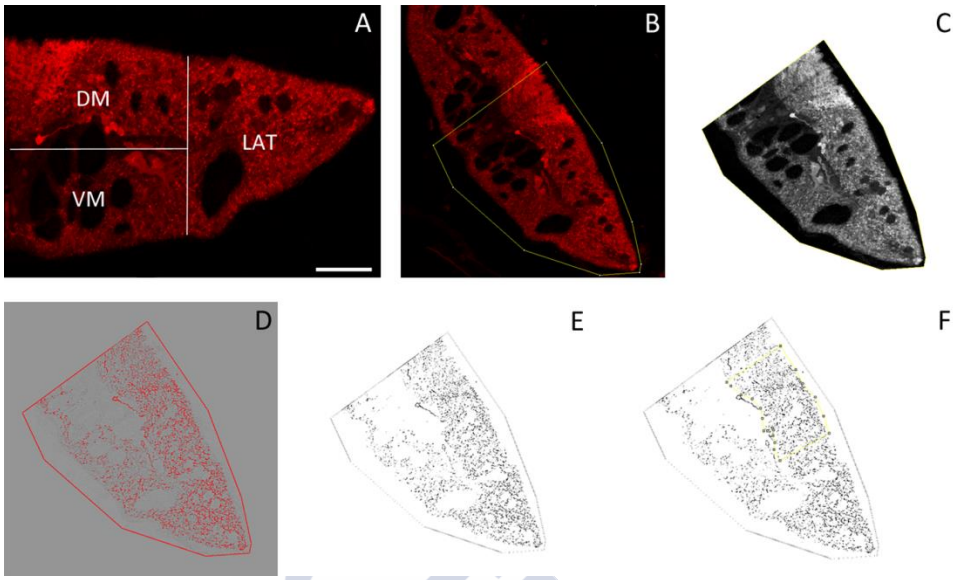

Figure 2

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DE COMPOSTELA

Chapter 4

Plasticity in the spinal glutamatergic system to restore the function after spinal cord injury in lampreys

PLASTICITY IN THE SPINAL GLUTAMATERGIC SYSTEM TO RESTORE THE FUNCTION AFTER SPINAL CORD INJURY IN LAMPREYS

Introduction

Spinal cord injury (SCI) leads to permanent disability and to an irreversible loss of movement and sensitivity below the lesion site in mammals, including humans. It has become a great health and economic problem due to the lacking of an adequate treatment. In contrast to mammals, it is well known that most of the anamniote vertebrates are capable of spinal cord regeneration and movement recovering (Tanaka and Ferretti, 2009). Among them, lampreys have been used as a model of spinal cord regeneration since the 1950's (Maron, 1959) and are the unique vertebrate that satisfies the five criteria established by the NIH to consider the functional regeneration complete (Guth et al., 1980; Rodicio and Barreiro-Iglesias, 2012). Therefore, lampreys have become a vertebrate model for the study of spinal cord regeneration.

The vertebrate locomotion is generated by the central pattern generator (CPG) networks in the spinal cord. The brainstem command centres regulate the spinal networks via reticulospinal (RS) neurons (revision in Dubuc et al., 2008). Functional spinal cord regeneration requires both, regeneration of damaged axons and formation of synaptic contacts. The study of the regeneration of descending axons in lampreys has been facilitated due to the large, identifiable spinal cord-projecting neurons of the lamprey brainstem. This has enabled investigators to test variation in the regenerative abilities of descending neurons after SCI (Davis and McClellan, 1994a, b; Jacobs et al., 1997). Jacobs and co-

workers (1997) reported that about 50% of the identified descending neurons of lampreys spontaneously regenerate their axons after a complete transection of the cord. Zhang and McClellan, (1999) observed that a 78% of the projections of the unidentified descending brain neurons regenerated after SCI. Damaged axons of intrinsic spinal cord neurons can also regenerate their axons across the lesion site (Yin and Selzer, 1983; Rouse and McClellan, 1997; Armstrong et al., 2003) and regenerated axons establish synaptic contacts below the lesion site (Mackler and Selzer, 1985). In addition, it has been suggested that intrinsic spinal neurons relay descending drive to locomotor network to caudal regions after SCI in lampreys, since the function at a certain spinal level is restored before the descending RS axons reach that level of the cord (Davis and McClellan, 1994b; Rouse and McClellan, 1997), indicating a role for intraspinal neurons even more important after SCI than in normal conditions. Since not all damaged axons regenerate, there is an increasing interest in knowing the compensatory changes that take place in the locomotor networks below the lesion site necessary to achieve locomotor recovery (Cooke and Parker 2009).

Glutamate mediates excitatory neurotransmission and is used by neurons in the command centres, RS neurons and neurons in the spinal cord that excite motor neurons and other interneurons (Grillner et al., 2008; Kiehn et al., 2008). In addition, glutamate release in response to lesion causes glutamate excitotoxicity which can lead to neuronal and oligodendrocyte death (Liu et al., 1991; 1999; Xu et al., 2004) impeding successful regeneration or making it difficult. While the short term changes in glutamate levels after injury are well documented (Liu et al., 1991; McAdoo et al., 1999) even in lamprey (see chapter 2), surprisingly, much less is known about the long term changes occurring at the level of spinal cord populations. Most of these studies refer to the lumbar transection of the spinal cord of neonate rats after weight-supported treadmill training (Cantoria et al., 2011) or to other neurotransmitter systems in lamprey

(serotonergic: Cohen et al., 2005; Cornide-Petronio et al., 2011, 2014) or in zebrafish (dopaminergic and serotonergic: Kuscha et al., 2012).

Identifying the changes that occur in the spinal cord after locomotion recovering in regenerating-vertebrates may offer clues to determine key events that lead to the recovery of function and help to design therapies to restore function in non-regenerating vertebrates. Here, we take advantage of the knowledge of the spinal cord glutamatergic system of the sea lamprey (see chapter 1) as well as of the labelling of the dendrites, somata and axons of the neurons of lampreys by anti-glutamate antibodies (Villar-Cerviño et al., 2011, 2013; chapter 1) to perform analysis that would not be possible in other species, since, as explained in chapter 1, the use of anti-glutamate antibodies in mammals is problematic because the metabolic pool of glutamate (Ottersen and Storm-Mathisen, 1985) and because staining of perikarya was somewhat inconsistent (Walberg et al., 1990). Moreover, the antibodies against vesicular glutamate transporters (VGLUTs) stain the glutamatergic terminals but only a few somata (Kaneko et al., 2002). The specificity of this approximation in lampreys has been validated by comparison between the distribution of glutamate immunoreactive neurons and VGLUT-expressing neurons, (Villar-Cerviño et al., 2011, 2013; chapter 1). The purpose of the present study was to quantify the changes that occur in the glutamatergic fibres after a complete spinal cord transection and during the regeneration in mature sea lamprey larvae. Since we have previously reported an immediate loss of glutamate immunoreactivity in neurons close to the lesion site (see chapter 2) we also quantify the glutamatergic neuronal populations in the grey matter to determine whether they recuperate glutamate immunoreactivity when the functional recovering is achieved. Quantification studies could serve to decipher the overall mechanisms of plasticity that take place during the regenerative process. Here, we report that processes of plasticity occur in the glutamatergic system during spinal cord regeneration in the sea

lamprey, since many of the glutamatergic spinal cell populations do not recover after SCI.

Material and methods

Ethical statement

All experiments were approved by the Bioethics Committee at the University of Santiago de Compostela and conformed to the European Union and the Spanish guidelines on animal care and experimentation.

Animals

Mature and developmentally stable larval sea lampreys, *Petromyzon marinus* L. (n= 25; between 80 and 156 mm in body length, 5 to 7 years of age), were used in the present study. Animals were collected from the river Ulla (Galicia, north-western Spain) with permission from the Xunta de Galicia and maintained in aerated fresh water aquaria at 15 ºC with a bed of river sediment until their use for experimental procedures.

Surgery

Animals were deeply anaesthetized by immersion in 0.1% tricaine methanesulphonate (MS-222 ; Sigma, St. Louis, MO) in Ringer solution (pH 7.4) of the following composition: 137 mM NaCl, 2.9 mM KCl, 2.1 mM $CaCl_2$, 2 mM HEPES. A complete transection of the spinal cord at the level of the 5th gill was performed as previously described (Cornide-Petronio et al., 2011, 2014; see chapters 2 and 3).

Tissue collection and processing

Control animals (n=5; without lesion) and injured animals after the different recovery periods [2, 4, 10 and 24 weeks after the injury (n=5 for each group)] were deeply anaesthetized with 0.1% MS-222 in Ringer solution and killed by decapitation. The region of the body comprised between the 4th and the 6th gills was fixed by immersion in 5% glutaraldehyde and 1% sodium metabisulfite (MB) in 0.05 M Tris-buffered saline (TBS; pH 7.4) for 20 hours. After fixation, the tissue was washed and embedded in Neg 50™ (Microm International GmbH, Walldorf, Germany), frozen in liquid nitrogen-cooled isopentane, sectioned on a cryostat in the transverse plane (14 µm thick) and mounted on Superfrost ® Plus glass slides (Menzel, Braunschweig, Germany).

Immunofluorescence

For immunofluorescence, sections were pretreated with 0.2% $NaBH_4$ in deionized water for 45 minutes to quench autofluorescence. Sections were incubated with a rabbit polyclonal anti-glutamate antibody (Immunosolution; 1:4.500) or a mouse monoclonal anti-glutamate antibody (Swant; 1:1000) in 0.05 M TBS with 1% MB during 3 days at 4ºC. After rinsing in TBS with 1% MB, sections were incubated for 1 hour at room temperature with Cy3- conjugated goat anti-rabbit immunoglobulin (Chemicon, Temecula, CA; 1:100) or fluorescein conjugated goat anti-mouse immunoglobulin (Millipore, Temecula, CA; 1:100) rinsed in TBS and mounted with Mowiol. All antibodies were diluted in TBS (pH 7.4) containing 0.2% Triton X-100 and 15% normal goat serum.

Antibodies

The polyclonal anti-glutamate antibody was raised in rabbit against a glutamate- glutaraldehyde-porcine thyroglobulin conjugate. The antibody has been tested by the supplier in sections of retina and cerebellum from various vertebrates, as well as in dot blot immunoassays with a variety of amino acid-protein conjugates. These include the standard 20 amino acids found in proteins, the non-protein amino acids D-serine, D-alanine and D-aspartate, GABA and the glycine containing tripeptide glutathione, which did not yield significant cross reactivity. This antibody has been developed by Dr David V. Pow (University of Newcastle, New South Wales, Australia) and used in previous studies of the lamprey brain and spinal cord (Villar-Cerviño et al., 2011, 2013; see chapters 2 and 3). This antibody did not stain any sea lamprey brain native protein band (Villar-Cerviño et al., 2011).

The mouse monoclonal anti-glutamate antibody was raised against glutaraldehyde-linked L-glutamate-bovine serum albumin (BSA) conjugate by P. Streit (Liu et al., 1989), and this clone was made commercially available through Swant. This antibody has been characterized with respect to cross reactivity by antibody dilution experiments as well as by absorption experiments (Adám and Csillag, 2006). In addition, the staining pattern obtained with both monoclonal and polyclonal anti-glutamate antibodies in this study and in sections of the brain and retina (unpublished observations) were the same.

Image acquisition

The sections were photographed and analysed with the spectral confocal microscopes TCS-SP2 and SP5 (Leica, Wetzlar, Germany). From the site of injury, ten spinal cord hemisections were photographed rostrally

and caudally. One out of three consecutive sections was photographed, to avoid counting twice the same cell. In control animals, 20 sections at the level of the 5th gill were photographed. Photographs were made at 20x magnification with 1.5 zoom, to visualize the hemisection of the spinal cord. Stacks of photographs were processed with LCS Lite and LAS software (Leica) to make a Z projection of the stack to generate a single tiff file of the photomicrograph. Contrast and brightness were minimally adjusted with Fiji software (Image J, NIH). Figure plates were made and lettering added using Adobe Photoshop CS4 (Adobe Systems, San José, CA). Schematic drawings were made using Corel Suite X5 (Corel, Ottawa, Canada).

Quantification of cells and fibres

Five types of glutamatergic cells were quantified: dorsomedial (DM), dorsolateral (DL), large lateral (LL), small lateral (SM) and cerebrospinal fluid-contacting cells (CSFc). The quantification of the cells was made manually, using LCS Lite and Fiji software. The following correction was made in the quantification of the cells: the step size of the confocal images was multiplied by the number of planes of the images (for each photograph) to know the number of glutamatergic cells that were observed in the section thickness. Then, for each section, the number of glutamatergic cells that would be observed in 450 µm was inferred. Finally, the average number of the glutamatergic cells was calculated for each cell type and animal. The quantification of processes (profiles) was made using Fiji software as it was previously explained (see chapter 3). Briefly, the white matter of the spinal cord was divided into three different regions [DM, ventromedial (VM) and lateral (LAT)] (Fig. 1A) and the glutamate immunoreactive (-ir) profiles were counted for each region in each hemisection. The plug-in Feature J Hessian of Fiji software was used for the quantification of the profiles. The number of profiles was

quantified in 10 hemisections (1 out of 3; 14 µm thick) of the 400 - 450 µm rostral and caudal to the site of injury. Then, the average number of the positive profiles was calculated for each region and animal. The quantification of the profiles was made at each time point of study after the injury, as these time points correspond to different moments of the axonal regeneration. During the first 2 weeks post-lesion (wpl) there is an axonal retraction of up to 2 mm (Roederer et al., 1983; Yin and Selzer, 1983; Zhang et al., 2005). After that, the descending axons start to grow reaching the rostral stump at 4 wpl and between 5-7 wpl the descending axons enter the caudal stump (Yin and Selzer, 1983). Therefore, at 10 wpl most of the regenerating axons might be re-innervating the caudal stump and at 24 wpl the regeneration could be considered complete. The quantification of the cells was made in control and 24 wpl larvae since the observation of the glutamatergic cells before and after the complete regeneration was the truly interesting issue.

Statistical analysis

Statistical analysis was carried out by GraphPad Prism 5 (GraphPad software, La Jolla, CA). Variability of values was given by standard error of the mean (SEM). To test the normality of the data, the Kolmogorov-Smirnov test was used. As all the data passed the normality test, an unpaired Student t test was used for the statistical analysis of the data from the quantification of the cells and a one-way ANOVA with Bonferroni's multiple comparison tests for the quantification of the profiles. In the figures, different levels of significance are represented with different numbers of asterisks. One asterisk means significant ($0.01 < p$ value < 0.05); two asterisks mean very significant ($0.001 < p$ value < 0.01) and three asterisks mean extremely significant (p value < 0.001).

Results

Glutamate immunoreactivity in the spinal cord

In this work, the changes in the glutamate immunoreactivity during the spinal cord regeneration after a complete transection of the cord in the sea lamprey were studied.

As previously described (see chapter 1), glutamate immunoreactivity was widely distributed in the spinal cord of the sea lamprey. Processes of neurons were shown in the dorsal, ventral and lateral columns of the white matter, being more abundant in the dorsal column (Fig. 1A). Regarding the glutamatergic cells, two main populations were observed in the grey matter (dorsal and lateral) together with the CSFc and the primary sensory dorsal cells (Fig. 1B). DM and DL cells are distinguished in the dorsal population and SL and LL cells in the lateral population. In the white matter, individually identified cells were also observed (edge cells and cells associated to Mauthner axons, Müller axons and medium-sized dorsolateral axons) (Fig. 1B). The dorsal cells and the cells of the white matter do not appear in all the sections in normal animals and some of them are even observed occasionally. Because of that we decided not to quantify these cells since the quantification might be biased.

Quantitative changes in the glutamatergic cells during spinal cord regeneration

As it was explained in Material and Methods section, five types of glutamatergic cells were quantified: DM, DL, LL, SL and CSFc cells. The number of glutamatergic cells was quantified in hemisections of spinal cord both, rostral and caudal to the site of injury in control (unlesioned) and 24 wpl animals (Fig. 2A-C). Quantification of the average number of

glutamatergic cells per hemisection in the DM population showed no significant differences both, rostral (unlesioned: 51.7 ± 4.1; 24 wpl: 30.8 ± 9.3) and caudal (unlesioned: 60.7 ± 14.5; 24 wpl: 31.9 ± 5.3) to de site of injury (Fig. 3A, B).

In the DL population of the rostral stump of the cord, quantification of the average number of glutamatergic cells per hemisection indicated a significant decrease at 24 wpl as compared to control animals (unlesioned: 124.6 ± 10.7; 24 wpl: 74.8 ± 19.7; Student t test, $p = 0.0442$) (Fig. 3C). The same was observed in the DL population of the caudal stump of the cord (unlesioned: 165.3 ± 23.2; 24 wpl: 79.2 ± 23.2; Student t test, $p = 0.0290$) (Fig. 3D).

In the rostral stump of the cord, no significant differences were observed in the LL and the SL glutamatergic cell populations (unlesioned: 69.3 ± 13.5; 24 wpl: 43.6 ± 20.9; unlesioned: 267.3 ± 21.1; 24 wpl: 178.9 ± 50.4, respectively) (Fig. 4A, C). However, in the caudal stump of the spinal cord, quantification of the average number of the LL and SL glutamatergic cells per hemisection indicated a significant decrease at 24 wpl as compared to control animals (unlesioned: 72.4 ± 11.9; 24 wpl: 23.2 ± 9.7; Student t test, $p = 0.0124$; unlesioned: 300.1 ± 26.1; 24 wpl: 154.7 ± 50.4; Student t test, $p = 0.0245$, respectively) (Fig. 4B, D).

Quantification of the average number of glutamatergic cells per hemisection in the CSFc cells indicated a significant decrease at 24 wpl as compared to control animals both, rostral (unlesioned: 189.9 ± 20.7; 24 wpl: 49.9 ± 21.3; Student t test, $p = 0.0011$) and caudal (unlesioned: 235.1 ± 19.2; 24 wpl: 45.5 ± 19.3; Student t test, $p < 0.0001$) to de site of injury (Fig. 4E, F).

Quantitative changes in the glutamate immunoreactive processes during spinal cord regeneration

The number of glutamate-ir profiles was quantified in hemisections of the spinal cord both, rostral and caudal to the site of injury in control animals and in 2, 4, 10 and 24 wpl larvae (Fig. 5). As it was stated in the Material and Methods section, the white matter of the cord was divided into three different regions that were quantified for each hemisection: DM, VM and LAT (Fig. 1A). In general, in all the regions studied, the number of profiles decreased at 2 wpl and at 10 wpl the number of profiles became similar to that of the control (unlesioned) larvae (Fig. 5B-I).

In the DM region of the rostral stump of the cord, quantification of the average number of glutamate-ir profiles per hemisection indicated a significant decrease at 2 wpl and 4 wpl as compared to control samples (unlesioned: 884.1 ± 161.9; 2wpl: 271.8 ± 23.8; 4 wpl: 393.7 ± 53.2; ANOVA, $p = 0.0006$) (Fig. 6A). Between 2 wpl and 10 wpl larvae were also significant differences (10 wpl: 755.6 ± 55.4) (Fig. 6A). At 10 wpl and 24 wpl, the average number of glutamate-ir profiles per hemisection was similar to that of unlesioned larvae (24 wpl: 608.1 ± 91.6). The same results, regarding the significant differences, were observed for the DM region of the caudal stump of the spinal cord (unlesioned: 848.8 ± 133.4; 2 wpl: 287.0 ± 34.3; 4 wpl: 386.4 ± 61.5; 10 wpl: 709.7 ± 90.3; 24 wpl: 600.3 ± 96.6; ANOVA, $p = 0.0015$) (Fig. 6B).

In the VM region of the rostral stump of the cord, quantification of the average number of glutamate-ir profiles per hemisection indicated a significant decrease at 2 wpl as compared to control samples (unlesioned: 508.7 ± 100.9; 2 wpl: 183.1 ± 7.4; ANOVA, $p = 0.0023$) (Fig. 6C). In addition, a significant decrease in the average number of glutamate-ir profiles per hemisection was observed at 2 wpl as compared to 10 wpl and 24 wpl larvae (10 wpl: 488.8 ± 42.5; 24 wpl: 462.5 ± 60.3) (Fig. 6C). At 4 wpl, 10 wpl and 24 wpl there were not significant differences with

unlesioned values (4 wpl: 291.6 ± 32.9). In the VM region of the caudal stump of the cord, quantification of the average number of glutamate-ir profiles per hemisection indicated a significant decrease at 2 wpl as compared to control samples (unlesioned: 495.5 ± 92.8; 2 wpl: 178.5 ± 6.9; ANOVA, p = 0.0081) (Fig. 6D). No significant differences were observed at 4 wpl, 10 wpl and 24 wpl as compared to unlesioned values (4 wpl: 328.3 ± 17.6; 10 wpl: 388.3 ± 43.9; 24 wpl: 424.6 ± 67.0).

In the LAT region of the rostral stump of the cord, quantification of the average number of glutamate-ir profiles per hemisection indicated a significant decrease at 2 wpl and 4 wpl as compared to control samples (unlesioned: 934.0 ± 122.4; 2wpl: 317.0 ± 32.2; 4 wpl: 427.8 ± 65.9; ANOVA, p = 0.0002) (Fig. 6E). A significant decrease in the average number of glutamate-ir profiles per hemisection was also observed at 2 wpl as compared to 10 wpl (10 wpl: 752.8 ± 76.9) (Fig. 6E). At 10 wpl and 24 wpl, the average number of glutamate-ir profiles per hemisection was similar to that of unlesioned larvae (24 wpl: 611.6 ± 81.4). In the LAT region of the caudal stump of the cord, quantification of the average number of glutamate-ir profiles per hemisection indicated a significant decrease at 2 wpl and 4 wpl as compared to control samples (unlesioned: 967.0 ± 67.9; 2wpl: 361.5 ± 46.3; 4 wpl: 384.3 ± 49.2; ANOVA, $p < 0.0001$) (Fig. 6F). Moreover, a significant decrease in the average number of glutamate-ir profiles per hemisection was observed at 2 wpl and 4 wpl as compared to 10 wpl and 24 wpl (10 wpl: 733.9 ± 80.8; 24 wpl: 718.0 ± 85.8) (Fig. 6F). At 10 wpl and 24 wpl, the average number of glutamate-ir profiles per hemisection was similar to that of unlesioned larvae and no significant differences were observed between them.

Discussion

How glutamate expression varies in the spinal cord after SCI and during regeneration has not been investigated in spite of the importance that glutamatergic signalling has in the circuits underlying locomotion (for review see Grillner and Wallén, 2007). Our results show, for the first time, the overall changes in the expression of glutamate in cells and fibres in the spinal cord after SCI and during regeneration in the sea lamprey. Despite the recovery of locomotion, the results indicate that most of the neuronal populations caudally and some rostrally to the site of injury do not recuperate whereas the number of processes does recover in all the regions studied both, rostrally and caudally to the site of injury.

The number of the glutamate-ir cells from the DM, DL, LL and SL spinal grey populations and CSFc cells has been quantified in unlesioned animals and at 24 wpl. Our results show that cells of all the populations except for the DL and CSFc cells in the rostral stump, and only the DM cells in the caudal stump of the cord recover similar levels to that of normal animals at 24 wpl, indicating that the recuperation of glutamate expression in the glutamatergic populations is not complete. Incomplete restoration of the levels of ascending and descending intraspinal neurons has been observed at 8 wpl in lampreys (Armstrong et al., 2003). In contrast, it has been observed an increase of the number of descending propriospinal neurons at 32 wpl (Rouse and McClellan, 1997). All of these results together indicate variability in the grade of recovery of the different neuronal populations.

Our results also indicate that cells in the rostral stump recover glutamate expression to a greater degree than in the caudal stump. Further, diversity in the recovery of glutamate expression among the different cell populations has been also observed. Thus, DM cells present similar levels of glutamate expression at 24 wpl both, rostrally and caudally, whereas CSFc cells show very and extremely significant

differences in glutamate levels at 24 wpl in the rostral and caudal stumps, respectively. DL cells show also significant differences in both stumps, and the rest of the cell populations recover glutamate levels only rostrally.

The results observed for the SL and DL population suggest that a lack of innervation and/or targets could produce a decrease in glutamate synthesis. SL cells are supposed to be the excitatory interneurons (EINs) which participate in the CPG (see chapter 1). The EINs have a descending axon which can give ascending branches and are excited by RS axons (Buchanan and Grillner, 1987; for revision see Grillner and Wallén, 2007). So, the lesion will lead to a loss of targets and innervation in both stumps. However, only the rostral stump recovers glutamate expression at 24 wpl. Although not all the RS axons regenerate, our results show a recover of the glutamatergic fibres at 24 wpl. The regenerating descending RS axons reach the rostral stump before than the caudal stump establishing new synapses with the intraspinal cells of the proximal area. The time that lasts the re-innervation of the caudal stump might be enough to cause a lack of recover of the glutamate synthesis or even the delayed degeneration or death of the glutamate positive cells in the distal stump. The same response is observed in the LL cell population. The DL cells have an ascending axon that crosses the midline ventrally and a dendrite to the DC (see chapter 1), and lose also their targets and their innervation from the DC. In this population lack of recovery of glutamate expression is observed in both stumps at 24 wpl. This indicates that DL cells could be more vulnerable to lack of innervation and/or targets. Different response to the lesion has been also observed among different RS neurons, which present different probabilities of regeneration (David and McClellan, 1994a, b; Jacobs et al., 1997). DM cells recover the glutamate expression to normal levels at 24 wpl rostrally and caudally to the site of injury. These cells are supposed to take part in sensory relay (see chapter 1) so that they might be involved in local intrasegmental circuits, which are less affected by the lesion.

The fate of the neurons that do not express glutamate at 24 wpl is not known. Recently, an almost complete lack of cell death has been observed in spinal intrinsic neurons one month after SCI (Shifman et al., 2012). However, delayed cell death has been reported for the descending giant RS cells, which axons fail to regenerate, at 6 months after SCI, and it has been suggested that the lack of targets and their trophic support is responsible for the delayed cell death (Shifman et al., 2008). Whether the glutamate-ir neurons that do not recover glutamate expression at 24 wpl suffer a delayed degenerative process or not could be clarified by further investigations.

A great decrease in the number of glutamate-expressing CSFc cells has been observed in both stumps when the functional recuperation of the spinal cord has been achieved. In addition to cell death and loss of the glutamatergic phenotype, glutamate release to the CSF extended until 24 wpl can account for the decrease of the number of glutamate-expressing CFSc cells. Long lasting high glutamate levels in the CSF have been observed in amyotrophic lateral sclerosis (Spreux-Varoquaux, 2002) and motor neuron disease (Shaw et al., 1995) patients. However, its meaning is not well understood and further studies would be necessary to clarify this issue.

The spinal glutamatergic processes belong to intrinsic dendrites and axons of the spinal glutamatergic neurons (see Chapter 1), and to the ascending and descending systems that control the locomotion: DC and spinobulbar (SB) system and RS and vestibulospinal (VS) systems, respectively. As processes have not been correlated to the cell they belong to, we discuss our results based on the knowledge about the white matter fascicles in which the different axons run in the spinal cord of the lamprey. The giant and medium-sized axons of some of the identified RS cells (Müller cells, Mauthner cells and V cells) are not glutamate-ir (see chapter 1). So, among the RS descending axons, we refer only to the small

unidentified glutamate-ir ones, which have been quantified. Otherwise, the ability of regeneration of the giant RS axons has been well studied before (Rovainen, 1976; Jacobs et al., 1997; Shifman et al., 2008).

In all the regions studied, a significant decrease in the number of profiles is observed at 2 wpl. This is in agreement with functional studies, in which until 2 wpl there is not locomotor muscle activity (Davis and McClellan, 1994a, b; Zhang and McClellan, 1999). From 10 wpl the regeneration of descending axons is considered complete (McClellan, 1994). Our results indicate that the number of profiles at 10 wpl is similar to that of the control samples, which is also in line with the functional phases of recovery observed in these previous studies. The recovery of glutamate expression might be related to the restoration of the damaged structures. The restoration of the processes could be due to true regeneration, which has been observed in unidentified descending RS axons (Zhang and McClellan, 1999) or to sprouting and axonal/dendritic branching, which apparently might occur in processes of intraspinal glutamatergic neurons (present results) and also in giant RS axons, in which splitting into multiple branches has been observed (Rovainen, 1976). In this work, the DM region comprises the DC as well as the descending trigeminal tract, which is located next to the DC (Calton et al., 1998) and processes of the DM and DL cells. In addition, some descending axons from the mesencephalic reticular nucleus (MRN) and the antero-lateral vagal group (ALV) which project to the medial (dorsal and/or ventral) spinal region (Shaw et al., 2010) might also descend in this region. Axons of the primary sensory dorsal cells and dorsal root ganglion cells constitute the DC (Tang and Selzer, 1979; Dubuc et al., 1993). In our results, the number of profiles in the DM region of the cord was significantly decreased during the first 4 wpl both, rostrally and caudally. Degeneration of the DC after SCI in lampreys has been observed only rostrally (Rovainen, 1976; Selzer, 1976 and 1979); in spite of this, the lesion affect also the caudal stump and glutamate immunoreactivity is significantly decreased in both stumps. From 10 wpl

onwards, the number of glutamate-ir profiles in the DM region of the white matter becomes similar to that of unlesioned animals. Regeneration of the axons of the dorsal cells, although limited, has been observed before (Selzer, 1978; Yin and Selzer, 1983; Armstrong et al., 2003) and DM glutamatergic cells recover their levels after SCI (see above). The regenerative capacity of ganglion cells remains unknown. In contrast, previous studies have shown that the projections from the ALV do not recover to normal levels even at 32 wpl, whereas axons of the MRN show normal levels at 32 wpl (Davis and McClellan, 1994b). These results suggest that apart from regenerative mechanisms, sprouting of dendrites and axons might be necessary for the recovery of the glutamate-ir profiles.

The number of profiles in the LAT region of the white matter was significantly decreased both, rostrally and caudally to the site of injury during the first 4 wpl. A recover in the number of profiles is observed at 10 wpl both, rostrally and caudally to the site of injury. Most of the processes of the LAT region correspond to small glutamatergic spinal-projecting neurons that have been observed in some rhombencephalic nuclei (Villar-Cerviño et al., 2013): anterior, middle and posterior rhombencephalic reticular nucleus (ARRN, MRRN, PRRN, respectively) as well as postero-lateral vagal group (PLV), which projections run in the lateral column (Shaw et al., 2010). True regeneration of most of the small unidentified RS neurons has been demonstrated by retrograde double labelling at 8-16 weeks after SCI in lampreys (Zhang and McClellan, 1999), and projections from the ARRN, MRRN and PRRN recover the best (Davis and McClellan, 1994b). In addition, ascending axons from giant interneurons and edge cells (Rovainen, 1967; Rovainen, 1979; Selzer, 1979; Buchanan and Einum, 2008) run also in the lateral tracts and have been shown to regenerate (Yin and Selzer, 1983; Armstrong et al., 2003). This is in agreement with the present results, in which the number of glutamatergic processes of the LAT spinal region is recovered from 10 wpl onwards. The small RS axons in the lateral tracts seem to be critical for the initiation of

locomotion in normal and spinal cord-transected lampreys (McClellan, 1988, 1994; Shaw et al., 2010). Thus, regeneration of glutamatergic descending axons in the lateral tracts appears to be crucial for the recovery of the function. The presence also of local interneurons with axonal branching might contribute to the recovery of the number of profiles. However, the most important contribution to the regeneration and restoration of the number of profiles in the lateral tracts appears to be the small unidentified RS axons.

The number of profiles in the VM region of the white matter is significantly decreased both, rostrally and caudally to the site of injury during the first 2 wpl. The number of profiles in this region is the first in return to normal levels, at 4 wpl in both stumps of the cord. Projections from the MRN and the ALV nuclei run in the medial spinal fascicles (Shaw et al., 2010). As it has been stated above, previous studies have shown that the projections from the ALV do not recover to normal levels whereas axons of the MRN show normal levels at 32 wpl (Davis and McClellan, 1994b). Therefore, the early recovery observed here should be due to other projections. Processes of DL, LL and SL glutamatergic cells course mainly in the ventromedial and lateral columns of the spinal cord (see chapter 1). Cohen and Baker, (1988) have suggested that short fibres regenerate better than long ones. It is possible that most of the glutamate-ir restored processes of the VM region belong to local interneurons, leading to a faster recover in the number of profiles quantified in this region. However, we have seen here that most of the local glutamatergic interneurons do not recover at 24 wpl, especially caudally to the site of injury. This suggests that processes of local neurons that recover glutamate expression suffer a process of sprouting, generating new branches and leading to the restoration of the number of glutamate-ir processes, especially in the caudal stump of the cord.

Although the quantification of the profiles, do not show significant differences when the functional recovery is established, differences in the glutamate-ir neurons indicate that mechanisms of plasticity are present and may play an important role in the restoration of the glutamatergic spinal circuits following SCI in lampreys. Even in an animal model that fully recovers the motor function after SCI, the recovery of the glutamate expression pattern in the spinal cord is not complete. Regenerative and mechanisms of plasticity seem to be responsible for the recovery of both extrinsic and intrinsic circuits. It has been proposed that plasticity in intrinsic spinal circuits is critical for hindlimb locomotor recovery after SCI in mammals (Rossignol and Frigon, 2011). All these findings support the use of the lamprey as a reliable animal model for the study of the recovery of the neuronal populations and processes biochemically characterized following SCI, since processes of plasticity seem to play an important role to restore the function, as it occurs in vertebrates with limited regenerative capacity, such as mammals.

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Abbreviations

ALV	Antero-lateral vagal
ARRN	Anterior rhombencephalic reticular nucleus
CPG	Central pattern generator
CSFc	Cerebrospinal fluid-contacting
DC	Dorsal column
DM	Dorsomedial
DL	Dorsolateral
-ir	Immunoreactive
LAT	Lateral
LL	Large lateral
MRN	Mesencephalic reticular nucleus
MRRN	Middle rhombencephalic reticular nucleus
PLV	Postero-lateral vagal

PRRN	Posterior rhombencephalic reticular nucleus
RS	Reticulospinal
SB	Spinobulbar
SCI	Spinal cord injury
SL	Small lateral
VGLUT	Vesicular glutamate transporter
VM	Ventromedial
VS	Vestibulospinal
wpl	Weeks post-lesion

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Figures

Figure 1. Confocal photomicrograph (A) and schematic draw (B) of transverse sections of the spinal cord of the sea lamprey showing glutamate immunoreactivity in control animals. **A:** Hemisection of the spinal cord showing the 3 regions in which the white matter was divided to quantify the glutamate immunoreactive (-ir) profiles. Dorsomedial (DM), ventromedial (VM) and lateral (LAT). DC indicates the dorsal column. **B:** Major glutamate-ir cell types in the larval spinal cord. Primary sensory neurons or dorsal cells (black asterisks); dorsal interneurons (black squares); cells of the lateral population (gray circles); cerebrospinal fluid-contacting cells (CSFc) cells (black circles); edge cells (gray squares); cells associated to reticulospinal and Mauthner axons (black stars). Scale bars = 75 µm (B); 50 µm (A).

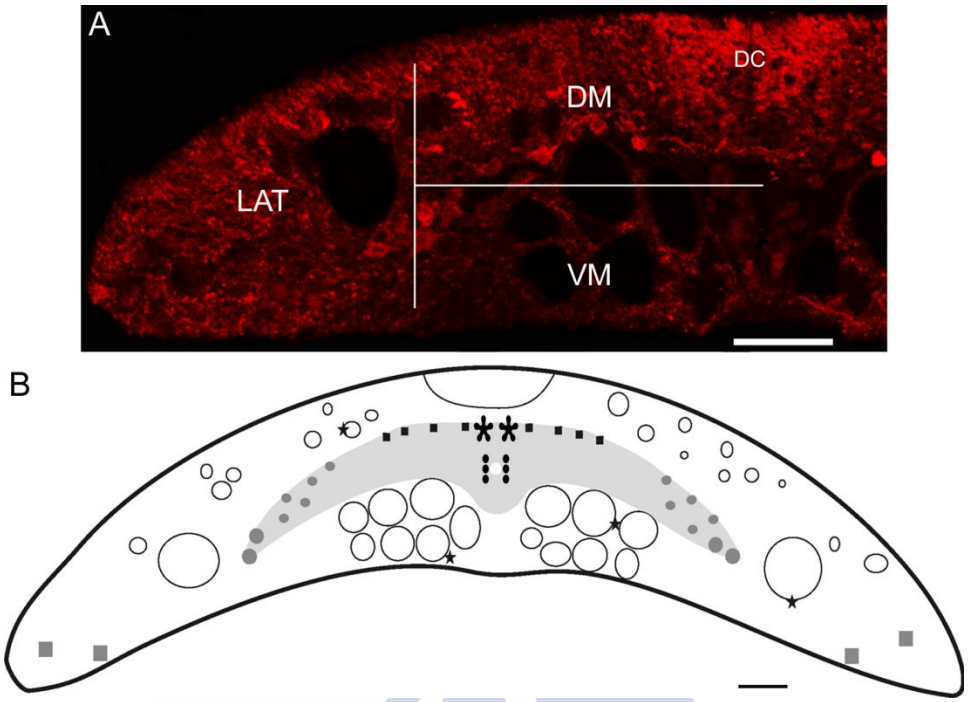

Figure 1

Figure 2. Confocal photomicrographs of transverse sections of the spinal cord of the sea lamprey showing glutamate immunoreactivity. **A:** Glutamate-ir cells in a control larva**B, C:** Glutamate-ir cells in the rostral (B) and caudal (C) stumps at 24 wpl. Dorsal interneurons (arrowheads), lateral interneurons (arrows), CSFc cells (outlined arrowheads). Star points to the central canal. In all figures dorsal is at the top. Lateral is on the right except for B, in which lateral is on the left. Scale bars = 25 µm.

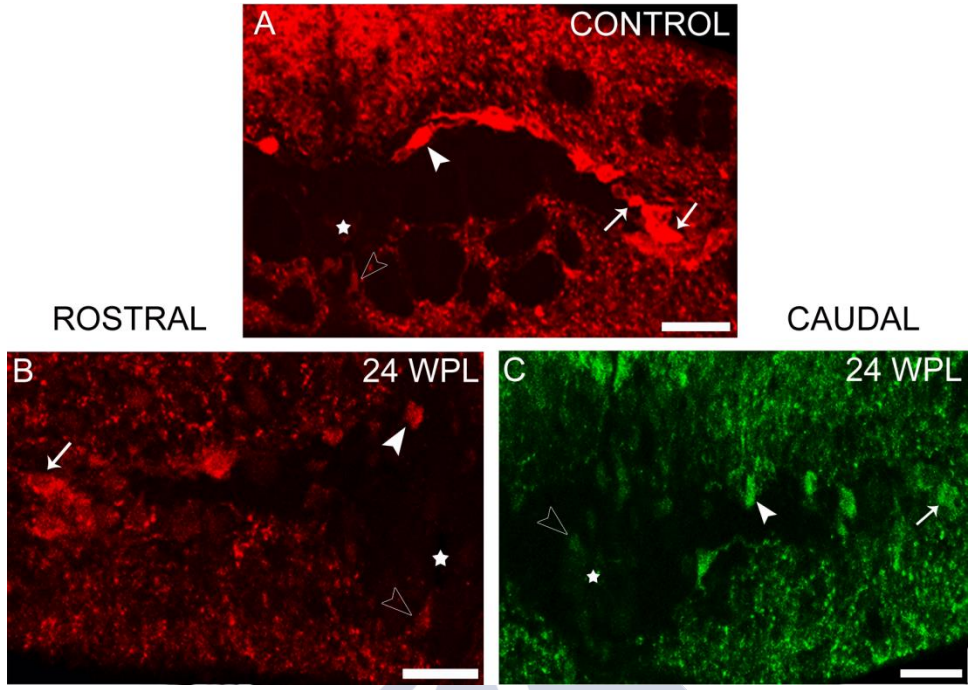

Figure 2

Figure 3. Graphics showing the numbers of the glutamate-ir cells per section of the dorsal populations in the rostral and caudal stumps of the cord in control larvae and at 24 wpl. **A, B:** Numbers of glutamate-ir DM neurons per section in the rostral (A) and caudal (B) stumps of the cord. **C, D:** Numbers of glutamate-ir DL neurons per section in the rostral (C) and caudal (D) stumps of the cord. The asterisk indicates significant differences of the means (*p* value < 0.05).

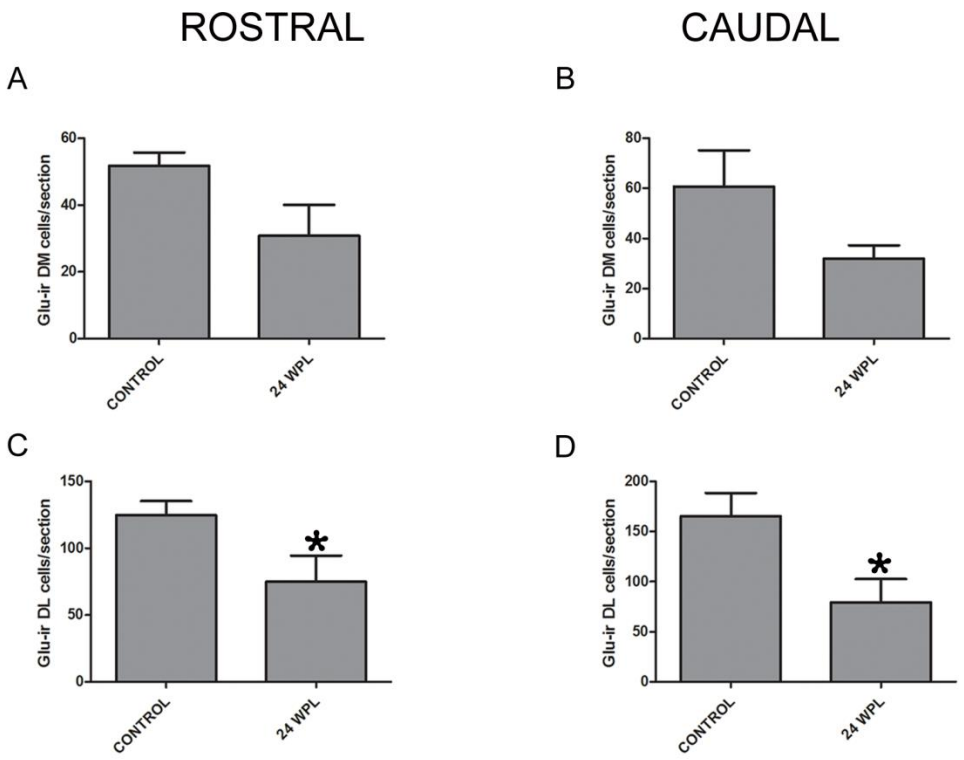
ROSTRAL
CAUDAL
A
B
C
D
Glu-ir DM cells/section
Glu-ir DL cells/section
CONTROL
24 WPL
*

Figure 3

Figure 4. Graphics showing the numbers of the glutamate-ir cells per section of the lateral populations and the CSFc cells in the rostral and caudal stumps of the cord in control larvae and at 24 wpl. **A, B:** Numbers of glutamate-ir LL neurons per section in the rostral (A) and caudal (B) stumps of the cord. **C, D:** Numbers of glutamate-ir SL neurons per section in the rostral (C) and caudal (D) stumps of the cord. **E, F:** Numbers of glutamate-ir CSFc cells per section in the rostral (E) and caudal (F) stumps of the cord. One asterisk means significant ($0.01 < p$ value < 0.05); two asterisks mean very significant ($0.001 < p$ value < 0.01) and three asterisks mean extremely significant (p value < 0.001).

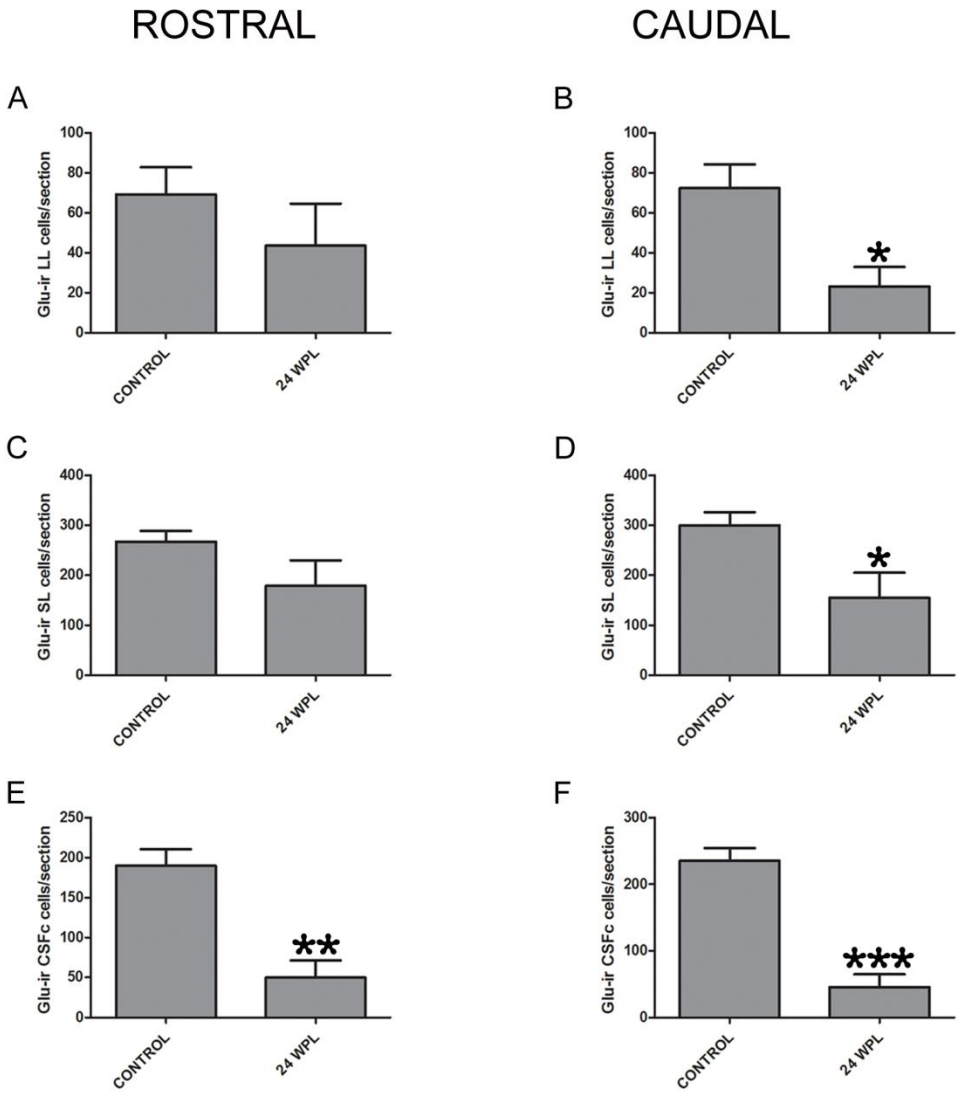
ROSTRAL
CAUDAL
A
B
C
D
E
F
Glu-ir LL cells/section
Glu-ir SL cells/section
Glu-ir CSFc cells/section
CONTROL
24 WPL
*
**
***

Figure 4

Figure 5. Confocal photomicrographs of transverse sections of the spinal cord of the sea lamprey showing glutamate immunoreactivity. **A:** Glutamate-ir in a control larva. DC indicates the dorsal column. Star points to the central canal. **B, C:** Glutamate-ir fibres in the rostral (B) and caudal (C) stumps at 2 wpl. **D, E:** Glutamate-ir fibres in the rostral (D) and caudal (E) stumps at 4 wpl. **F, G:** Glutamate-ir fibres in the rostral (F) and caudal (G) stumps at 10 wpl. **H, I:** Glutamate-ir fibres in the rostral (H) and caudal (I) stumps at 24 wpl. In all figures dorsal is at the top. Lateral is on the left except for B, D, G and I, in which lateral is on the left. Scale bars = 50 µm (B, C, D, E, F, G, H, I); 25 µm (A).

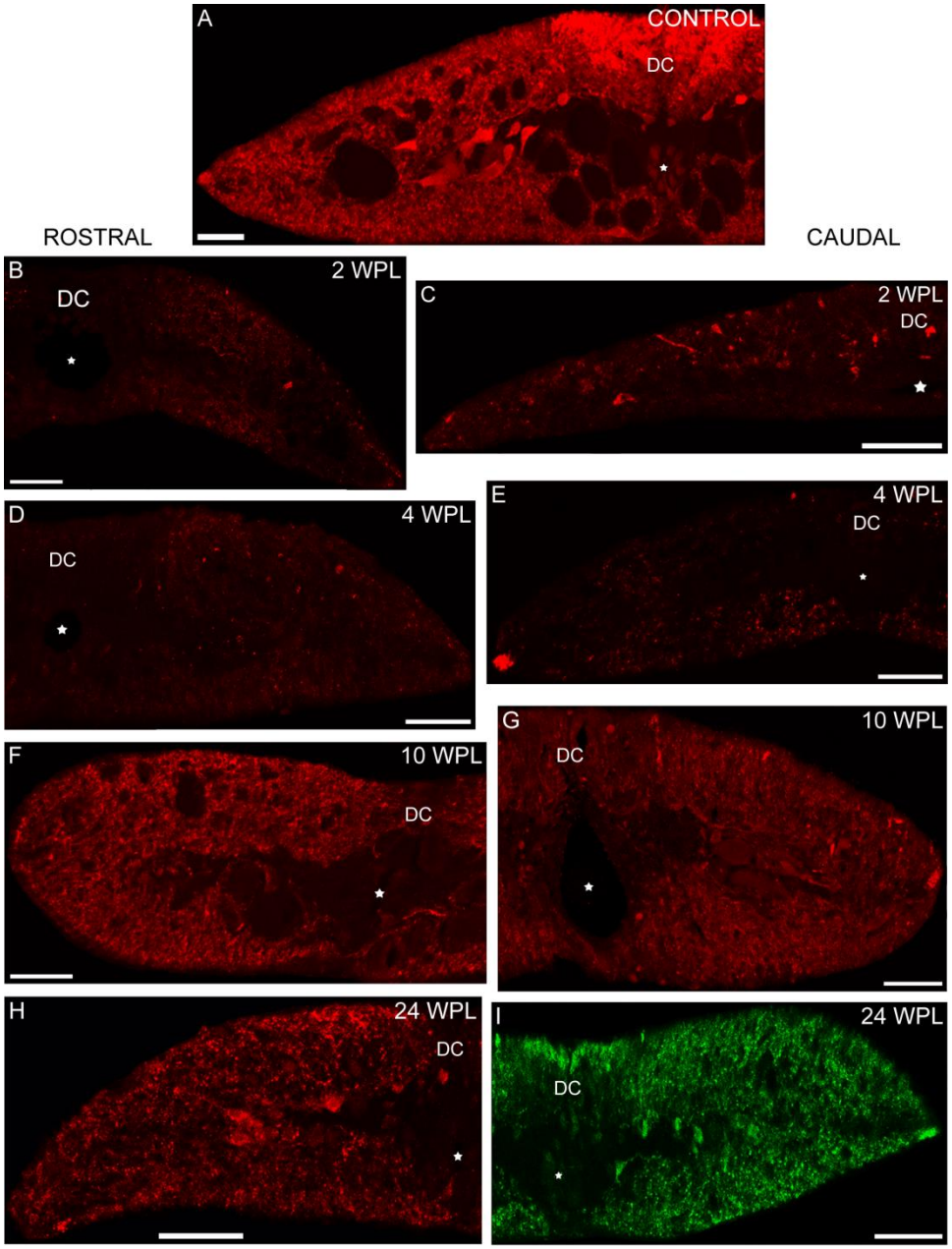
A
CONTROL
DC
ROSTRAL
CAUDAL
B
2 WPL
DC
C
2 WPL
DC
D
4 WPL
DC
E
4 WPL
DC
F
10 WPL
DC
G
10 WPL
DC
H
24 WPL
DC
I
24 WPL
DC

Figure 5

Figure 6. Graphics showing the numbers of the glutamate-ir profiles per section in the rostral and caudal stumps of the cord. **A, B:** Numbers of glutamate-ir DM profiles per section in the rostral (A) and caudal (B) stumps of the cord. **C, D:** Numbers of glutamate-ir VM profiles per section in the rostral (C) and caudal (D) stumps of the cord. **E, F:** Numbers of glutamate-ir LAT profiles per section in the rostral (E) and caudal (F) stumps of the cord. One asterisk means significant ($0.01 < p$ value < 0.05); two asterisks mean very significant ($0.001 < p$ value < 0.01) and three asterisks mean extremely significant (p value < 0.001).

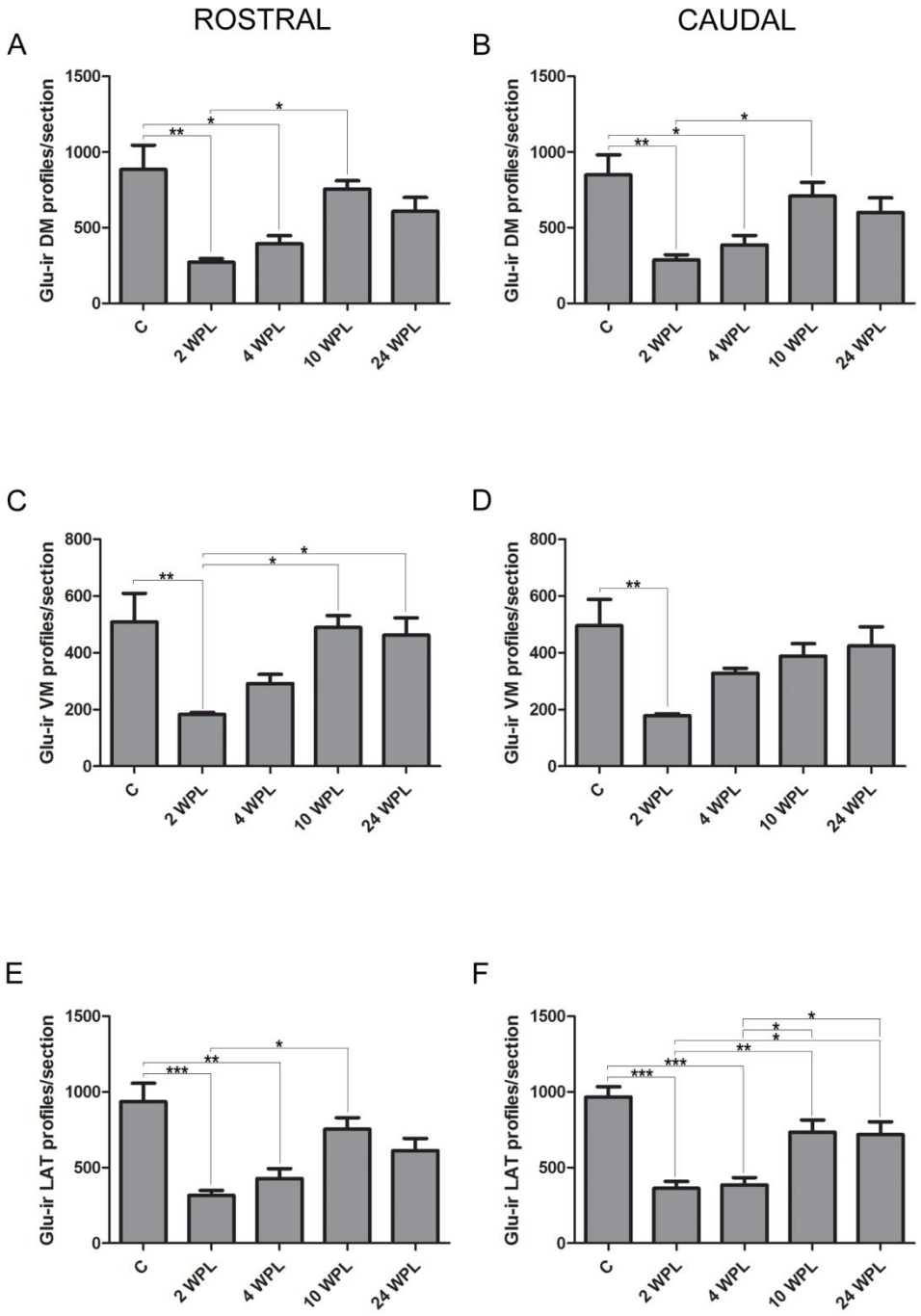
ROSTRAL
CAUDAL
A
B
C
D
E
F
Glu-ir DM profiles/section
Glu-ir VM profiles/section
Glu-ir LAT profiles/section
1500
1000
500
0
800
600
400
200
C
2 WPL
4 WPL
10 WPL
24 WPL
*
**
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Figure 6

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General discussion

GENERAL DISCUSSION

General discussion

Glutamate plays a key role in the spinal circuits underlying locomotion (Roberts et al., 1998; Higashijima et al., 2004). The central nervous system (CNS) of lampreys follows the general pattern of that of vertebrates. Further, the lamprey is the vertebrate in which the spinal networks have been described in more detail, based on electrophysiological studies (Buchanan, 2001; Grillner, 2003) and has been used as a model for the study of the spinal networks underlying locomotion for years (revision in Grillner and Wallén, 2007). Because of its phylogenetic position, lampreys are also very useful for study the basic organization and evolution of the neurochemical systems of vertebrates. Moreover, lampreys have also become a reliable animal model in studies of spinal cord regeneration because, in contrast to mammals, they have the ability of spontaneously recover locomotor function following spinal cord injury (SCI) (for revision see Shifman et al., 2007).

The main aims of this thesis project have been the anatomical characterization of the spinal glutamatergic system and the investigation of short and long term changes in this system following SCI in the sea lamprey. *In situ* hybridization and immunofluorescence techniques have been used to characterize and demonstrate the glutamatergic populations and fibres or the presence of neurons and astrocytes. To show the actively transport of glutamate into the astrocytes, pharmacological techniques have been used.

In this project, we report for the first time a comprehensive study of the expression and distribution of the vesicular glutamate transporter (VGLUT) mRNA and the glutamate immunoreactivity in neurons of the

spinal cord of lampreys, respectively. Our results support the electrophysiological characterization of the excitatory interneurons that participate in the spinal locomotor networks as glutamatergic (Buchanan and Grillner, 1987), as well as of other identifiable spinal cells such as primary sensory dorsal cells and giant caudal interneurons (Rovainen, 1967; Christenson et al., 1988b; el Manira et al., 1996), since these cells expressed VGLUT mRNA. In addition, this work raises the number of glutamatergic spinal cell types by two: cerebrospinal fluid-contacting (CSFc) cells and white matter interneurons. The latter may be involved in the circuits underlying locomotion, indicating that the types of excitatory interneurons that take place in the spinal locomotor circuits are higher than previously thought. So far, only grey matter excitatory interneurons have been implicated in these circuits (revision in Grillner et al., 2008). The results obtained in the study of the colocalization between glutamate and GABA or glycine indicate that glutamatergic cells are neurochemically more complex than previously thought, and that it is not a feature of developmental cells, since the cells that show colocalization in larvae also show colocalization in adult stage. Two examples of colocalization are especially striking: all the glutamate immunoreactive (-ir) CSFc cells are also GABA-ir, with differences in the distribution of both immunoreactivities. Differential distribution of neurotransmitters in the same cell could mean different use by the cell of these neurotransmitters, as have been observed in rat motor neurons (Herzog et al., 2004; Nishimaru et al., 2005). Ventral edge cells have been characterized as glycinergic (Villar-Cerviño et al., 2008). Electrophysiological studies indicate that there are two types of edge cells, excitatory ipsilateral-projecting and inhibitory contralateral-projecting edge cells (Rovainen, 1974; Viana di Prisco et al., 1990). Our results are in agreement with the fact that some edge cells are glutamatergic. However, colocalization of glutamate and glycine has been observed in all the ventral putative glycinergic edge cells, revealing a more complex functionality of these cells.

Glutamate molecule exerts harmful actions in the first days after a spinal injury. Following SCI in mammals, a massive release of glutamate to excitotoxic levels has been observed (Panter et al., 1990; Liu et al., 1999; McAdoo et al., 1999; Xu et al., 2004) and leads to neuronal and oligodendrocyte death (Liu et al., 1999; Xu et al., 2004). It has been shown that the rise in glutamate levels is due to astrocyte uptake dysfunction. Down-regulation or lack of glutamate transporter (GLT) in surviving astrocytes, apoptosis of GLT-expressing astrocytes, and, especially, reversed glutamate uptake have been described (Szatkowski et al., 1990; McAdoo et al., 2000; Rossi et al., 2000; Lepore et al., 2011). In this project, we report for the first time the short term response in terms of glutamate expression and the astrocytic response following SCI in a regenerating vertebrate, the sea lamprey, in which a lack of cell death has been observed during the first days after SCI (Shifman et al., 2012). Our results show that there is also a massive release of glutamate from neurons after SCI in lampreys. However, in contrast to mammals, glutamate transporters of astrocytes of lampreys continue working in a proper way after SCI. Therefore, they uptake the glutamate released, protecting neurons and other cells from death. Knowing how glutamate transporters of lampreys maintain their functionality could provide new therapies and targets to protect the injured spinal cord from the secondary injury.

The glutamatergic spinal system is involved in the initiation of locomotion and in the sensory pathways that give feedback to the reticulospinal (RS) cells (revision in Dubuc et al., 2008). Glutamate is used by neurons in the command centres, RS neurons and neurons in the spinal cord that excite motor neurons and other interneurons (Grillner et al., 2008; Kiehn et al., 2008). Therefore, its importance after a SCI for recovering of functional locomotion is clearly seen. We report here for the first time, the changes in the spinal glutamatergic system after SCI and during regeneration in the sea lamprey. Previous studies have been mainly focused on the regeneration of the giant RS glutamatergic

descending axons (Rovainen, 1976; Selzer, 1978; Jacobs et al., 1997; Shifman et al., 2008), although a few works about the regeneration of the unidentified RS axons have been also made (Davis and McClellan, 1994a, b; Zhang and McClellan, 1999). Our results indicate that plasticity mechanisms are present in the spinal glutamatergic system to restore the function after SCI. Thus, whereas the number of fibres recuperates to normal levels at 4-10 weeks post lesion (wpl) both, rostrally and caudally to the site of injury, some of the glutamatergic neuronal populations do not recover at 24 wpl, especially in the caudal stump of the cord. This indicates that the recuperation of glutamate expression in the glutamatergic populations is not complete. Incomplete restoration of the levels of ascending and descending intraspinal neurons has been also observed in lampreys (Armstrong et al., 2003). The recovery of the number of glutamate-ir profiles could be due to true regeneration, which has been observed in unidentified descending RS axons (Zhang and McClellan, 1999) or to sprouting and axonal/dendritic branching, which apparently might occur in processes of intraspinal glutamatergic neurons (see chapter 4) and also in giant RS axons, in which splitting into multiple branches has been observed (Rovainen, 1976). Thus, restoration of the function relies not only on regenerating but also on plasticity mechanisms even in an animal capable of recover the locomotor function after SCI. In mammals, it has been observed that plasticity in intrinsic spinal circuits is critical for hindlimb locomotor recovery after SCI (Rossignol and Frigon, 2011).

All these findings suggest that lampreys constitute a reliable model for studying the molecular mechanisms that underlie the spinal cord regeneration, since the plasticity processes seem to be the basis of their regenerative ability as it occurs in vertebrates with limited regeneration capacity, such as mammals.

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Summary and conclusions

SUMMARY AND CONCLUSIONS

Summary

Spinal cord injury (SCI) consists in a lesion within the spinal cord that leads to disruption of axonal bundles conveying sensory and motor information. SCI results in an irreversible loss of sensitivity and function below the lesion site in non-regenerating vertebrates, such as mammals, including humans. The injured axons are not able to regrow through the site of injury or re-establish synaptic contacts.

In the pathophysiology of SCI, there are two differentiated stages. The primary injury corresponds with the initial alterations of the tissue and is due to the lesion itself. It is characterised by disruption of the meninges and the blood brain barrier, haemorrhage and massive death of neurons, astrocytes and oligodendrocytes by necrosis. During the secondary injury process, the damage of the primary events spreads to distal regions and great amounts of neurotransmitters, such as glutamate are released. Glutamate is excitotoxic and causes excessive calcium influx inside the cells which leads to neuronal, astrocyte and oligodendrocyte death by apoptosis producing also loss of myelin. In addition, astrocytes become reactive and constitute the glial scar which, in long term, results in a physical and chemical barrier that prevents the axonal growth. The inflammatory response leads to the activation and infiltration of neutrophils, macrophages and microglia leading to a massive alteration of the nervous tissue.

Between 250,000 – 500,000 people suffer SCI every year around the world, and about 1,000 people in our country. Because of this, SCI

constitutes a great social and economic problem, which makes the research on SCI truly important for the society.

The sea lamprey, *Petromyzon marinus*, belongs to the cyclostomes or agnathans, the most ancient group of extant vertebrates. Therefore, lampreys hold a key phylogenetic position between urochordates, the immediately related invertebrates, and gnathostomes (jawed vertebrates). Thus, they are unique species to study the evolution of morphological as well as molecular and genomic characters at the base of the Craniates and throughout the evolution of this phylum. During the last years, the whole genome of the sea lamprey has been sequenced and published (http://www.ensembl.org) allowing the completion of the evolutionary studies from a genomic point of view. Lampreys have become a model in comparative neuroanatomy and in the study of the networks that underlie the locomotion in vertebrates. The central nervous system (CNS) of lampreys follows the general pattern of that of vertebrates but has much fewer neurons. Further, the isolated brainstem-spinal cord of lampreys can be maintained alive *in vitro* for several days and the loco motor pattern can be elicited by electric stimulation of the diencephalic and mesencephalic locomotor regions. Due to these features, the lamprey is the vertebrate in which the spinal networks that underlie locomotion have been described in more detail, based on electrophysiological studies. On the other hand, lampreys are also an animal model for studies of spinal cord regeneration and other central nervous system disorders. In contrast to mammals, lampreys are capable of recover normal-appearing locomotion after a complete transection of the cord with axonal pathway and synaptic specificity. In fact, the lamprey is the unique vertebrate that meets all the five criteria proposed by the NIH to demonstrate functional regeneration in the spinal cord:

1. The experimental lesion must cause disconnection of nerve processes.
2. Processes of CNS neurons must bridge the level of injury.
3. The regenerated fibres must make junctional contacts.
4. The regenerated fibres must generate post-junctional responses.
5. Changes in function must derive from regenerated connections.

In spite of the regenerative capacity of the CNS of lampreys, plasticity mechanisms are also important to achieve the restoration of the function, as it is observed in non-regenerating vertebrates. Finally, not all the axons of lampreys regenerate in the same way, which allow the study the intrinsic molecular mechanisms that underlie the ability of the neurons to regenerate in the same permissive environment after SCI.

The spinal cord of the lamprey is flattened and transparent, lacks myelin and the blood vessels do not enter its parenchyma. There are different neuron types within the spinal cord, such as motor neurons, dorsal cells, edge cells and several types of intrinsic neurons. The spinal cord has an intrinsic circuit that generates a coordinated rhythmic output that controls the locomotion, without any sensory influence. This circuit is called the central pattern generator (CPG) and is present in all vertebrates, including humans. The CPG is found along the entire length of the cord in lampreys. It is constituted by several types of neurons, including the excitatory glutamatergic interneurons, which participate in the rhythmic activation of other interneurons and motor neurons, and in the rhythm generation itself, as well as the small inhibitory interneurons which are implicated in the left-right alternating locomotor pattern. The CPG is controlled by descending systems which in turn receive feedback information from ascending systems about the state of the locomotor networks and about sensory signals generated during movements. The descending systems to the spinal cord in lampreys are the reticulospinal system (RS), which includes the ipsilateral giant RS axons of the cord, as well as other axons, and the vestibulospinal system (VS) that projects only

to the rostral spinal cord. The ascending systems in lampreys are the dorsal column that carries sensory information from the skin to the brainstem, and the spinal lemniscal pathway, which convey sensory signals and information about the motor pattern to the brain.

Glutamate is the major excitatory neurotransmitter in the vertebrate CNS. Glutamatergic systems, including metabolism, release mechanisms, receptors and transporters are implicated in nearly all aspects of normal brain function, development and ageing as well as in most neurological diseases. Because of this, it is of great importance that glutamate is at the correct concentration, in the correct place and at the correct time. One of the main problems is produced when glutamate transporters do not function in a correct way. High levels of extracellular glutamate results in an over-stimulation of the glutamate receptors that leads to an increase in the Na^+ and Ca^{2+} influx, energy consumption, free radical production and, consequently, to neuronal death. A defect in the glutamate uptake has been suggested to participate in numerous neurological diseases, such as ischemia, traumatic brain and spinal cord injury, amyotrophic lateral sclerosis, Alzheimer's disease or epilepsy, among others.

Despite the importance of glutamate in the CNS, the distribution of the glutamatergic populations in the brain and the spinal cord of most vertebrates is still unknown. In the sea lamprey, most of the works that characterized the excitatory (glutamatergic) transmission in some identified cells of the CNS of lampreys were physiological until the last years. Immunohistochemical studies of our group have described glutamate immunoreactive (-ir) neurons in the retina and in the dorsal column nucleus. Most recently, the lamprey vesicular glutamate transporter (VGLUT) have been characterized by our group providing a new tool to describe the glutamatergic neurons in this animal model. Thus, comprehensive *in situ* hybridization studies detecting VGLUT mRNA in the forebrain and brainstem of the sea lamprey have been published

recently. It has been found that glutamatergic populations are widely distributed along the lamprey brain. However, the distribution of the glutamatergic populations in the spinal cord of the lamprey has remained unknown.

We aimed to increase our knowledge about the spinal glutamatergic system in lampreys in normal conditions, after a spinal cord injury and during the spinal regeneration. Therefore, in this thesis project we focused on (1) the characterization of the glutamatergic neuronal populations in the spinal cord of lampreys and its comparison with that of γ-aminobutyric acid (GABA) and glycine, (2) the investigation of the massive glutamate release and the subsequent astrocyte response after SCI, and (3) the analysis of the changes in the spinal glutamatergic system during spinal cord regeneration. In addition, we designed a method for the quantification of neuronal processes and their comparison in different experimental conditions.

CHAPTER 1: The glutamatergic neurons in the spinal cord of the sea lamprey: an in situ hybridization and immunohistochemical study

Glutamate is the main excitatory neurotransmitter involved in spinal cord circuits in vertebrates. In spite of this, in most vertebrates the distribution of glutamatergic spinal neurons is still unknown. Lampreys, which belong to the most ancient group of extant vertebrates, have been extensively used as a model to investigate the neuronal circuits underlying locomotion. Glutamatergic circuits have been characterized on the basis of the excitatory responses elicited on postsynaptic neurons. However, the presence of glutamatergic neurochemical markers in spinal neurons has not been investigated. In this study, we report for the first time the expression of a lamprey VGLUT mRNA in the spinal cord of the sea lamprey. We also study the distribution of glutamate immunoreactivity in

perikarya and fibres. The largest glutamatergic neurons found in the grey matter were the dorsal cells and caudal giant cells. Two additional VGLUT-positive grey matter populations were also observed: one dorsomedial population constituted by small cells and one lateral population, which was constituted by small and large cells. Around the central canal, some cerebrospinal fluid-contacting cells also expressed VGLUT. In the white matter, some edge cells and some cells associated with giant axons (Müller and Mauthner axons) and the dorsolateral fascicle expressed VGLUT. Large lateral cells and the cells associated with reticulospinal axons are in a key position to receive descending inputs involved in the control of locomotion. We also compared the distribution of glutamate immunoreactivity with that of the neurotransmitters GABA and glycine. Colocalization of glutamate and GABA or glycine was observed in some small spinal cells. These results confirm the glutamatergic nature of various neuronal populations, and reveal new small-celled glutamatergic populations. Colocalization study predicts that some glutamatergic neurons would exert complex actions on postsynaptic neurons.

CHAPTER 2: Neuronal release and successful astrocyte uptake of glutamate following complete spinal cord transection in lampreys

In contrast to mammals, the spinal cord of lampreys spontaneously recovers from a complete spinal cord injury (SCI). Understanding the differences between lampreys and mammals in their response to SCI could provide valuable information to propose new therapies. Unique properties of the astrocytes of lampreys probably contribute to the success of spinal cord regeneration. Following SCI in mammals, a massive release of glutamate to excitotoxic levels, which leads to neuronal and oligodendrocyte death, has been observed. It has been shown that the rise in glutamate levels is due to astrocyte uptake dysfunction. The main aim of this chapter was to investigate the release of glutamate and the

subsequent astrocyte response during the first week following a complete spinal cord transection in the sea lamprey, in which a lack of cell death has been observed during the first days after SCI. The immunofluorescence detection of glutamate, Hu (a neuronal marker) and cytokeratins (a lamprey glial marker) has allowed us to show, for the first time in any vertebrate, the glutamate release from neurons and the response of astrocytes during the first days following SCI. Our results show that spinal injury caused the immediate loss of glutamate immunoreactivity in neurons close to the lesion site. This is in agreement with the rise in extracellular glutamate levels observed in mammals after SCI. Strikingly, astrocytes showed glutamate immunoreactivity after SCI, which is not observed with a previous treatment with an inhibitor of the glutamate transporters, DL-*threo*-β-Benzyloxyaspartic acid (DL-TBOA). This indicates that, in contrast to mammals, glutamate transporters of astrocytes of lampreys continue working properly after SCI. Thus, the astrocytes actively uptake the glutamate released providing protection from death to neurons. How glutamate transporters of lampreys maintain this impressive capacity of actively uptake glutamate following SCI remains unknown. Investigation on this topic could provide new targets to prevent the secondary injury following injury to the CNS.

CHAPTER 3: Confocal microscopy used for the semiautomatic quantification of the changes in aminoacidergic fibres during spinal cord regeneration

Spinal cord injury causes permanent functional deficits and an irreversible loss of function in mammals. An adequate treatment does not exist so far and, therefore, it constitutes a public health and economic problem. Changes in the different spinal neurotransmitters systems, including the aminoacidergic neurotransmitters, have been observed after the injury. In this work, we describe a very useful method to quantify the changes in the number of fibres in the spinal neurotransmitters systems

during the regeneration of the spinal cord in lampreys. This method combines confocal microscopy together with the use of appropriate computer software. Confocal microscopy permits to obtain high-resolution images, allowing the distinction between two immunofluorescence profiles that are very close to each other. We have combined high- resolution images of confocal microscopy with the use of available free software (Fiji and the software's plug-in Feature J). This computer software allows making a semiautomated quantification of the number of positive profiles in photomicrographs. Thus, in this chapter, we show a protocol to detect aminoacidergic neurotransmitters in the CNS of lampreys using glutaraldehyde tissue fixation, and to quantify the neuronal fibres during spinal cord regeneration. Establishing standard protocols for this type of studies will provide a tool to compare results when analysing the changes in different neurotransmitter systems at different time points after injury. Moreover, this method can also be used with bright-field microscopy and be adapted to other experimental conditions and animal models.

CHAPTER 4: Plasticity in the spinal glutamatergic system to restore the function after spinal cord injury in lampreys

In contrast to mammals, it is well known that most of the anamniote vertebrates are capable of spinal cord regeneration and movement recover. Among them, lampreys are the unique vertebrate that satisfies the five criteria established by the NIH to consider the functional regeneration complete. The vertebrate locomotion is generated by the central pattern generator networks within the spinal cord. Glutamate mediates excitatory neurotransmission and is used by neurons in the brain command centres, reticulospinal neurons and neurons in the spinal cord that excite motor neurons and other interneurons. While the short term changes in glutamate levels after injury

are well documented, much less is known about the long term changes occurring at the level of spinal cord populations. The purpose of this chapter was to quantify the changes that occur in the glutamatergic neurons and fibres during the regeneration of the spinal cord in mature sea lamprey larvae. We used glutamate immunofluorescence to detect the glutamate immunoreactive (-ir) cells and fibres, and the semiautomated quantification method described in chapter 3 to quantify the glutamate-ir fibres at different time points after the injury (2, 4, 10 and 24 weeks post lesion; wpl). The quantification of the glutamate-ir cells was made manually in normal animals and at 24 wpl. Briefly, a significant decrease in the number of fibres was observed at 2 wpl, whereas at later time points the expression gradually increased returning to normal levels. However, significant changes were observed in the number of cells at 24 wpl in most of the populations. The results obtained indicate that plasticity mechanisms, and not only regenerative processes, are present and may play an important role in the restoration of the glutamatergic spinal circuits following SCI in lampreys. These properties make lampreys a more reliable animal model for CNS regenerative capacity in vertebrates, as the plasticity processes seem to be the basis of the restricted regenerative ability of vertebrates with limited regeneration capacity, such as mammals.

Developmentally stable larvae, downstream migrating young adult and upstream migrating adult sea lampreys, *Petromyzon marinus,* were used in this thesis project.

In situ hybridization technique was used to demonstrate the presence of the VGLUT mRNA. Digoxygenin-labelled riboprobes were synthesized using the amplified fragments of the cloned sequences (Villar-Cerviño et al., 2010) as templates, following standard protocols. *In situ* hybridizations were conducted using standard protocols, with an RNAse A

treatment added to the post-hybridization washings. Staining was conducted in BM Purple at 37ºC until the signal was clearly visible.

For inhibition of the glutamate transporters, anesthesiated larvae were placed dorsal side up in a Sylgard-lined dish filled with Ringer solution. The skin, the muscles and the meninx of the region comprised between the third and the seventh gill were carefully removed exposing the spinal cord. The animals were maintained in 30 µM DL-TBOA diluted in dimethyl sulfoxide (DMSO) in Ringer during 4 h.

Immunofluorescence protocols were used to detect glutamate-GABA-, glycine-, Hu- and cytokeratin-immunoreactivities in normal conditions or after spinal cord injury. After deeply anesthesia, larvae were placed dorsal side up in a Sylgard-lined dish filled with Ringer solution. The spinal cord was exposed by a transverse incision from the dorsal midline at the level of the fifth gill, a complete spinal cord transection was performed with a scalpel and the spinal cord cut ends were visualized under a microscope. After surgery, lampreys were maintained on ice for 1 h to allow the wound to air dry. After this time, the animals were allowed to recover in aerated freshwater tanks at 19.5 ºC. Each transected animal was examined 24 h after surgery to confirm that there was no movement caudal to site of injury. For detection of the different markers, sections were incubated with a mixture of a rabbit polyclonal anti-glutamate antibody and a mouse monoclonal anti-GABA or anti-cytokeratin or anti-HuC/HuD antibodies or with a mixture of a rabbit polyclonal anti-glycine and a mouse monoclonal anti-glutamate antibody. For indirect immunofluorescence detection of each molecule, Cy3-conjugated goat anti-rabbit and fluorescein-conjugated goat anti-mouse immunoglobulins were used.

The quantification of profiles was made using the plug-in Feature J for Fiji software. For statistical analysis, the program GraphPad Prism was used and the Kolmogorov-Smirnov normality test, Student t and ANOVA

with Bonferroni's multiple comparisons tests were used to analyse the different data.

All experiments were approved by the Bioethics Committee at the University of Santiago de Compostela and conformed to the European Union and the Spanish guidelines on animal care and experimentation to minimize the pain and suffering.

Some of the chapters of this Doctoral Thesis have been already published as research articles or book chapters with the following references:

- Chapter 1: PLoS ONE. 2012. 7:e47898. doi: 10.1371/journal.pone.0047898.
- Chapter 2: this chapter belongs to a research article, in which information about other neurotransmitters, that are not the aim of this thesis, is provided. Glia. 2014. 62:1254–1269.
- Chapter 3: published as a book chapter in: *Confocal and multiphoton laser-scanning microscopy of neuronal tissue: Applications and quantitative image analysis*. 2014. Eds: Bakota L and Brandt R. Springer, New York pp. 239-250.
- Chapter 4: this chapter will be submitted for publishing in a short while.

Conclusions

The analysis of the results obtained in the present Thesis led us to the following conclusions:

1. The characterization of the glutamatergic cell populations of the spinal cord of the sea lamprey has raised by two the number of glutamatergic cell types: cerebrospinal fluid-contacting cells and white matter interneurons.

2. VGLUT expression has also been found in dorsal cells, giant interneurons, edge cells and two neuronal populations of the grey matter: dorsal and lateral, which is in agreement with previous electrophysiological studies.

3. The pattern of VGLUT expression and glutamate immunoreactivity in the spinal cord is similar in larvae and adults, indicating that no major changes occur in this system during metamorphosis.

4. Colocalization of glutamate and GABA is observed in cells of the dorsal and lateral grey populations and in cerebrospinal fluid-contacting cells, whereas colocalization of glutamate and glycine is observed also in those cell types and in ventral edge cells. These findings suggest that glutamatergic cells of the lamprey spinal cord are neurochemically more complex than previously thought.

5. Spinal cord injury leads to a decrease in the expression of glutamate in spinal neurons and to its accumulation in astrocytes in the regions adjacent to the site of injury, during the first week following injury in lampreys.
6. Treatment with DL-TBOA, a glutamate transporter inhibitor, prevents glutamate accumulation by astrocytes. This indicates that astrocytes actively uptake glutamate released by neurons following spinal cord injury.

7. In contrast to the observed in mammals, astrocytes of lampreys have a high capacity of glutamate uptake after spinal cord injury, which is maintained during the first days after a complete transection of the cord. This feature could contribute to the impressive regenerative and survival ability of the spinal cord cells of lampreys.
8. A method to standardize studies of quantification of neurotransmitter fibres densities during spinal cord regeneration has been developed. Establishment of standard protocols for this kind of studies provides a meaningful way to compare the results obtained in different studies.
9. In the rostral stump of the cord, glutamate immunoreactive neurons recover similar levels to that of normal animals at 24 weeks post-lesion, except for the dorsolateral and cerebrospinal fluid-contacting cells, whereas in the caudal stump of the cord, only glutamate immunoreactive dorsomedial cells recover their levels.
10. A significant decrease in the number of glutamate immunoreactive processes is observed 2 weeks after a complete transection of the cord both, rostrally and caudally to the site of injury in all spinal regions studied. The number of glutamate immunoreactive processes returns to levels similar to those found in control animals at 10 weeks post-lesion, except for the ventromedial region, which recovers at 4 weeks after the lesion in both stumps of the cord.
11. The recovery of the spinal glutamatergic circuits is due to mechanisms of regeneration and plasticity.

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Resumen y conclusiones

RESUMEN Y CONCLUSIONES

Resumen

La lesión medular (LM) consiste en un daño producido en la médula espinal que da lugar a la interrupción de los tractos de axones encargados de llevar información sensitiva y motora. En vertebrados que no son capaces de regenerar, como los mamíferos, incluyendo a los humanos, la LM provoca, de forma irreversible, la pérdida de sensibilidad y de función por debajo del lugar de la lesión. Los axones dañados no pueden volver a crecer a través del lugar de la lesión ni restablecer los contactos sinápticos.

Desde el punto de vista fisiopatológico, en la LM se pueden distinguir dos etapas. El daño primario hace mención a las alteraciones iniciales del tejido y es debido a la propia lesión. Se caracteriza por la rotura de las meninges y de la barrera hematoencefálica, hemorragia y muerte masiva de neuronas, astrocitos y oligodendrocitos por necrosis. Durante el proceso de daño secundario, las lesiones producidas por los procesos primarios se van extendiendo hasta zonas distales y se liberan grandes cantidades de neurotransmisores, como glutamato. El glutamato es excitotóxico; su liberación masiva causa un excesivo flujo de calcio hacia el interior de las células que da lugar a la muerte por apoptosis de neuronas, astrocitos y oligodendrocitos, produciéndose también la pérdida de la mielina. Por otro lado, los astrocitos se transforman en astrocitos reactivos y construyen la cicatriz glial que, a largo plazo, resulta ser una barrera física y química que impide el crecimiento de los axones. Además, tiene lugar una respuesta inflamatoria que produce la

activación e infiltración de neutrófilos, macrófagos y microglía llevando a una alteración masiva del tejido nervioso.

Entre 250.000 – 500.000 personas sufren una lesión medular cada año en todo el mundo y, aproximadamente, 1.000 personas en nuestro país. Debido a esto, la LM constituye un gran problema social y económico, por lo que la investigación en este campo tiene una gran importancia para la sociedad.

La lamprea de mar, *Petromyzon marinus*, pertenece a los ciclóstomos o agnatos, el grupo más antiguo de vertebrados actuales. Por ello, la lamprea ocupa una posición filogenética clave entre los urocordados (los invertebrados más inmediatos) y los gnatóstomos (vertebrados mandibulados). Así, se trata de una especie única para el estudio de la evolución de las características morfológicas así como de las bases moleculares y genómicas a lo largo de la evolución del filo de los Craneados. En los últimos años, se ha secuenciado y publicado el genoma completo de la lamprea de mar (http://www.ensembl.org) permitiendo la realización de estudios evolutivos desde un punto de vista genómico. La lamprea es también un modelo en el campo de la neuroanatomía comparada y en el estudio de las redes neuronales que participan en la locomoción en los vertebrados. El sistema nervioso central (SNC) de la lamprea sigue el patrón general del de todos los vertebrados aunque tiene una cantidad de neuronas mucho menor. Esto permite estudiar los circuitos neuronales más fácilmente que en otros vertebrados. Además, el cerebro y la médula espinal se pueden mantener aislados vivos *in vitro* durante varios días y es posible conseguir el patrón motor mediante la estimulación eléctrica de las áreas locomotoras diencefálica y mesencefálica. Gracias a estas características, la lamprea es el vertebrado en que con más detalle se han descrito las redes espinales que subyacen a la locomoción, basándose en estudios electrofisiológicos. Por otro lado, las lampreas son

también animales modelo en estudios de regeneración de médula espinal y de otras enfermedades del sistema nervioso. A diferencia de lo que ocurre en mamíferos, las lampreas son capaces de recuperar una locomoción normal después de una sección completa de la médula, observándose una alta especificidad en las rutas de regeneración de los axones y en los nuevos contactos sinápticos. De hecho, la lamprea es el único vertebrado que cumple los cinco criterios propuestos por el NIH para demostrar la existencia de regeneración funcional de la médula espinal:

1. La lesión experimental debe causar la desconexión de las prolongaciones nerviosas.
2. Las prolongaciones de las neuronas del SNC deben superar el nivel de la lesión.
3. Las fibras regeneradas deben establecer contactos sinápticos.
4. Las fibras regeneradas deben generar respuestas post-sinápticas.
5. Los cambios en la función deben derivarse de las conexiones regeneradas.

A pesar de la capacidad de regeneración del SNC de la lamprea, los mecanismos de plasticidad también son importantes para conseguir la restauración de la función, al igual de lo que se observa en vertebrados sin capacidad de regeneración. De hecho, no todos los axones de las lampreas regeneran de la misma manera, lo que permite estudiar los mecanismos moleculares intrínsecos que subyacen a la capacidad regenerativa de las neuronas en el mismo ambiente permisivo tras una LM.

La médula espinal de la lamprea es transparente y aplanada, carece de mielina y los vasos sanguíneos no entran en el parénquima medular. Existen distintos tipos neuronales en la médula espinal: motoneuronas, células dorsales, células del borde y numerosas clases de neuronas intrínsecas. La médula espinal presenta un circuito intrínseco

que, sin influencia sensitiva, es capaz de generar una salida coordinada rítmica que controla la locomoción. Este circuito se conoce como generador de patrones centrales (GPC) y se encuentra en todos los vertebrados, incluidos los humanos. El GPC en la lamprea se encuentra a lo largo de la médula espinal. Está constituido por diversos tipos de neuronas, incluyendo las interneuronas glutamatérgicas excitadoras que participan en la activación rítmica de otras interneuronas, de las motoneuronas y en la generación del propio ritmo y las interneuronas inhibidoras pequeñas que están implicadas en el patrón motor de alternancia derecha-izquierda. El GPC está controlado por los sistemas descendentes que, a su vez, reciben señales procedentes de los sistemas ascendentes que les proporcionan información acerca del estado de las redes locomotoras o de señales sensitivas generadas durante el movimiento. Los sistemas descendentes que proyectan a la médula espinal en la lamprea son el reticuloespinal (RS), que da lugar a los axones gigantes ipsilaterales de la médula y el vestibuloespinal (VS) que proyecta únicamente a la médula espinal rostral. Los sistemas ascendentes en lampreas son la columna dorsal, que transporta información sensitiva desde la piel al tronco cerebral y el tracto espino lemnisco que conduce señales sensitivas e información sobre el patrón motor a diferentes regiones cerebrales.

El glutamato es el principal neurotransmisor excitador del SNC de vertebrados. Los sistemas glutamatérgicos, incluidos el metabolismo, mecanismos de liberación, receptores y transportadores están implicados en prácticamente todos los aspectos de la función normal cerebral así como en el desarrollo, envejecimiento y en la mayoría de las enfermedades neurológicas. Por todo ello, es de especial importancia que el glutamato se encuentre a la concentración adecuada en el lugar y momento adecuados. Uno de los mayores problemas se produce cuando los transportadores no funcionan correctamente. Niveles muy elevados de glutamato extracelular dan lugar a una sobre-estimulación

de los receptores de glutamato que provoca un aumento en los flujos de Na^+ y Ca^{2+} hacia el interior celular, en el consumo de energía, en la producción de radicales libres y, en consecuencia, la muerte neuronal. En numerosas enfermedades neurológicas como isquemia, lesión cerebral y medular traumática, esclerosis lateral amiotrófica, enfermedad de Alzheimer o epilepsia, entre otras, se ha sugerido la participación de un defecto en la captación de glutamato por los transportadores.

A pesar de la importancia del glutamato en el SNC, la distribución de las poblaciones glutamatérgicas en el cerebro y la médula espinal de la mayoría de los vertebrados se desconoce. En la lamprea, hasta estos últimos años, la mayoría de los trabajos que caracterizaban la transmisión excitadora (glutamatérgica) se habían realizado mediante técnicas fisiológicas en determinadas neuronas identificadas. Algunos estudios inmunohistoquímicos de nuestro grupo habían descrito neuronas inmunorreactivas a glutamato (glu-ir) en la retina y en el núcleo de la columna dorsal. Recientemente, nuestro grupo ha caracterizado el transportador vesicular de glutamato (VGLUT) de la lamprea, proporcionando así una nueva herramienta para la descripción de las neuronas glutamatérgicas en este modelo animal. De este modo, últimamente se han publicado estudios detallados de hibridación *in situ* de la expresión de mRNA del VGLUT en el cerebro de la lamprea. Se ha visto que las poblaciones glutamatérgicas se encuentran ampliamente distribuidas a lo largo del cerebro de la lamprea. Sin embargo, la distribución de las poblaciones glutamatérgicas de la médula espinal de la lamprea ha continuado siendo desconocida.

Nuestro objetivo en este proyecto es aumentar el conocimiento del sistema glutamatérgico espinal de la lamprea en condiciones normales, después de una lesión de médula espinal y durante la regeneración medular. Por ello, nos hemos centrado en (1) la caracterización de las poblaciones glutamatérgicas espinales en la médula espinal de la lamprea y su comparación con las poblaciones

GABAérgicas y glicinérgicas, (2) la investigación de la liberación masiva de glutamato y la consiguiente respuesta en los astrocitos tras una LM y (3) el análisis de los cambios producidos en el sistema glutamatérgico espinal durante la regeneración medular. Además, hemos diseñado un protocolo para cuantificar las prolongaciones neuronales y compararlas en diferentes condiciones experimentales.

CAPÍTULO 1: Las neuronas glutamatérgicas en la médula espinal de la lamprea de mar: un estudio de hibridación *in situ* e inmunohistoquímica.

El glutamato es el principal neurotransmisor excitador implicado en los circuitos medulares de vertebrados. A pesar de esto, la distribución de neuronas glutamatérgicas en la médula espinal se desconoce en la mayoría de los vertebrados. La lamprea, que pertenece al grupo más antiguo de los vertebrados actuales, se ha utilizado ampliamente como modelo para la investigación de los circuitos neuronales que subyacen a la locomoción. Hasta el momento, los circuitos glutamatérgicos se han caracterizado en base a las respuestas excitadoras obtenidas en neuronas post-sinápticas. Sin embargo, la presencia de marcadores neuroquímicos no se ha investigado. En este trabajo, se describe por primera vez la expresión del mRNA del VGLUT de la lamprea en la médula espinal de la lamprea. Además, también se estudia la distribución de la inmunorreactividad a glutamato en los somas y las fibras. Las neuronas más grandes que se han encontrado en la sustancia gris son las células dorsales y las interneuronas gigantes caudales. Otras dos poblaciones positivas para VGLUT se han observado en la sustancia gris: población dorsomedial, formada por células pequeñas y población lateral, formada por células grandes y pequeñas. Alrededor del canal central, algunas células que contactan con el líquido cerebroespinal expresan también VGLUT. En la sustancia blanca, algunas células del borde y células asociadas a los axones gigantes (axones de Müller y de Mauthner) o a los

axones del fascículo dorsolateral son positivas para VGLUT. Las células laterales grandes y las que están asociadas a los axones reticuloespinales se encuentran en una posición clave para recibir información acerca del control de la locomoción. En este estudio se compara también la distribución de la inmunorreactividad a glutamato con las de los neurotransmisores ácido γ-aminobutírico (GABA) y glicina. Colocalización de glutamato con GABA o glicina se ha observado en varios tipos de células espinales pequeñas. Estos resultados confirman la naturaleza glutamatérgica de varias poblaciones neuronales y revelan la existencia de dos nuevas poblaciones de células pequeñas. El estudio de colocalización pone de manifiesto que algunas neuronas glutamatérgicas podrían ejercer funciones más complejas de lo esperado en las neuronas post-sinápticas.

CAPÍTULO 2: Liberación neuronal y captación de glutamato por los astrocitos tras una sección medular completa en lampreas.

A diferencia de lo que ocurre en mamíferos, la médula espinal de la lamprea se recupera espontáneamente después de una LM completa. Entender las diferencias entre lampreas y mamíferos en la respuesta a una LM, puede proporcionar una valiosa información para proponer nuevas terapias. Es posible que características únicas de los astrocitos de la lamprea contribuyan al éxito de la regeneración de la médula espinal. Después de una LM en mamíferos, se produce una liberación masiva de glutamato, alcanzando niveles extracelulares excitotóxicos, que llevan a la muerte de neuronas y oligodendrocitos. Se ha mostrado que el aumento en los niveles de glutamato es debido a una disfunción en la captación del mismo por los astrocitos. El principal objetivo de este capítulo es investigar la liberación de glutamato y la consiguiente respuesta de los astrocitos durante la primera semana después de una sección completa de la médula espinal en la lamprea, animal en el que

se ha descrito una ausencia de muerte celular en los primeros días tras una LM. A través de la detección de glutamato, Hu (marcador neuronal) y citoqueratinas (marcador glial de lamprea) hemos podido mostrar, por primera vez en un vertebrado, la liberación de glutamato desde las neuronas y la respuesta de los astrocitos en los primeros días tras una LM. Nuestros resultados evidencian que la LM provoca una pérdida inmediata de la inmunorreactividad a glutamato en las neuronas cercanas al lugar de la lesión. Esto está de acuerdo con el aumento de los niveles extracelulares de glutamato observados en mamíferos después de una LM. Sorprendentemente, los astrocitos de la lamprea presentan inmunorreactivididad a glutamato después de la LM, que no se observa si se ha hecho un tratamiento previo con un inhibidor de los transportadores de glutamato, ácido DL-treo-β-Benciloxiaspártico (DL-TBOA). Esto indica que, al contrario de lo observado en mamíferos, los transportadores de glutamato de los astrocitos de la lamprea continúan funcionando correctamente después de una LM. Así, los astrocitos captan activamente el glutamato liberado protegiendo a las neuronas de la muerte. Cómo los transportadores de glutamato de las lampreas mantienen la capacidad de captar activamente el glutamato después de una LM se desconoce. Profundizar en la investigación en este campo podría aportar nuevas dianas terapéuticas para intentar evitar el daño secundario que se produce tras una lesión en el SNC.

CAPÍTULO 3: Microscopía confocal utilizada para la cuantificación semiautomática de los cambios en las fibras aminoacidérgicas durante la regeneración de la médula espinal

La lesión de médula espinal provoca déficits funcionales permanentes, así como una pérdida irreversible de la función en mamíferos. Hasta el momento no existe un tratamiento adecuado, por lo que se trata de un gran problema económico y de salud pública.

Después de una LM, se han observado cambios en los diferentes sistemas espinales de neurotransmisores aminoacidérgicos. En este trabajo, se describe un método muy útil para cuantificar los cambios en el número de fibras de los sistemas espinales de neurotrasmisores durante la regeneración de la médula espinal en la lamprea. Este protocolo combina la microscopía confocal con el uso de un software apropiado. La microscopía confocal permite obtener imágenes de alta resolución en las que se puede distinguir entre dos puntos inmunofluorescentes muy cercanos. De este modo, se combinan imágenes de alta resolución con la utilización de un software libre (Fiji con el plug-in Feature J). Este programa permite hacer una cuantificación semiautomática del número de puntos positivos en las microfotografías. De este modo, en este capítulo se muestra un protocolo para detectar neurotransmisores aminoacidérgicos en el SNC de la lamprea (utilizando glutaraldehido como fijador del tejido) y para cuantificar las fibras neuronales durante la regeneración medular. Establecer protocolos estándar para este tipo de estudios provee de una herramienta que permite comparar los resultados cuando se trata de analizar cambios en diferentes sistemas de neurotransmisores y a diferentes tiempos tras la lesión. Además, este método se puede usar también con imágenes de microscopía de campo claro y se puede adaptar a otras condiciones experimentales y otros modelos animales.

CAPÍTULO 4: Plasticidad en el sistema glutamatérgico espinal para restablecer la función después de una lesión de médula espinal en lampreas

A diferencia de mamíferos, se sabe que la mayoría de los vertebrados anamniotas son capaces de regenerar la médula espinal y de recuperar el movimiento. Entre ellos, la lamprea es el único vertebrado que cumple los cinco criterios establecidos por el NIH para

considerar que la regeneración funcional es completa. La locomoción en los vertebrados es generada por las redes espinales del generador de patrones centrales. La neurotransmisión excitadora mediada por glutamato es utilizada por los centros de mando del cerebro, las neuronas reticuloespinales y algunas neuronas de la médula espinal para excitar a las motoneuronas y a otras interneuronas. Los cambios que se producen a corto plazo en los niveles de glutamato tras una LM están bien documentados. Sin embargo, se sabe mucho menos de los cambios que ocurren a largo plazo en las poblaciones medulares. El propósito de este capítulo es cuantificar los cambios que se producen en las neuronas y fibras glutamatérgicas durante la regeneración de la médula espinal en larvas maduras de lamprea de mar. Para detectar las células y fibras glu-ir se ha utilizado inmunofluorescencia a glutamato, mientras que para cuantificar las fibras glu-ir a diferentes tiempos después de la lesión (2, 4, 10 y 24 semanas post lesión; spl) se ha utilizado el protocolo descrito en el capítulo 3. La cuantificación de las células glu-ir se ha hecho manualmente en animales control y a 24 spl. Se observa una disminución significativa del número de fibras a las 2 spl que se va recuperando hasta alcanzar niveles normales posteriormente. Sin embargo, en la mayoría de las poblaciones celulares se observa una disminución significativa a las 24 spl. Estos resultados indican que mecanismos de plasticidad, y no únicamente procesos regenerativos, están presentes y además parecen jugar un papel muy importante en la recuperación de los circuitos espinales glutamatérgicos después de una LM en lampreas. Esta característica hace que las lampreas sean un modelo fiable para el estudio de la capacidad de regeneración del SNC de los vertebrados, ya que los mecanismos de plasticidad parecen ser la base de la limitada capacidad de regeneración que se observa en los vertebrados con regeneración restringida, como los mamíferos.

En este proyecto de tesis se han utilizado larvas en un estadío de desarrollo estable, adultos jóvenes y adultos de lamprea de mar, *Petromyzon marinus*.

Para demostrar la presencia de mRNA del VGLUT de lamprea se realizó la técnica de hibridación *in situ*. Se sintetizaron ribosondas marcadas con digoxigenina (DIG), utilizando como cebadores fragmentos amplificados de la secuencia clonada, siguiendo protocolos estándar. Las hibridaciones in situ se realizaron siguiendo protocolos estándar, añadiendo un tratamiento con RNAsa A a los lavados de post-hibridación. El teñido se realizó con BM Purple a 37ºC hasta que la señal se hizo claramente visible.

Para inhibir los transportadores de glutamato, larvas previamente anestesiadas se colocan con la región dorsal hacia arriba en una placa con una base de Sylgard y llena de solución Ringer. Se quitan cuidadosamente la piel, los músculos y las meninges de la región comprendida entre la tercera y la séptima branquia dejando expuesta la médula espinal. Los animales se mantienen en 30 µM DL-TBOA diluido en dimetil sulfóxido (DMSO) en Ringer durante 4h.

Se utilizaron protocolos de inmunofluorescencia para detectar inmunorreactividades a glutamato, GABA, glicina, Hu y citoqueratinas en condiciones normales o tras una lesión de médula espinal. Tras una anestesia profunda, las larvas se colocan con la región dorsal hacia arriba en una placa con una base de Sylgard y llena de Ringer. La médula espinal se expone a través de una incisión desde la línea media dorsal a nivel de la quinta branquia y se secciona completamente con un bisturí. A continuación, los extremos cortados de la médula se visualizan bajo una lupa. Después de la cirugía, las lampreas se mantienen en hielo durante 1 h para permitir que la herida se seque al aire. Tras este periodo, se deja que los animales se recuperen en tanques aireados con agua dulce a 19,5ºC. Los animales con la médula

seccionada se examinaron 24 h después de la cirugía para confirmar que no se producía ningún movimiento caudal al lugar de la lesión. Para la detección de los distintos marcadores, las secciones se incubaron en mezclas de anticuerpo policlonal de conejo anti-glutamato con anticuerpo monoclonal de ratón anti-GABA o anti-citoqueratinas o anti-HuC/HuD o con una mezcla de anticuerpo policlonal de conejo anti-glicina y anticuerpo monoclonal de ratón anti-glutamato. Para la detección de los anticuerpos por inmunofluorescencia indirecta se utilizaron inmunoglobulina G de cabra anti-conejo conjugada con Cy3 e inmunoglobulina G de cabra anti-ratón conjugada con fluoresceína.

La cuantificación de las fibras se realizó utilizando el plug-in Feature J para el software Fiji. El análisis estadístico se realizó con el programa GraphPad Prism y los tests estadísticos usados para analizar los datos fueron: test de normalidad Kolmogorov-Smirnov, t Student, ANOVA y test de comparaciones múltiples de Bonferroni.

Todos los experimentos realizados en este proyecto han sido aprobados por el Comité de Bioética de la Universidad de Santiago de Compostela y cumplen la normativa de la Unión Europea y de España sobre cuidado y experimentación animal para minimizar el dolor y el sufrimiento.

Algunos de los capítulos de esta Tesis Doctoral han sido publicados en formato de artículo científico o de capítulo de libro, con las siguientes referencias:

- Capítulo 1: PLoS ONE. 2012. 7:e47898. doi: 10.1371/journal.pone.0047898.

- Capítulo 2: este capítulo pertenece a un artículo científico en el que se aporta información acerca de otros neurotransmisores que no son objeto de estudio de esta tesis. Glia. 2014. 62:1254–1269.

- Capítulo 3: ha sido publicado como capítulo de un libro en: *Confocal and multiphoton laser-scanning microscopy of neuronal tissue: Applications and quantitative image analysis*. 2014. Eds: Bakota L and Brandt R. Springer, New York pp. 239-250

- Capítulo 4: este capítulo se va a enviar a una revista científica para que sea publicado.

Conclusiones

El análisis de los resultados obtenidos en esta Tesis permite extraer las siguientes conclusiones:

1. La caracterización de las poblaciones celulares glutamatérgicas de la médula espinal de la lamprea de mar ha aumentado el número de tipos celulares glutamatérgicos en dos: células que contactan con el líquido cerebroespinal e interneuronas de la sustancia blanca.

2. Se ha encontrado además expresión de VGLUT en las células dorsales, interneuronas gigantes, células del borde y en dos poblaciones neuronales de la sustancia gris: dorsal y lateral, lo que concuerda con estudios electrofisiológicos previos.

3. El patrón de expresión de VGLUT e inmunorreactividad a glutamato en la médula espinal es similar en larvas y adultos, lo que indica que no se producen grandes cambios en este sistema durante la metamorfosis.

4. Se ha observado colocalización de glutamato y GABA en células de las poblaciones dorsal y lateral de la sustancia gris y en células que contactan con el líquido cerebroespinal, mientras que colocalización entre glutamato y glicina se ha observado además en las células del borde ventrales. Esto sugiere que las células glutamatérgicas de la médula espinal de la lamprea son neuroquímicamente más complejas de lo que se creía.

5. La lesión medular da lugar a una disminución en la expresión de glutamato en las neuronas espinales y a su acumulación en los astrocitos en las regiones adyacentes al lugar de la lesión durante la primera semana después de la lesión en lampreas.

6. El tratamiento con DL-TBOA, inhibidor del transportador de glutamato, impide la acumulación de glutamato por los astrocitos. Esto indica que los astrocitos captan de forma activa el glutamato liberado por las neuronas después de una lesión medular.

7. Al contrario de lo que ocurre en mamíferos, los astrocitos de las lampreas presentan una alta capacidad de captación de glutamato después de una lesión medular que se mantiene durante los primeros días después de una sección medular completa. Esta característica podría contribuir a la sorprendente capacidad regenerativa y a la supervivencia de las células de la médula espinal de las lampreas.

8. Se ha desarrollado un método para estandarizar los estudios de cuantificación de la densidad de fibras de los diferentes neurotransmisores durante la regeneración de la médula espinal. Establecer protocolos estándar para este tipo de trabajos proporciona herramientas útiles para comparar los resultados obtenidos en diferentes estudios.

9. Las neuronas inmunorreactivas a glutamato de la región rostral a la lesión recuperan niveles similares a los observados en animales normales 24 semanas después de la lesión, excepto las células dorsolaterales y las que contactan con el líquido cerebroespinal, mientras que en la región caudal únicamente las células dorsomediales recuperan sus niveles.

10. Dos semanas después de la sección medular, se observa una disminución significativa del número de fibras inmunorreactivas a glutamato rostral y caudalmente al lugar de la lesión en todas las regiones estudiadas. El número de fibras inmunorreactivas a glutamato vuelve a niveles similares a los de los controles 10

semanas después de la lesión, excepto en la región ventromedial que se recupera 4 semanas tras la lesión.

11. La recuperación en los circuitos intraespinales se debe a procesos de regeneración y plasticidad.