RESEARCH ARTICLE

Neuronal Release and Successful Astrocyte Uptake of Aminoacidergic Neurotransmitters After Spinal Cord Injury in Lampreys

Blanca Fernández-López, Silvia María Valle-Maroto, Antón Barreiro-Iglesias, and María Celina Rodicio

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. The main aim of our study was to investigate, in the sea lamprey, the release of aminoacidergic neurotransmitters and the subsequent astrocyte uptake of these neurotransmitters during the first week following a complete SCI by detecting glutamate, GABA, glycine, Hu and cytokeratin immunoreactivities. This is the first time that aminoacidergic neurotransmitter release from neurons and the subsequent astrocytic response after SCI are analysed by immunocytochemistry in any vertebrate. Spinal injury caused the immediate loss of glutamate, GABA and glycine immunoreactivities in neurons close to the lesion site (except for the cerebrospinal fluid-contacting GABA cells). Only after SCI, astrocytes showed glutamate, GABA and glycine immunoreactivity. Treatment with an inhibitor of glutamate transporters (DL-TBOA) showed that neuronal glutamate was actively transported into astrocytes after SCI. Moreover, after SCI, a massive accumulation of inhibitory neurotransmitters around some reticulospinal axons was observed. Presence of GABA accumulation significantly correlated with a higher survival ability of these neurons. Our data show that, in contrast to mammals, astrocytes of lampreys have a high capacity to actively uptake glutamate after SCI. GABA may play a protective role that could explain the higher regenerative and survival ability of specific descending neurons of lampreys.

GLIA 2014;62:1254–1269

Key words: glia, glutamate, glycine, GABA, spinal cord injury, cytokeratin, regeneration

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 (Cohen et al., 1986; Wood and Cohen, 1979; Yin and Selzer, 1983), fishes (Bernstein and Gelderd, 1970; Bunt and Fill-Moebs, 1984; Becker et al., 1997; Coggeshall et al., 1982) and amphibians (Davis et al., 1990; Lee, 1982) 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

View this article online at wileyonlinelibrary.com. DOI: 10.1002/glia.22678

Published online April 15, 2014 in Wiley Online Library (wileyonlinelibrary.com). Accepted for publication Apr 3, 2014.

Address correspondence to María Celina Rodicio, Departamento de Biología Celular y Ecología, Edificio CIBUS, Campus Vida, Universidad de Santiago de Compostela, CP. 15782, Santiago de Compostela, A Coruña, Spain. E-mail: mcelina.rodicio@usc.es

From the Department of Cell Biology and Ecology, CIBUS, University of Santiago de Compostela, 15782, Santiago de Compostela, Spain.

Blanca Fernández-López and Silvia María Valle-Maroto contributed equally to this work.

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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 aminoacidergic neurotransmitters and a subsequent astrocyte reaction. Reactive astrocytosis after CNS injury appears to be a conserved phenomenon throughout vertebrate evolution (Larner et al., 1995), which has 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, γ-aminobutyric acid (GABA), and glycine (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 amino acid neurotransmitters 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). An increase in glycine and GABA extracellular levels after SCI has been also reported (Demediuk et al., 1989; Panter et al., 1990). As previously suggested (Panter et al., 1990), extracellular glycine could contribute to glutamate excitotoxicity, since it is a coagonist of the *N*-methyl-D-aspartate (NMDA) glutamate receptor (Ransom and Stec, 1988). In contrast, GABA has been shown to have neuroprotective effects (Han et al., 2008).

The process of aminoacidergic neurotransmitter 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 aminoacidergic neurotransmitters in response to SCI and the subsequent astrocytic response in the sea lamprey. Specifically, we 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, glycine and GABA immunoreactivities that take place in the first week after SCI; (3) the colocalization of glutamate, glycine and GABA with cytokeratins to investigate whether any of these neurotransmitters 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 and that, after SCI, GABA could play a role favouring the survival of specific descending neurons.

Materials 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, northwestern Spain) with permission from the Xunta de Galicia and maintained in aerated fresh water aquaria at 15°C 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 h 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°C. Lampreys are known to be able to recover normal appearing locomotion at this

temperature (Cohen et al., 1999). Each transected animal was examined 24 h after surgery to confirm that there was no movement caudal to 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^{TM} (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 mouse monoclonal anti-GABA antibody (Sigma; 1: 1,200) or a mouse monoclonal anti-glutamate antibody (Swant, Bellinzona, Switzerland; 1:1,000) and a rabbit polyclonal anti-glycine antibody (Immunosolution, Jesmond, Australia; 1:3,000) or with a cocktail of a rabbit polyclonal anti-GABA antibody (Affiniti, Mamhead, UK; 1:500) or a rabbit polyclonal anti-glutamate antibody (Immunosolution; 1:4.500) or a rabbit polyclonal anti-glycine antibody 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 4°C. After rinsing in TBS with 1% MB, sections were incubated for 1 h 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 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 Csillaq, 2006) and it has been used in a previous study of lamprey spinal cord (Fernández-López et al., 2012).

The polyclonal anti-glutamate antibody was raised in rabbit against a glutamate- glutaraldehyde-porcine thyroglobin 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 (Fernández-López et al., 2012; Villar-Cerviño et al., 2011, 2013). This antibody did not stain any sea lamprey brain native protein band (Villar-Cerviño et al., 2011).

The polyclonal anti-glycine antibody was raised against a glycine-porcine thyroglobin conjugate and 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 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 reactivity. This antibody has been developed by Dr. David V. Pow (University of Newcastle, Australia) and used in a number of studies on glycinergic neurons of the retina, brain, and spinal cord of the sea lamprey (Villar-Cerviño et al., 2008a, b, 2011).

The monoclonal anti-GABA antibody was raised against GABA conjugated to BSA with glutaraldehyde and was evaluated by the supplier for activity and specificity by use of dot blot immunoassay. No cross-reaction was observed with BSA, L-α-aminobutiric acid, L-glutamic acid, L- aspartatic acid, glycine, δ-aminovaleric acid, L-theorine, L-glutamine, taurine, putrescine, L-alanine or carnosine. The antibody showed weak cross-reaction with β-alanine. This antibody has been used in previous studies of the sea lamprey nervous system (Barreiro-Iglesias et al., 2009a, c; Valle-Maroto et al., 2011; Villar-Cerviño et al., 2008a,b).

The specificity of the polyclonal anti-GABA antiserum has been characterized by ELISA by the supplier against conjugates with BSA-glutaraldehyde. This antibody has been used in previous studies of lampreys (Meléndez-Ferro et al., 2002; Villar-Cerviño et al., 2006).

Moreover, the anti-glycine, the polyclonal anti-GABA 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, 2008b).

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 h (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 and Measurements

After immunofluorescence, the sections were photographed and analyzed 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.

The presence of inhibitory neurotransmitter accumulations around descending axons (*halos*) was observed in larvae processed between 1 and 3 dpl (n = 20). The percentage of axons with glycine or GABA *halos* was calculated for the dorsal fascicle, lateral fascicle, longitudinal medial fascicle and the Mauthner axons as follows: 10 alternative 14 µm sections of the first 300 µm rostral from the site of injury were analyzed per animal. The mean number of *halos* for each fascicle or Mauther axons was then calculated for each animal. The percentage of *halos* per fascicle or Mauthner axons was calculated based on the total number of axons of the fascicles or Mauthner axons (14 axons in the dorsal fascicle, 12 axons in the lateral fascicle, 18 axons in the longitudinal medial fascicle and 2 Mauthner axons). The final percentages were calculated as mean ± SEM from the data from the 20 animals. The correlation (Pearson test) between the presence of inhibitory neurotransmitter *halos* and the survival ability of the descending neurons of each fascicle [see Shifman et al., 2008: Dorsal fascicle (95.7 %), lateral fascicle (94.5 %), longitudinal medial fascicle (56.7%) and Mauthner axons (29.0%)] was then calculated. The statistical analysis was carried out with GraphPad (La Jolla, CA).

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 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, GABA, glycine and cytokeratins in cells of the spinal cord of lampreys has been already described [glutamate (Fernández-López et al., 2012; Mahmood et al., 2009; Shupliakov et al., 1992), GABA (Batueva et al., 1990; Brodin et al., 1990; Meléndez-Ferro et al., 2003; Ruiz et al., 2004; Robertson et al., 2007), glycine (Gustafsson et al., 2002; Shupliakov et al., 1996; Villar-Cerviño et al., 2008b), cytokeratins (Lurie et al., 1994, Merrick et al., 1995)] and can be observed in Fig. 1. Briefly, in control larval sea lampreys glutamate (Fig.1A, E–G), GABA (Fig.1B,H,I), and glycine (Fig.1C,J-L) immunoreactivities were observed in cells of the dorsal (Fig. 1K,I) and lateral (Fig. 1E,F,I,J, grey populations and in cerebrospinal fluid contacting (CSFc) cells (Fig. 1G,H,L). As previously reported, colocalization of glutamate and GABA or glycine (Fernández-López et al., 2012), and glycine and GABA (Villar-Cerviño et al., 2008b) was observed in cells of some of these populations. Dorsal primary sensory cells (not shown) and individual cells located in the white matter (Fig. 1F), including edge cells, were glutamate immunoreactive (-ir). Ventral edge cells were also glycine-ir. The anti-cytokeratin antibody labeled the cell bodies of astrocytes located in the grey matter (Fig. 1N), the lateral funiculus (Fig. 1M), the perimeter of the giant axons and also the astrocytic processes that course radially (Fig. 1D,M). No cells around the central canal showed cytokeratin expression (Fig.1D). Figure 2 shows colocalization of all the aminoacidergic neurotransmitters with Hu (neuronal marker) (Fig. 2A-C″) and the absence of colocalization of these neurotransmitters with cytokeratins (Fig. 2D-F″) in control animals. This indicates that these neurotransmitters are probably present in astrocytes at very low levels, which precludes the observation of immunolabeling in control animals.

Spinal cord injury led to a clear decrease in the expression of glutamate, glycine, and GABA in neurons and to their accumulation in astrocytes in the regions adjacent to the lesion site. Glutamate, glycine, and GABA 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, 5, and 6.

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 fibers 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-A″). 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)].

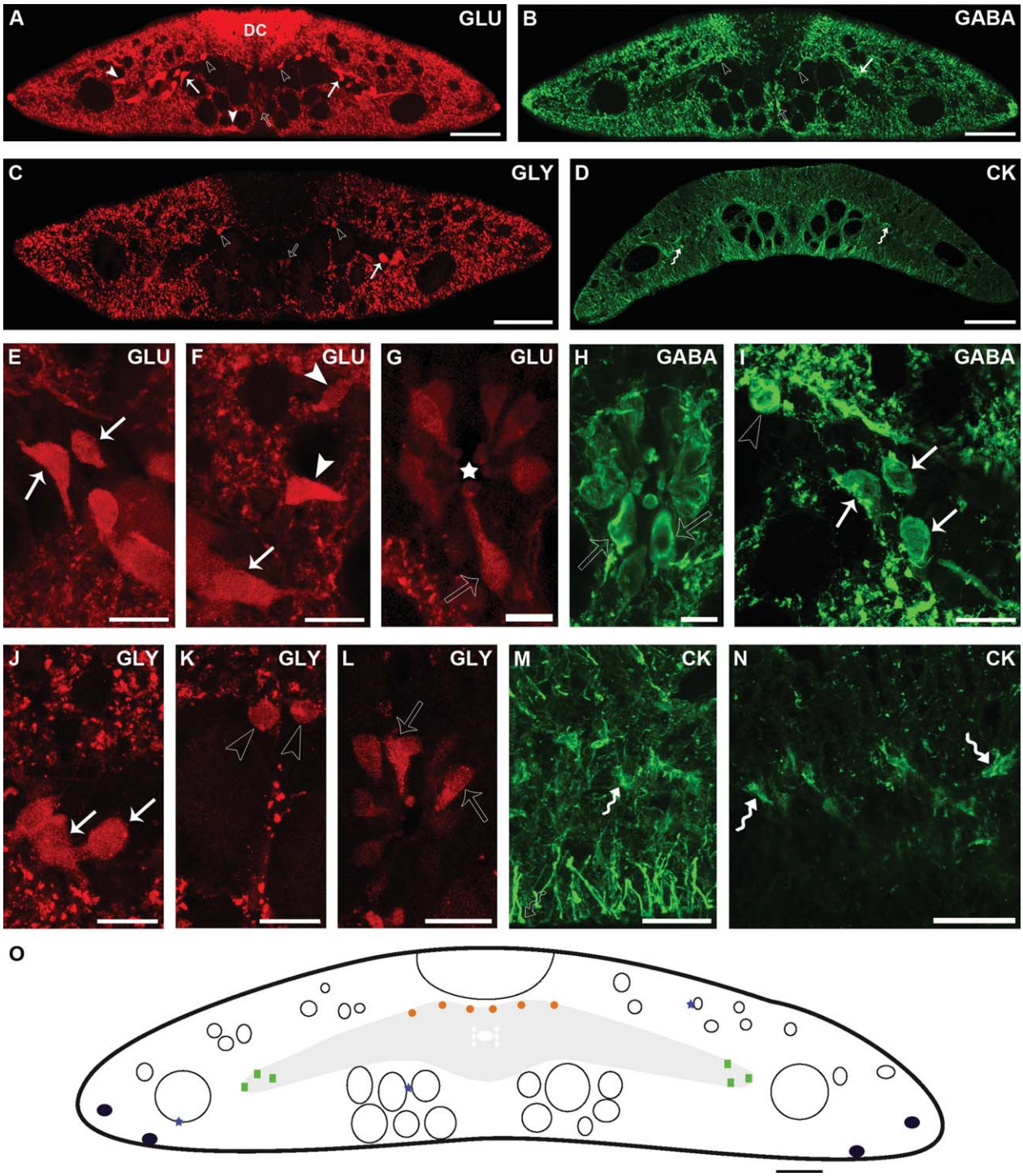

FIGURE 1: Confocal photomicrographs of transverse sections of the spinal cord of the sea lamprey showing glutamate, GABA, glycine and cytokeratin (CK) immunoreactivities in control animals. **A:** Glutamate immunoreactivity. Outlined arrowheads point to neurons of the dorsal population. Arrows point 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:** GABA immunoreactivity. **C:** Glycine immunoreactivity. **D:** CK immunoreactivity. Curved arrows point to astrocytes. Note the absence of CK immunoreactivity in cells around the central canal. **E-L:** Details of glutamate-ir (E: Lateral population cells. **F:** Lateral cell and cells located in the white matter. **G:** CSF-c cells. Star indicates the central canal.), GABA-ir (**H:** CSFc cells. **I:** Lateral and dorsal population cells.) and glycine-ir (**J:** Lateral population cells. **K:** Dorsal grey population cells. **L:** CSFc cells.) neurons **M, N:** Details of CK immunoreactivity. M: Astrocyte in the lateral funiculus. Note the astrocytic radial processes. Outlined curved arrow points to an astrocyte endfoot. N: Astrocytes located in the grey matter. **O:** 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 except for J, K in which lateral is on the right. Scale bars = 75 μm (A-D, O); 15 μm (E, F, I-N); 10 μm (G); 7.5 μm (H).

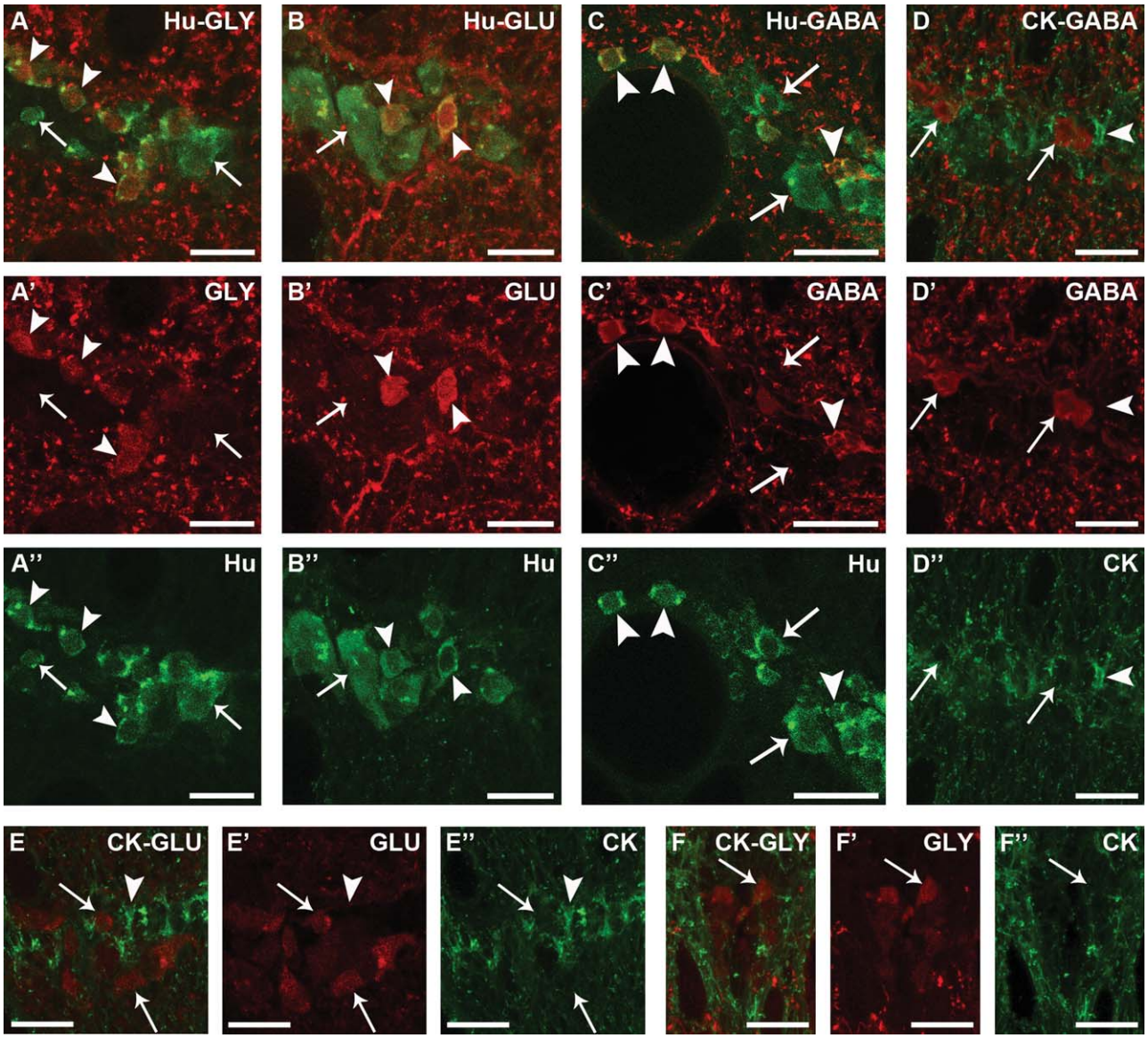

FIGURE 2: High magnification photomicrographs showing details of double labeled neurons (arrowheads) for glutamate, glycine or GABA and Hu and absence of colocalization between glutamate, glycine or GABA and CK in the spinal cord of control lampreys. A-A″: Glycine-ir neurons. Arrows point to Hu single labeled neurons. B-B″: Glutamate-ir neurons. C-C″: GABA-ir neurons. D-D″: GABA-ir single labeled neurons (arrows) and single labeled astrocytes immunoreactive to cytokeratins (arrowheads). E-E″: Glutamate-ir single labeled neurons (arrows) and singled labeled astrocytes immunoreactive to cytokeratins. F-F″: Glycine-ir single labeled neurons (arrow). In all figures dorsal is at the top. Lateral is on the right, except for E-E″ in which lateral is on the left. A–F: Overlay; A′, F′: Glycine; B′, E′: Glutamate; C′, D′: GABA; A″, B″, C″: Hu; D″, E″, F″: CK. Scale bars = 25 μm (C-C″); 20 μm (A-B″, D-F″).

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 from the lesion site. Glutamate-ir primary sensory dorsal cells were observed from

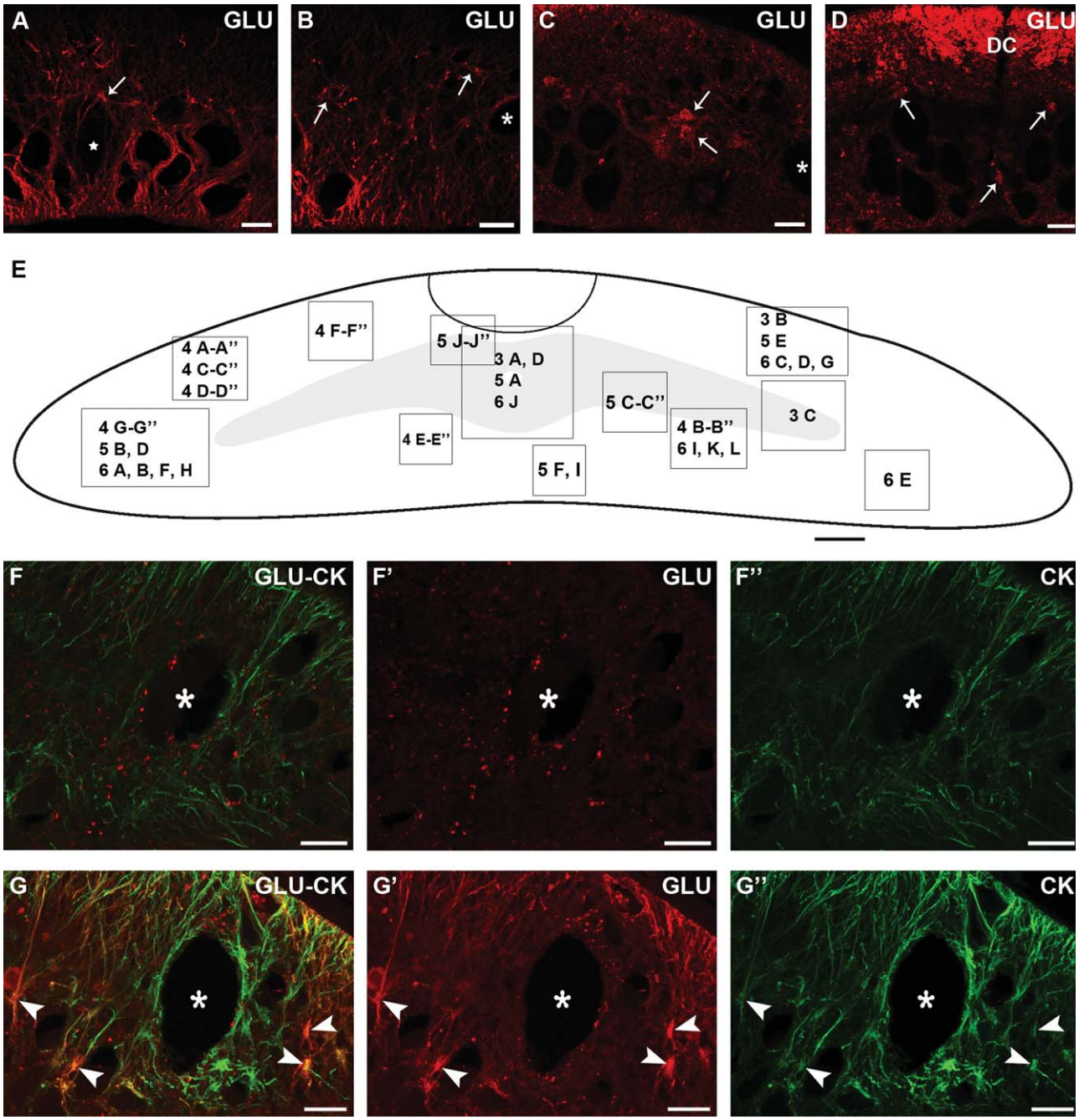

FIGURE 3: Confocal photomicrographs of transverse sections of spinal cord showing details of glutamate immunoreactivity at 0 dpl in transected animals (A-D), in DL-TBOA treated samples (F-F″) and in not treated samples (G-G″). A, B: Neurons and fibers glutamate-negative in the adjacent region to the lesion site, rostral (A) and caudal (B). Note the presence of glutamate-ir astrocytes (arrows). C, D: Glutamate-ir neurons (arrows) and fibers 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 Figs. 3, 4, 5, and 6. 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″).

600 to 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.

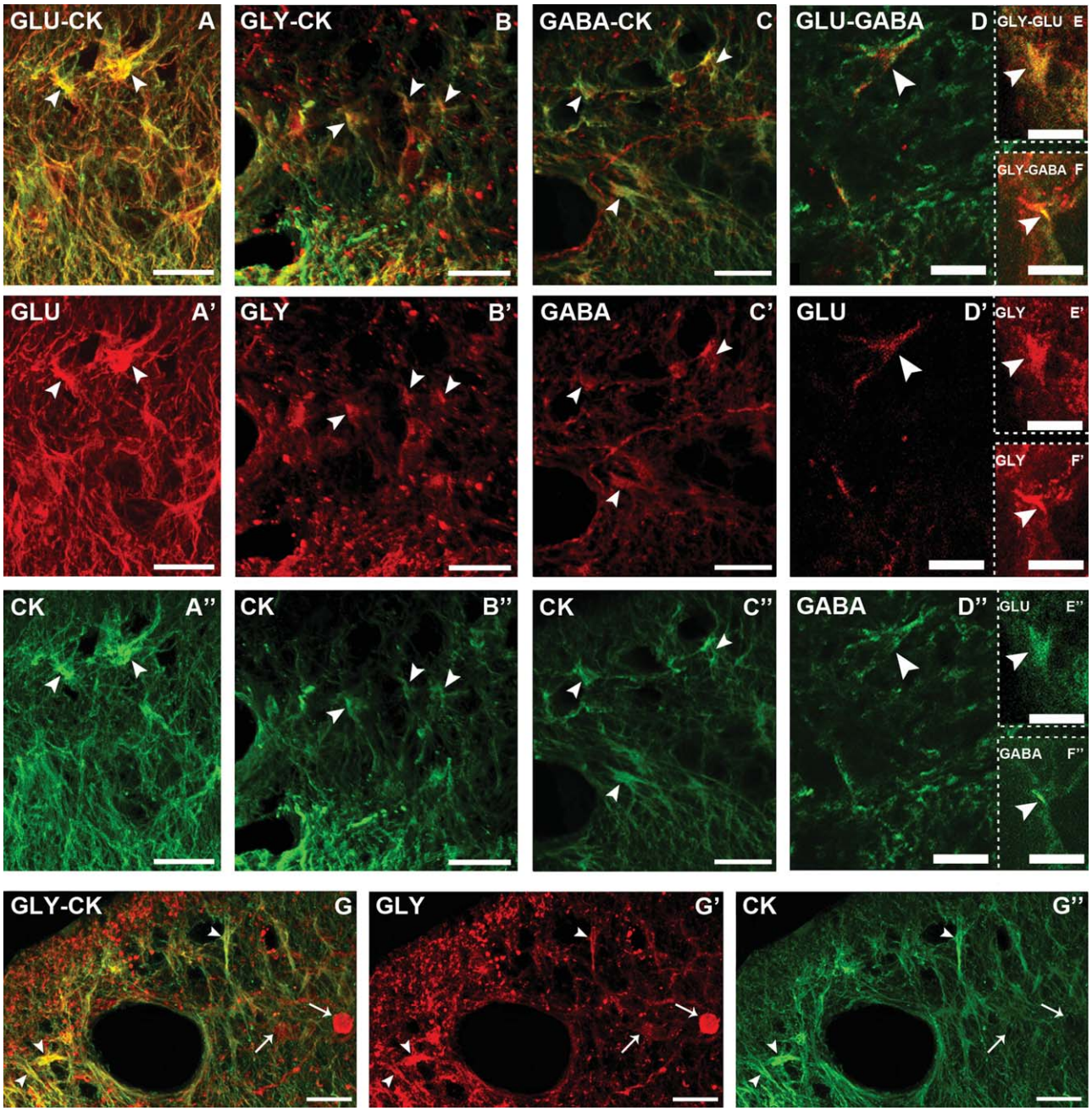

FIGURE 4: High magnification photomicrographs of transverse sections of spinal cord showing details of double immunolabeled astrocytes (arrowheads) for glutamate, glycine or GABA and CK in larvae processed immediately after the injury. A-A″: Glutamate-ir astrocytes. B-B″: Glycine-ir astrocytes. C-C″: GABA-ir astrocytes. D-D″: Double labeled astrocyte for glutamate and GABA. E-E″: Double labeled astrocyte for glutamate and glycine. F-F″: Double labeled astrocyte for glycine and GABA. Note that GABA is only express in part of the astrocyte. G-G″: Glycine-ir astrocytes and neurons (arrows). In all figures dorsal is at the top. Lateral is on the left except for B-B″, in which lateral is on the right. A, B, C, D, E, F, G: Overlay; A′, D′, E″: Glutamate; B′, E′, F′, G′: Glycine; C′, D″, F″: GABA; A″, B″, C″, G″: CK. Scale bars = 20 μm (A-C″, G-G″); 10 μm (D-F″).

At 0 dpl, glycine was also released from neurons and neuronal processes, whereas astrocytes showed strong glycine immunoreactivity rostrally and caudally in the first 300 μm from the injury site (Fig. 4B-B″). After the first 300 μm from the lesion site, glycine immunoreactivity was maintained in neurons and neuronal processes. Between 300 and 350 μm from the site of injury, glycine immunoreactivity was observed both in neurons and astrocytes in the same spinal sections (Fig. 4G-G″). Glycine-ir grey (dorsomedial, lateral and CSFc) and white matter (ventral edge cells) cell populations were clearly observed from 350 μm to the site of injury. Caudally from 350 μm to the site of injury onwards, glycine-ir ventral edge cells not

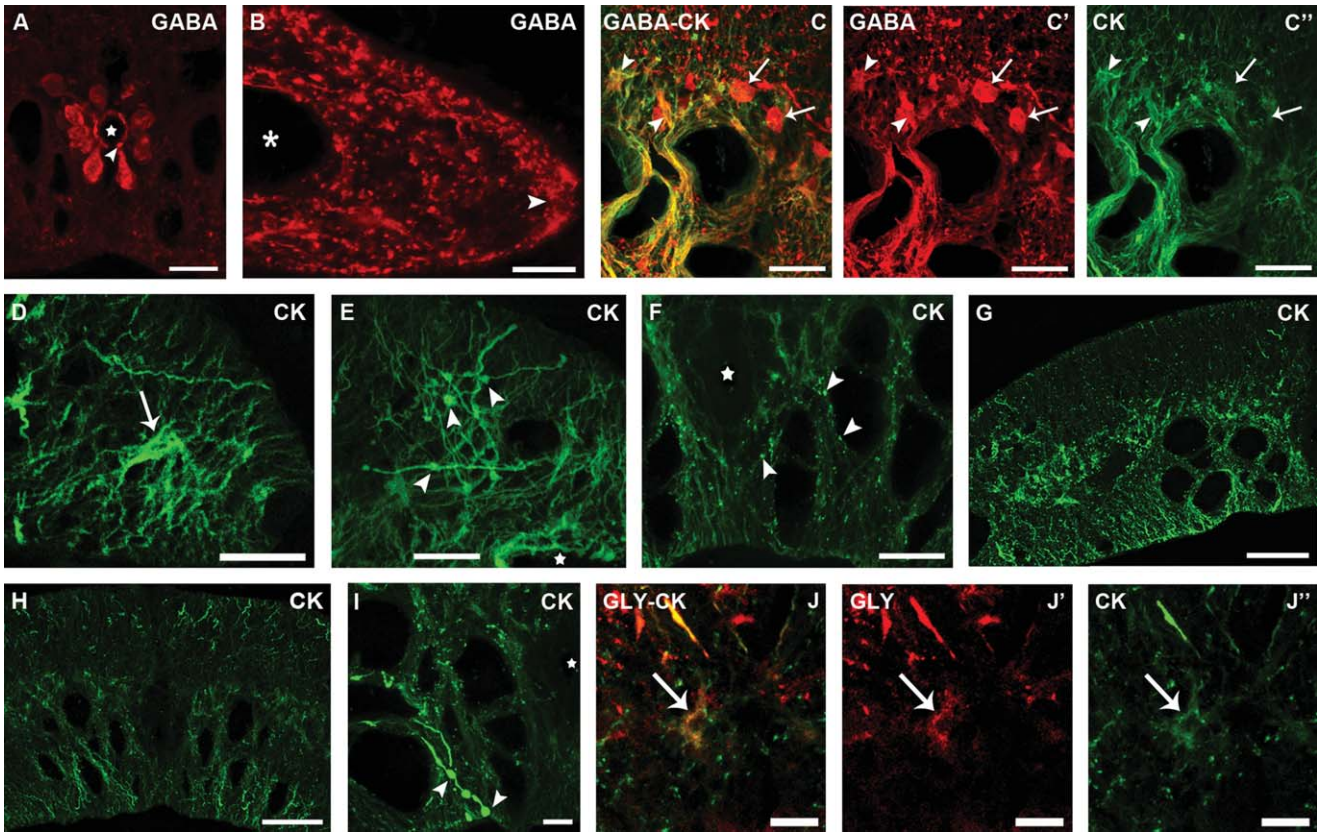

FIGURE 5: Confocal photomicrographs of transverse sections showing details of GABA and CK immunoreactivities and double immunolabeled astrocytes (arrowheads) for glycine or GABA and CK. A: GABA-ir CSFc cells. Arrowhead points to a flattened apical process. The star indicates the central canal. B: GABA immunoreactivity in the marginal neuropil (arrowhead). The asterisk indicates the Mauthner axon. C-C″: GABA-ir astrocytes and neurons (arrows). D: CK-ir reactive astrocyte (arrow). E: CK-ir astrocyte processes with beaded appearance (arrowheads). The star indicates the Mauthner axon. F: CK immunoreactivity with a dotted (arrowheads) appearance. Star indicates the central canal. G, H: CK immunoreactivity beyond the adjacent region to the injury site, rostral (J) and caudal (K). I: CK-ir processes with beaded appearance (arrowheads). The star indicates the central canal. J-J″: Glycine-ir astrocytes 1dpl. All photomicrographs are from larvae processed immediately after the injury except J-J″. In all figures dorsal is at the top. Lateral is on the right except for G, I and J-J″, in which lateral is on the left. C, J: Overlay; J′: Glycine; C′: GABA; C″, J″: CK. Scale bars = 40 μm (G, H); 20 μm (A-F); 10 μm (I-J″).

always showed colocalization with glutamate immunoreactivity (see above). Therefore, caudally ventral edge cells appear to maintain glycine expression better than glutamate expression.

At 0 dpl, GABA was released from most neuronal processes and populations, except for the CSFc GABA cells, in the first 400 μm rostrally and caudally from the site of injury. In these regions, astrocytes showed GABA immunoreactivity after the injury (Fig. 4C-C″). Moreover, astrocytes showing colocalization between two neurotransmitters were also observed at this time point after the lesion (Fig. 4D-D″: glutamate and GABA; 4E-E″: glycine and glutamate; 4F-F″: glycine and GABA). In contrast, the CSFc cells maintained their GABA immunoreactivity. The apical processes of CSFc cells were flattened and strongly GABA-ir in the first sections from the injury site. Moreover, they appeared to be lining the central canal (Fig. 5A). The marginal neuropil, which is formed by the lateral processes of the CSFc cells, was still GABA-ir after injury (Fig. 5B). It is noteworthy that, unlike that observed for the GABA-ir CSFc cells, the glutamate- and glycine-ir CSFc cells lost their immunoreactivity immediately after the lesion. Beyond the first 400 μm from the lesion site, other neuronal bodies and processes (apart from the CSFc cells) began to maintain their GABA immunoreactivity. Between 400 and 450 μm from the site of injury, GABA-ir astrocytes and neurons could be observed in the same spinal cord sections (Fig. 5C-C″). Between 400 and 600 μm from the site of injury, GABA immunoreactivity in the dorsal and lateral grey matter populations and processes was slightly lower than that of control samples. From the first 600 μm from the site of injury, the pattern of GABA immunoreactivity was similar to that observed in control larvae, with cells of all GABAergic populations being GABA-ir.

At 0 dpl, the complete spinal cord transection also led to changes in cytokeratin expression that extended for about 500 μm rostral and caudal from the lesion site. The astrocytes

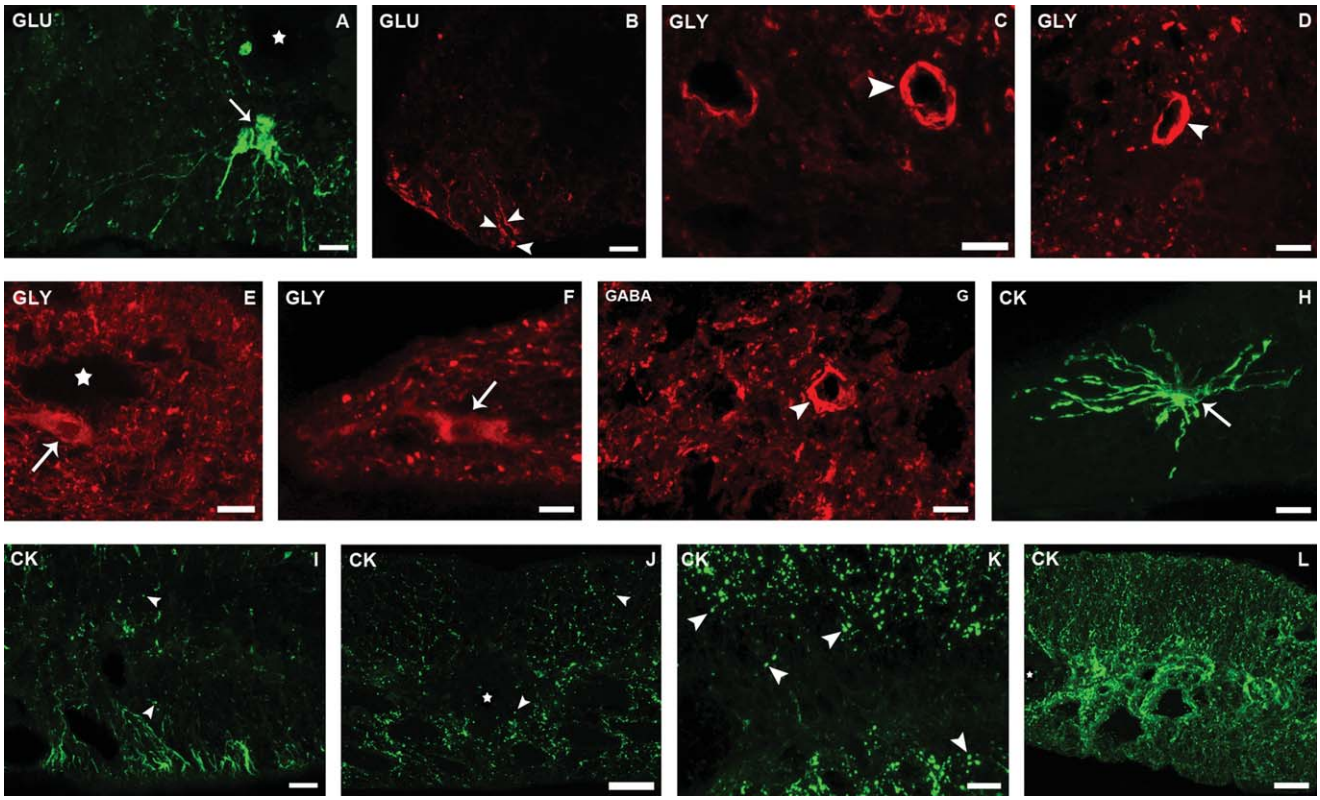

FIGURE 6: High magnification photomicrographs of transverse sections of spinal cord showing details of glutamate, glycine, GABA and CK immunoreactivities in larvae processed 1–7dpl. A: Glutamate-ir astrocyte (arrow) 3 dpl. The asterisk indicates the Mauthner axon. B: Glutamate-ir glial processes and end-feet (arrowheads) 7 dpl. C, D: Glycine-ir *halos* (arrowheads) surrounding axons of the dorsal (C) and lateral (D) funicles. E: Glycine-ir cell (arrow) associated to a Mauthner axon (asterisk). F: Glycine-ir lateral edge cell (arrow). G: GABA-ir *halo* (arrowhead) surrounding an axon of the dorsal funicle. H: CK-ir reactive astrocyte (arrow) 2 dpl. I: CK-ir processes of astrocytes (arrowheads) transversally cut, 1dpl J: CK-ir astrocyte 3 dpl. Note the faint CK immunoreactivity in the perimeningeal ends. K: CK-ir processes of astrocytes (arrowheads) transversally cut, 7dpl. L: CK immunoreactivity appearance at 7 dpl. Dorsal is at the top. Lateral is on the left except for C, E, I, K and L, in which lateral is on the right. Scale bars = 20 μm (A, B, C, D, E, F, G, H, I, K); 10 μm (J, L).

became reactive acquiring a more rounded profile with wider processes and showing intense immunoreactivity (Fig. 5D). Some processes of astrocytes showed a beaded appearance (Fig. 5F). From the first 500 μm from the site of injury onwards, the cytokeratin expression was rather similar to that observed in control animals [Fig. 5G (rostral) and 5H (caudal)], although, processes with beaded appearance were occasionally observed (Fig. 5I).

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. 6A) 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. 6B). 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.

Glycine immunoreactivity was observed in astrocytes until 1 dpl (Fig. 5J-J″), but they were glycine negative in larvae processed 2 to 7 dpl. Interestingly, in the first 300 μm from the site of injury, between 1 and 3 dpl, glycine appeared to accumulate progressively to form a *halo* around some descending axons in the dorsal (8.8% of the axons with *halos*) and lateral (10.1% of the axons with *halos*) funicles and occasionally around one of the giant axons coursing in the longitudinal medial fascicle (3.8% of the axons with *halos*) or around the Mauthner axon (7.7% of the Mauthner axons with *halos*) (Figs. 6C,D and 7A). These *halos* did not show cytokeratins immunoreactivity. The presence of glycine *halos* did not correlate significantly with the subsequent survival ability of the neurons of origin of these axons (Fig. 7C). A pattern of glycine immunoreactivity similar to that of control

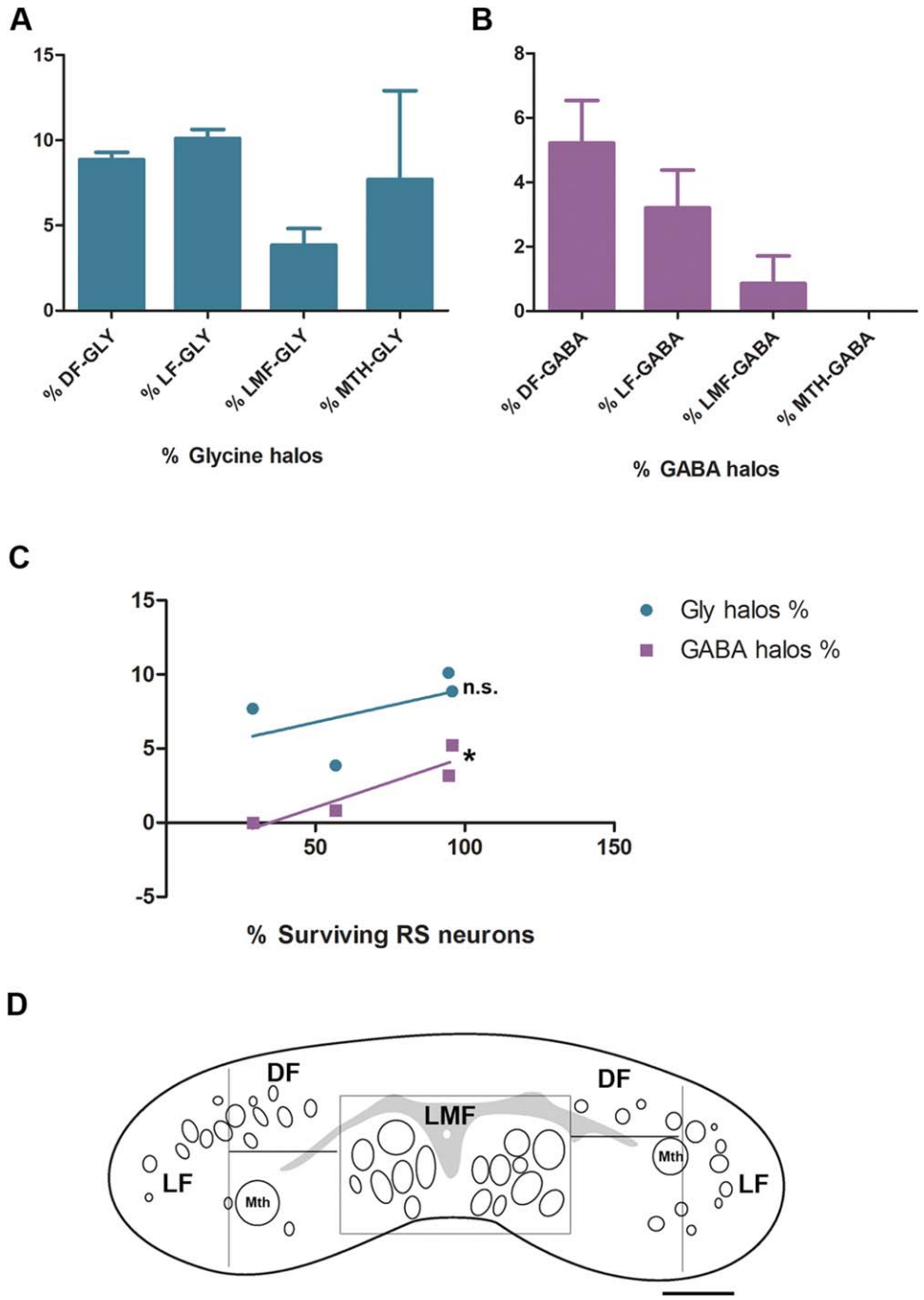

FIGURE 7: Percentages of inhibitory neurotransmitter *halos* surrounding the axons of the dorsal (DF), lateral (LF), longitudinal medial (LMF) fascicles and the Mauthner axons (MTH) of the spinal cord. A: Percentages of glycine-ir *halos*. B: Percentages of GABA-ir *halos*. C: For each of the fascicles, the percentage of glycine (blue circles) and GABA (purple squares) *halos* were plotted against the percentage of times the neurons originating the fascicles survive, as previously reported Shifman et al. (2008). The line represents the best fit linear regeression ($r^2 = 0.29$ for glycine-ir *halos*; $r^2 = 0.85$ for GABA-ir *halos*). RS (reticulospinal). The asterisk indicates a significant correlation ($P < 0.05$). D: Schematic drawing of the spinal cord showing the different axonal fascicles and the Mauthner axons (Mth). DF: dorsal fascicle; LMF: longitudinal medial fascicle; LF: lateral fascicle. Scale bar = 75 μm.

larvae was observed from 300 μm from the transection site in 1–4 dpl animals and from 80 μm in 7 dpl animals.

GABA immunoreactivity was not observed in neurons located in the first 100 to 150 μm from the injury site between 1 and 4 dpl, except for the CSFc cells that were GABA-ir. At 1 dpl, GABA-ir astrocytic processes were observed in the first 80 μm from the site of injury. At 2–7 dpl, GABA-ir was not observed in astrocytes. As observed for

glycine, from 1 dpl to 3 dpl, GABA-ir/cytokeratin negative *halos* were observed around descending axons (5.2% of the axons in the dorsal funicle, 3.2% of the axons in the lateral funicle, 0.8% of the giant axons in the medial longitudinal fascicle and 0% in the Mauthner axons) (Figs. 6G and 7B). A correlation between the presence of GABA-ir *halos* around reticulospinal axons and the survival ability of the neurons originating these axons (Shifman et al., 2008) was detected (Pearson correlation coefficient, $r = 0.92$, $P < 0.05$; Fig. 7C). A GABA pattern of expression similar to that of control larvae was observed from 100 to 140 μm from the site of injury and from the site of injury at 7 dpl.

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. 6H). From 1dpl, at the border of the lesion, processes of astrocytes transversally cut could be observed (Fig. 6I). These processes were more clearly observed at 2 dpl (Fig. 6J) and 7 dpl (Fig. 6K). 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. 6L).

Discussion

The short-term response in terms of aminoacidergic neurotransmitter expression following a complete SCI in the sea lamprey has been studied for the first time. All the substances studied [glutamate, GABA, glycine, 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 these aminoacidergic neurotransmitters by neurons and their capture by astrocytes. In animals without injury, immunoreactivity for these neurotransmitters 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 aminoacidergic neurotransmitters in astrocytes under normal conditions. Furthermore, it was observed that a particular cell population (CSFc cells) released glutamate and glycine but not GABA after the injury.

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.

Aminoacidergic Neurotransmitter 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 (Liu et al., 1999; McAdoo et al., 1999; Panter et al., 1990; 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 (Lepore et al., 2011; Olsen et al., 2010). It has been shown that glutamate release after CNS injury in mammals is mainly produced by means of reversed uptake (McAdoo et al., 2000; Rossi et al., 2000; Szatkowski et al., 1990), contributing to increase glutamate excitotoxicity. Lepore et al. (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 the first 24 h following SCI, glycine and GABA immunoreactivities were also observed in the astrocytes of the sea lamprey. In mammals, a rise in extracellular levels of glycine and GABA has been reported after SCI (Panter et al., 1990). It is known that glycine is also involved in excitatory glutamatergic neurotransmission (review in Zafra and Giménez, 2008). Glycine is a mandatory co-agonist for the activation of NMDA glutamate receptors (Johnson and Ascher, 1987). So, the increase in extracellular levels of glycine following SCI could potentiate excitotoxicity (Panter et al., 1990). Our observations demonstrating glycine uptake by astrocytes immediately after SCI indicate that this could help prevent the potentiation of glutamate excitotoxicity in lampreys.

In contrast to the toxic effects of high glutamate and glycine extracellular levels, GABA has been shown to exert neuroprotective effects (Chen-Xu et al., 2000; Han et al., 2008; O'Connell et al., 2001; Yang et al., 2000). In our study, GABA was observed to accumulate in astrocytes in the first 24 hours after SCI. Of the 3 aminoacidergic neurotransmitters studied here, GABA was the first one to recover the immunoreactivity pattern observed in control samples, which may help to achieve functional recovery. Actually, a recent study has shown that raised endogenous GABA levels after a complete SCI in lampreys can be related to a better recovery of function (Svensson et al., 2013).

Sustained Expression of GABA in CSFc Cells

In contrast to other cell types and neurotransmitters, the CSFc GABAergic cells of lampreys do not lose GABA immunoreactivity after SCI in the regions around the injury site. Interestingly, their strong GABA-ir apical dendrites show a change of morphology. Their apical portions become flattened and appear to line almost all the central canal perimeter. In contrast, in the eel, no morphological changes of the apical dendrites have been reported (Dervan and Roberts, 2003). The persistence of GABA immunoreactivity in these cells raises the question of whether GABA released by CSFc cells may influence regeneration in lampreys. It could act by offering protection or by promoting regeneration; both actions being not exclusive. The CSFc cells are the first GABAergic cells detected in the developing spinal cord (Meléndez-Ferro et al., 2003). GABA acts as a factor that modulates several essential developmental processes (for a revision see Owens and Kriegstein 2002), most but all of them being necessary to achieve regeneration. The specific persistence of GABA expression in CSFc cells suggests that, after SCI in the sea lamprey, GABA could play a role similar to that played during development to achieve regeneration.

The function of the apical dendrites of the CSFc cells is not clear. They appear to be chemosensitive (Huang et al., 2006) by detecting changes in the CSF composition, but other functions such as mechanoreception or secretion have also been proposed (Vígh et al., 2004). The flattening and rearrangement of the apical dendrites to line part of the central canal perimeter indicates that they respond to injury and that they could be playing new roles during regeneration. For example, forming a physical barrier between the interior of the central canal and the extracellular spaces of the spinal cord. It could also be related to other changes in the central canal. For example, the Reissner's fibre of the central canal is interrupted after injury and its newly produced material is accumulated at the site of injury (Barreiro-Iglesias et al., 2009b). The Reissner's fibre contains, binds and transports away developmental and molecular signals such as subcommissural organ-spodin, dopamine and serotonin (Caprile et al., 2003; Gobron et al., 2000). So, their accumulation could be related to the changes in the apical dendrites of CSFc cells.

Accumulation of Inhibitory Neurotransmitters around Identifiable Reticulospinal Descending Axons

A surprising result of our study is the observation of intense glycine and GABA immunoreactive *halos* around some of the reticulospinal descending axons in response to injury. The reticulospinal system plays a predominant role in the control of locomotion (Grillner et al., 2008). The strong accumulation of inhibitory neurotransmitters around reticulospinal axons close to the injury site suggests that they could play a role in the fate of reticulospinal neurons. It is known that different identifiable reticulospinal neurons of lampreys have different regenerative (Jacobs et al., 1997) and survival abilities after SCI (Barreiro-Iglesias and Shifman, 2012; Shifman et al., 2008). Here, we determined the proportion of axons having inhibitory *halos* around them in different types of descending axons to compare these data with the percentages of times that the corresponding descending neurons survive several weeks after SCI. Rovainen reported the stereotypic location of identifiable reticulospinal axons in the spinal cord of lampreys (Rovainen, 1976; Fig. 7D). The survival ability of the identifiable reticulospinal neurons has been reported by Shifman et al. (2008) 12 months after a spinal cord transection at the level of the 5th gill. The Mauthner cells, whose axons course in the lateral fascicle, and the Müller cells, whose axons course in the medial longitudinal fascicle, are

bad survivors (with a probability of survival/regeneration of less than 60%). However, the accessory Mauthner cells and other identifiable reticulospinal neurons, whose axons course mainly in the dorsal and also in the lateral fascicles, are good survivors (with a probability of survival/regeneration of more than 60%) (Shifman et al., 2008). A significant and positive correlation between the presence of GABA-ir *halos* around the reticulospinal axons and the survival ability of the corresponding reticulospinal neurons was observed. This strongly suggests a role for GABA in protecting these neurons at the site of axotomy. Interestingly, we have recently reported that caspase activation in descending axons at the site of injury precedes the activation of caspases in the neuronal bodies of these neurons (Barreiro-Iglesias and Shifman, in press). The accumulation of extracellular GABA around reticulospinal axons may be due to a regulated increase in the production and release of inhibitory neurotransmitters to hyperpolarize axons. Keeping the descending axons hyperpolarized may protect against excitotoxicity, counteracting harmful effects that could lead to retrograde neuronal death (Saransaari and Oja, 1997).

Conclusions and Future Perspectives

In summary, the results of our study demonstrate that the astrocytes of lampreys have a high capacity of glutamate uptake after SCI, which is maintained during the first days after a complete transection of the cord. In addition, CSFc cells maintain their GABA expression in spite of the injury and inhibitory neurotransmitters accumulate around the axons of good survivor/regenerator reticulospinal neurons. All these factors appear to contribute to the impressive regenerative and survival capacity of the spinal cord of lampreys.

Acknowledgment

Grant sponsor: Spanish Ministry of Science and Innovation; Grant number: BFU2010–17174; ABI was supported by a postdoctoral fellowship from the Xunta de Galicia.

The authors thank the staff of Ximonde Biological Station for providing lampreys used in this study and Dr. Selzer (Temple University) for providing the anti-cytokeratin antibody. They also thank the Microscopy Service (University of Santiago de Compostela) for confocal microscope facilities.

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