Neuropharmacology 131 (2018) 389–402

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Anatomical recovery of the GABAergic system after a complete spinal cord injury in lampreys

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ARTICLE INFO

Article history:
Received 26 April 2017
Received in revised form
26 December 2017
Accepted 4 January 2018
Available online 6 January 2018

Keywords:
GABA
GABAB
Complete spinal cord injury
Cerebrospinal fluid contacting-cell
Sea lamprey
Spinal cord plasticity

ABSTRACT

Lampreys recover locomotion spontaneously several weeks after a complete spinal cord injury. Dysfunction of the GABAergic system following SCI has been reported in mammalian models. So, it is of great interest to understand how the GABAergic system of lampreys adapts to the post-injury situation and how this relates to spontaneous recovery. The spinal cord of lampreys contains 3 populations of GABAergic neurons and most of the GABAergic innervation of the spinal cord comes from these local cells. GABAB receptors are expressed in the spinal cord of lampreys and they play important roles in the control of locomotion. The aims of the present study were to quantify: 1) the changes in the number of GABAergic neurons and innervation of the spinal cord and 2) the changes in the expression of the gabab receptor subunits b1 and b2 in the spinal cord of the sea lamprey after SCI. We performed complete spinal cord transections at the level of the fifth gill of mature larval lampreys and GABA immunohistochemistry or gabab *in situ* hybridization experiments. Animals were analysed up to 10 weeks post-lesion (wpl), when behavioural analyses showed that they recovered normal appearing locomotion (stage 6 in the Ayer's scale of locomotor recovery). We observed a significant decrease in the number of GABA-ir cells and fibres 1 h after lesion both rostral and caudal to the lesion site. GABA-ir cell numbers and innervation were recovered to control levels 1 to 2 wpl. At 1, 4 and 10 wpl the expression of gabab1 and gabab2 transcripts was significantly decreased in the spinal cord compared to control un-lesioned animals. This is the first study reporting the quantitative long-term changes in the number of GABAergic cells and fibres and in the expression of gabab receptors in the spinal cord of any vertebrate following a traumatic SCI. Our results show that in lampreys there is a full recovery of the GABAergic neurons and a decrease in the expression of gabab receptors when functional recovery is achieved.

1. Introduction

In humans, as in the other mammalian species, traumatic spinal cord injury (SCI) can cause permanent disability. Unlike mammals, lampreys recover locomotion spontaneously several weeks after a complete SCI (reviewed in Barreiro-Iglesias, 2012; Shifman et al., 2007; Rodicio and Barreiro-Iglesias, 2012; Barreiro-Iglesias, 2015). In lampreys, the recovery process involves the regeneration of descending spinal axons (Jacobs et al., 1997; Cornide-Petronio et al., 2011), the formation of synaptic connections between regenerated axons and neurons caudal to the lesion (Rovainen, 1976; Selzer, 1978; Wood and Cohen, 1979) and the production of new neurons in the spinal cord (Zhang et al., 2014). Recent studies have also shown how neurotransmitters systems in lampreys adapt to the post-SCI situation showing anatomical and functional plasticity: including the serotonergic (Cohen et al., 2005; Cornide-Petronio et al., 2014; Becker and Parker, 2015), aminoacidergic (Svensson et al., 2013; Fernández-López et al., 2014b, 2016) and dopaminergic (Fernández-López et al., 2015) systems. The spontaneous occurrence of these events makes lampreys an interesting model to study regeneration and plasticity after SCI to understand the events

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https://doi.org/10.1016/j.neuropharm.2018.01.006
0028-3908/

that lead to successful recovery.

GABA is the main inhibitory neurotransmitter in the central nervous system of vertebrates. In the spinal cord of rats, GABAergic neurons are present in almost all spinal cord laminae and around the central canal (Barber et al., 1982; Magoul et al., 1987). The rostral ventromedial medulla (RVM) contains GABAergic neurons that project mainly to the dorsal horn (Reichling and Basbaum, 1990; Antal et al., 1996; Hossaini et al., 2012; François et al., 2017), where they inhibit enkephalinergic/GABAergic interneurons, facilitating mechanical pain (François et al., 2017). Studies in fishes (Higashijima et al., 2004; Sueiro et al., 2004) and amphibians (Dale et al., 1987a, b; Roberts et al., 1987; Harper and Roberts, 1993) have reported different types of GABA immunoreactive (-ir) neurons in the spinal cord. The spinal cord of lampreys contains an intrinsic GABAergic component with 3 different types of GABA-ir cells in the grey matter (Batueva et al., 1990; Brodin et al., 1990; Meléndez-Ferro et al., 2003; Ruiz et al., 2004; Rodicio et al., 2008; Fernández-López et al., 2014b; Jalalvand et al., 2014; Villar-Cerviño et al., 2014) and an extrinsic component formed by the axons of GABAergic reticulospinal cells of the anterior and posterior rhombencephalic nuclei (Valle-Maroto et al., 2011).

In mammals, GABA plays important roles in both pre- and post-synaptic sites. GABAergic cells are involved in the transmission of somatosensory information to higher centres, in the propriospinal coordination of fore and hind limb movements and in reflex activities (Barber et al., 1982). GABAergic neurons produce synaptic inhibition via both GABAA and GABAB receptors in the spinal cord (Cazalets et al., 1994; Hwang and Yaksh, 1997; Munro et al., 2008 (Castro et al., 2011; Marchenko et al., 2015). In turtles, transmitter release by motoneurons is modulated by GABAB receptors (Castro et al., 2007). In *Xenopus laevis*, descending GABAergic neurons are responsible for tonic inhibition reducing locomotion (Lambert et al., 2004). This inhibition affects spinal motoneurons and premotor interneurons (Li et al., 2003). In the spinal locomotor network of lampreys, GABA is not required for burst generation but it plays a powerful modulatory role during locomotion (Homma and Rovainen, 1978; Tegnér et al., 1993; Grillner, 2003; Schmitt et al., 2004). Activation of GABAB receptors causes a reduction of burst frequency with a maintained well-coordinated locomotor activity (Tegnér et al., 1993; Schmitt et al., 2004). GABAB receptors play a role at the level of dendrites and somas of interneurons and motoneurons modifying the intrinsic membrane properties (Matsushima et al., 1993; El Manira and Bussières, 1997; Wikström and El Manira, 1998). Moreover, there is an inhibitory pre-synaptic GABAergic modulation through GABAB receptors in afferent fibres and axons of interneurons (Alford et al., 1991; Alford and Grillner, 1991; El Manira et al., 1996) and in sensory inputs (Parker et al., 1998).

Due to the importance of GABA in the modulation of spinal circuitries, alterations of the GABAergic system lead to an impairment of function. In mice, genetic ablation of GABAergic interneurons responsible for the phasic modulation results in motor deficits (Fink et al., 2014). In rodents, spinal injuries cause a reduction in the number of GABAergic interneurons (Zhang et al., 1994; Gwak et al., 2008; Meisner et al., 2010; for a review see Gwak and Hulsebosch, 2011). In cats, there is an increase in the expression of glutamate decarboxylase 67 (GAD67; the enzyme that catalyses the decarboxylation of glutamate to GABA), but not in the expression of GAD65 (a second isoform whose molecular weight is 65 kDa), below the site of injury several months after a complete thoracic SCI (Tillakaratne et al., 2000). The hypofunction of the GABAergic tone in the spinal dorsal horn is a key factor in central neuropathic pain (CNP) after SCI (Zhang et al., 1994; Drew et al., 2004; Liu et al., 2004; Gwak et al., 2006). No studies have looked at changes in the expression of GABAB receptor following a direct traumatic injury to the spinal cord. In rodents, other nerve injuries decrease the expression of GABAB receptors or gabab mRNA in the spinal cord (Castro-Lopes et al., 1995; Wang et al., 2011).

Despite the importance of the GABAergic system, the long-term anatomical changes that occur in this system after SCI have not been quantified in any vertebrate yet. The knowledge of how the GABAergic system responds to the injury sets the basis for further functional studies. Here, we studied the anatomical changes that occur in the spinal GABAergic system of lampreys following a complete SCI by analysing: 1) the changes in the number of GABAergic neurons in the spinal cord, 2) the changes in the GABAergic innervation of the spinal cord and 3) the changes in the expression of the gabab receptor subunits b1 and b2 in the spinal cord. Our results indicate that recovery of function in lampreys following SCI is associated with a fast and full recovery of the GABAergic cells and a sustained decrease in the expression of the gabab receptor.

2. Material and methods

2.1. Animals

Mature and developmentally stable larval sea lampreys (n = 89; between 5 and 7 years of age, 100-140 mm in body length), *Petromyzon marinus* L., were caught in the river Ulla (Galicia, Northwest Spain) and maintained in aerated fresh water aquaria at 15 °C with a bed of river sediment until their use in experimental procedures. Before the experiments, all animals were deeply anaesthetized with 0.1% tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO) in Ringer solution (pH 7.4; NaCl 137 mM, KCl 2.9 mM, $CaCl_2$ 2.1 mM, HEPES 2 mM). All experiments were approved by the Bioethics Committee at the University of Santiago de Compostela and the *Consellería do Medio Rural e do Mar* of the *Xunta de Galicia* (project code JLPV/IId; Spain) and were performed in accordance to European Union and Spanish guidelines on animal care and experimentation. During the experimental procedures, special effort was taken to minimize animal suffering and to reduce the use of animals.

2.2. Surgical procedures

Larval sea lampreys were randomly assigned to any of the following experimental groups: control un-lesioned animals and animals with a complete spinal cord transection. Animals with a complete spinal cord transection were analysed 1 h post-lesion (hpl), 1 week post-lesion (wpl), 2 wpl, 4 wpl, or 10 wpl. The complete spinal cord transection was performed as previously described (Barreiro-Iglesias et al., 2014). Briefly, the spinal cord was exposed from the dorsal midline at the level of the 5th gill, a complete spinal cord transection was performed with Castroviejo scissors and the spinal cord cut ends were visualized under the stereomicroscope. Then, the animals were kept on ice for 1 h to allow the wound to air dry. After this hour, the animals were returned to fresh water tanks. Animals were analysed 1 or 24 h after surgery to confirm that there was no movement caudal to the lesion site. Then, animals were allowed to recover in fresh water tanks at 19.5 °C.

2.3. Behavioural analyses

The behavioural recovery of the animals was analysed at 10 wpl based on the study of Ayers (1989) and following the protocol of Hoffman and Parker (2011). This qualitative assessment of locomotor function was made from video recordings of 5 min (camera:

Panasonic Full-HD HC-V110). The animals were placed in a plastic aquarium (36 × 23 × 10.5 cm) and swimming activity was initiated by lightly pinching the tail of the animal using a pair of forceps. The locomotor recovery of the animals was categorized in a scale of 1–6 (Ayers, 1989; Hoffman and Parker, 2011). Animals in stage 5 or 6 correspond to animals in which regeneration of axons through the site of injury has occurred based on activity evoked by stimulation across the lesion site in the isolated spinal cord (Hoffman and Parker, 2011). Two blinded experimenters independently evaluated each 10 wpl animal. Based on both analyses, a mean stage value of locomotor recovery was obtained for each animal.

2.4. Tissue processing

After the different recovery periods, control and lesioned larvae were deeply anaesthetized with 0.1% MS-222 (Sigma) in Ringer solution and killed by decapitation. For GABA immunofluorescence, the region of the body 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 h. For gabab *in situ* hybridization, the region of the body between the 4th and the 6th gills was fixed by immersion in 4% paraformaldehyde for 12 h.

After either type of 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). Then, the sections were used for immunofluorescence or *in situ* hybridization experiments.

2.5. GABA immunofluorescence

For immunofluorescence, sections were pre-treated with 0.2% NaBH4 in deionized water for 45 min to quench glutaraldehyde induced fluorescence. Then, the sections were incubated with a mouse monoclonal anti-GABA antibody (Sigma; 1: 1200; Cat# GB-69; RRID: AB_2314453) or with a rabbit polyclonal anti-GABA antibody (Biomol, UK; 1:500; Cat# BML-GA-1159; RRID: AB_2051478) in 0.05 M TBS with 1% MB during 3 days at 4 °C. After rinsing in TBS, sections were incubated for 1 h at room temperature with a Cy3-conjugated goat anti-rabbit immunoglobulin (Chemicon, Temecula, CA; 1:100; Cat# AP132C; RRID: AB_92489) or a fluorescein-conjugated goat anti-mouse immunoglobulin (Chemicon; 1:100; Cat# AQ303F; RRID: AB_92818), 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. GABA immunofluorescence was carried out in parallel in spinal cord sections of control un-lesioned and spinal transected animals.

2.6. Antibodies

The monoclonal anti-GABA antibody was raised against GABA conjugated to BSA with glutaraldehyde and was evaluated by the supplier for activity and specificity using a dot blot immunoassay. No cross-reaction was observed with BSA, L-γ-aminobutiric acid, L-glutamic acid, L-aspartatic acid, glycine, d-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, b; Villar-Cerviño et al., 2008a, b; Valle-Maroto et al., 2011; Fernández-López et al., 2012, 2014b).

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 in lampreys (Meléndez-Ferro et al., 2002; Villar-Cerviño et al., 2006; Fernández-López et al., 2014b).

Moreover, the polyclonal and the monoclonal anti-GABA antibodies were tested by Western blotting of lamprey brain protein extracts and they did not recognize any native protein in blots (Villar-Cerviño et al., 2006, 2008b). Both antibodies reveal the same neuronal populations in the sea lamprey CNS.

2.7. In situ hybridization

In situ hybridization for the detection of gabab1 and gabab2 transcripts was performed in transverse sections (14 μm thick) of the spinal cord of control un-lesioned and spinal cord transected animals. *In situ* hybridization with specific riboprobes for the gabab1 and gabab2 receptors was conducted as previously described (Romaus-Sanjurjo et al., 2016). Briefly, the spinal cord sections were incubated with the gabab1 or gabab2 DIG-labeled probes at 70 °C overnight and treated with RNAse A (Invitrogen, Massachusetts, USA) in the post-hybridization washes. Then, the sections were incubated with a sheep anti-DIG antibody conjugated to alkaline phosphatase (1:2000; Roche, Mannhein, Germany) overnight. Staining was conducted in BM Purple (Roche) at 37 °C. *In situ* hybridization experiments were performed in parallel with animals from the 4 different experimental groups and the colorimetric reaction was stopped simultaneously for all sections from the different groups of animals. As previously shown by our group, gabab1 and gabab2 *in situ* signals appear as a dotted labelling in sections of the sea lamprey central nervous system (Romaus-Sanjurjo et al., 2016).

2.8. Image acquisition

Spinal cord sections from the GABA immunofluorescence experiments were photographed and analysed by spectral confocal microscopy (models TCS-SP2 and SP5; Leica, Wetzlar, Germany). In control un-lesioned larvae, 4 sections rostral and 4 sections caudal to the level of the 5th gill were photographed. In lesioned animals, 4 sections (1 of each 6 consecutive sections) within the 150–550 μm rostral and 4 sections (1 of each 6 consecutive sections) within the 150–550 μm caudal from the site of injury were photographed. For each of the spinal cord sections from un-lesioned and lesioned animals, only one random half of the spinal cord was photographed. All photomicrographs were taken at 20x magnification (with a 1.5x digital zoom) without changing the amplifier gain or the offset to avoid the introduction of experimental variability. Stacks of confocal microphotographs were processed with LCS Lite (Leica) and Fiji (Image J, NIH, Bethesda, Maryland, USA) software to generate a Z projection of the stack and to compile a single tiff file of the photomicrograph.

An Olympus photomicroscope (AX-70; Provis) with a 20x Apochromatic 0.75 lens and equipped with a colour digital camera (Olympus DP70, Tokyo, Japan) was used to acquire images of spinal cord sections from the *in situ* hybridization experiments. In control un-lesioned larvae, 4 sections rostral and 4 sections caudal to the level of the 5th gill were photographed. In lesioned animals, 7 sections (1 of each 4 consecutive sections) within the 150–550 μm rostral and 7 sections (1 of each 4 consecutive sections) within the 150–550 μm caudal from the site of injury were photographed. Images were always taken with the same microscope and software settings. Again, only one half of the cord was photographed for each section.

After the quantifications, contrast and brightness were minimally adjusted with Adobe Photoshop CS4 (Adobe Systems, San José, CA, USA). Figure plates and lettering were generated using

Adobe Photoshop CS4 (Adobe Systems). Schematic drawings were made using Corel Suite 7 (Corel, Ottawa, Canada).

2.9. Quantification of GABA-ir cells and fibres

Three types of GABAergic cells were quantified: dorsal, lateral and cerebrospinal fluid-contacting (CSF-c) cells. Cells were quantified manually in the stacks of confocal microphotographs taken from the spinal cord sections as previously described (Fernández-López et al., 2016). Stereological counting was performed by disregarding the cells located in the first optical section of the confocal stack of each spinal cord section. The total number of cells was then calculated for each cell type and animal (rostral and caudal) based on the results obtained for each section.

The number of GABA-ir profiles (processes) was quantified using Fiji software, as previously described (Fernández-López et al., 2014a). Briefly, the white matter of the spinal cord was divided in three different regions (DM, VM and LAT) (Fig. 1A). The number of GABA-ir profiles was quantified in each region of each spinal cord section. The mean number of positive profiles per section was obtained for each region and animal (rostral and caudal). A threshold was established to have the most accurate images. To establish this threshold value, 10 random images with different profile densities were analysed before performing the actual quantifications and an optimal threshold value was selected. The same threshold was then used for all the photomicrographs. Imaging and quantification of cells and profiles were carried out by a blinded experimenter.

2.10. Quantification of the number of gabab1 and gabab2 positive profiles

We quantified the number of gabab1 and gabab2 positive profiles per spinal cord section in the 400 μm between 150 μm and 550 μm rostral and caudal to the site of lesion. 1 out of each 4 consecutive sections were analysed. The average number of positive profiles per section was then calculated for each animal (rostral and caudal). The semiautomatic quantification of the number of positive gabab1 and gabab2 profiles per spinal cord section was done as previously described for the quantification of 5-ht1a positive *in situ* profiles (Cornide-Petronio et al., 2014). This method is the same that was used for the quantification of positive immunofluorescence profiles (see above). Imaging and quantification of positive *in situ* profile numbers were carried out by a blinded experimenter.

2.11. Statistical analysis

Statistical analysis was carried out using Prism 6 (GraphPad software, La Jolla, CA). Data were presented as mean ± SEM. Normality of the data was determined by the Kolmogorov-Smirnov test and the homoscedasticity was determined by the Brown-Forsythe test. The data that were shown to be normally distributed and homoscedastic were analysed by a one-way ANOVA. Post-hoc Dunnett's multiple comparison tests were used to compare pairs of data. The data that were not normally distributed were analysed by a Kruskal-Wallis test and post-hoc Dunn's multiple

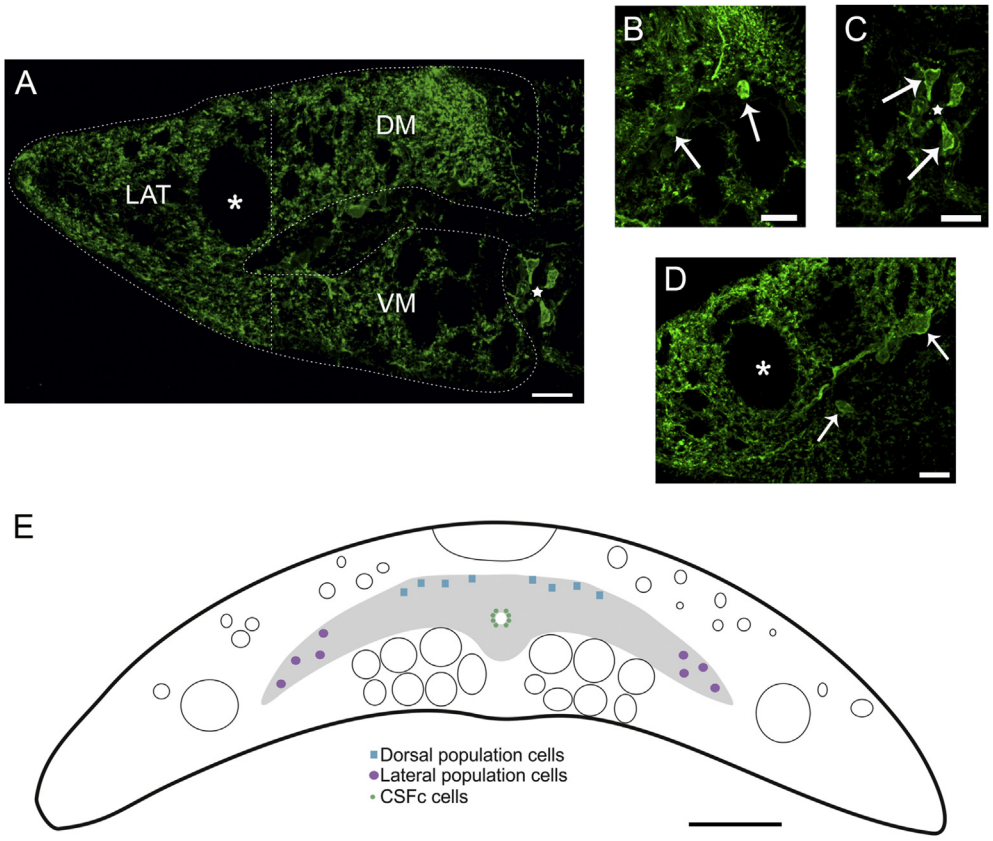

Fig. 1. A: Confocal photomicrograph of a transverse section of the spinal cord of a control larva showing the 3 regions of the white matter in which the number of GABA-ir profiles was quantified (dashed lines). Note that the highest density of fibres is seen in the dorsomedial region next to the dorsal column. **B-D**: Photomicrographs showing the 3 grey matter GABAergic cell populations. **B**: Cells of the dorsal population. **C**: Cerebrospinal fluid-contacting (CSF-c) cells. **D**: Cells of the lateral population. **E**: Schematic drawing of a transverse section of the spinal cord showing the location of GABA-ir neurons. Arrows indicate GABA-ir cells. Asterisks indicate the Mauthner axons. The star indicates the central canal. Dorsal is at the top in all photomicrographs and midline to the right. Abbreviations: DM, dorsomedial; LAT, lateral; VM, ventromedial. Scale bars: 150 μm (E); 40 μm (A); 20 μm (B-D).

comparisons test. The significance level was set at 0.05. In the figures, significance values were represented by a different number of asterisks: 1 asterisk (p value between 0.01 and 0.05), 2 asterisks (p value between 0.001 and 0.01), 3 asterisks (p value between 0.0001 and 0.001) and 4 asterisks (p value < .0001).

3. Results

3.1. Behavioural recovery of the animals

Between 1 hpl and 2 wpl the animals were only able to move the head and the body above the site of injury. At 4 wpl, the animals had some locomotor activity below the site of injury showing a few caudally propagating flexion waves. All animals recovered normal appearing locomotion at 10 wpl with all of them reaching stage 6 in the Ayers' scale of locomotor recovery (as an example see Suppl. Videos 1 to 3).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.neuropharm.2018.01.006.

3.2. The GABA-ir system in un-lesioned control animals

This study is focused on the changes that take place in the GABAergic spinal system following SCI and during the regenerative process. GABA immunoreactivity in the spinal cord of lampreys has been previously described in detail (Batueva et al., 1990; Brodin et al., 1990; Meléndez-Ferro et al., 2003; Ruiz et al., 2004; Robertson et al., 2007) and can be observed in Fig. 1. Briefly, in control larval sea lampreys at the level of the 5th gill, three different GABAergic cell populations can be observed in the spinal grey matter: dorsal, lateral and CSF-c cells (Fig. 1B-E). Occasionally, GABAergic cells are also observed in the white matter. Most of the GABAergic fibres in the spinal cord of lampreys run in the dorsolateral and ventral columns of the white matter, while they are scarce in the dorsal column (Fig. 1A). The highest density of GABAergic fibres is found in the region adjacent to the dorsal column (Fig. 1A). The distribution of GABAergic cells and fibres described here coincides with that observed in previous studies (see above).

3.3. Changes in GABAergic cell numbers and innervation following a complete spinal cord transection

The changes in GABA-ir cell numbers are shown in Fig. 2 and Table 1. As stated above, three GABAergic populations were quantified in the rostral and caudal stumps of the cord: dorsal, lateral and CSF-c cells. Rostral to the site of injury, we observed a significant 87.6%, 78.3% and 77.2%, reduction in the number of dorsal (Kruskal-Wallis, $p = .0027$), lateral (Kruskal-Wallis, $p = .0186$) and CSF-c (ANOVA, $p = .0001$) GABA-ir cells 1 hpl, respectively, as compared to control un-lesioned animals (Fig. 2A, B, H-J; Table 1). Caudal to the site of injury, we observed a significant 81.6%, 77.1% and 72.8%, reduction in the number of dorsal (ANOVA, $p = .0032$), lateral (ANOVA, $p = .0277$) and CSF-c (ANOVA, $p = .0011$) GABA-ir cells at 1 hpl, respectively, as compared to control un-lesioned animals (Fig. 2A, E, K-M; Table 1). The numbers of GABA-ir cells in most neuronal populations of the rostral and caudal stumps of spinal cord increased at 1 wpl and later and were not significantly different to control un-lesioned values (Fig. 2C, D, F-M; Table 1). Only the CSF-c population of the rostral stump still showed a significant reduction in the number of GABA-ir cells 1 wpl (Fig. 2J; Table 1). Once the cell populations were recovered, their anatomical distribution was the same to that observed in control animals.

The changes in GABA-ir innervation are shown in Fig. 3 and Table 2. In the rostral stump of the spinal cord, we observed a significant 92.8%, 89.5% and 85.6% decrease in the number of GABA-ir profiles in the dorsomedial (Kruskal-Wallis, $p = .0041$), ventromedial (Kruskal-Wallis, $p = .0019$) and lateral (ANOVA, $p < .0001$) zones at 1 hpl, respectively, as compared to control un-lesioned animals (Fig. 3A, B, H-J; Table 2). In the caudal stump of the spinal cord, we observed a significant 94.7%, 92% and 87.2% reduction in the number of GABA-ir profiles in the dorsomedial (ANOVA, $p < .0001$), ventromedial (ANOVA, $p < .0001$) and lateral (Kruskal-Wallis, $p = .0066$) zones at 1 hpl, respectively, as compared to control un-lesioned animals (Fig. 3A, E, K-M; Table 2). From 1 wpl onwards, the number of GABA-ir profiles in the three regions of the rostral and caudal stumps of the spinal cord increased (Fig. 3C, D, F-M; Table 2). From 1 wpl onwards, there were no significant differences with control un-lesioned larvae, except in the caudal ventromedial region that still showed a significant reduction in the number of GABA-ir profiles 1 wpl (Fig. 3C, D, F-M; Table 2).

3.4. Expression of the gabab1 and gabab2 subunits in the spinal cord of larval sea lamprey

As previously described for adult lampreys (Romaus-Sanjurjo et al., 2016), we observed expression of both gabab transcripts in the grey matter of control spinal cords and the colorimetric *in situ* signal appeared as a dotted labelling (Fig. 4). The pattern of expression of the gabab subunits observed in larval lampreys at the level of the 5th gill was the same as the one observed in our previous study in adult lampreys at more rostral levels (Romaus-Sanjurjo et al., 2016). Expression of gabab transcripts was observed in motor neurons and interneurons, which can be identified based on their different morphology and location in the grey matter [motor neurons are large or medium size cells that are only present in the ventrolateral region of the grey matter (Nieuwenhuys and Nicholson, 1998) (Fig. 4);]. Dorsal cells showed expression of the gabab transcripts (not shown) as well. Expression of both transcripts was also observed in a band of cells lateral to the grey matter, where only a few neurons are present and most of the cells are astrocytes (Retzius, 1893; see also Fig. 1D in Fernández-López et al., 2014a, b) (Fig. 4). Expression of both gabab transcripts was also observed around the central canal, which contains CSF-c and ependymoglial cells (Fig. 4).

3.5. Changes in gabab1 and gabab2 expression following a complete spinal cord transection

Quantification of the number of gabab1 positive *in situ* profiles per section showed significant differences between control animals and each of the experimental groups (Fig. 5; Table 3). Rostral to the site of injury, we observed a 48.3%, 37.2% and 45% reduction in the number of positive profiles at 1, 4 and 10 wpl, respectively, as compared to control un-lesioned animals (ANOVA, $p = .001$; Fig. 5A-E; Table 3). Caudal to the site of injury, we observed a 72.2%, 48.3% and 47.1% reduction in the number of positive profiles at 1, 4 and 10 wpl, respectively, as compared to control un-lesioned animals (ANOVA, $p = .0009$; Fig. 5B, F-I; Table 3).

Quantification of the number of gabab2 positive *in situ* profiles per section showed significant differences between control animals and each of the experimental groups (Fig. 6; Table 3). Rostral to the site of injury, we observed a 68.9%, 57.1% and 76.6% reduction in the number of positive profiles at 1, 4 and 10 wpl, respectively, as compared to control un-lesioned animals (ANOVA, $p < .0001$; Fig. 6A-E; Table 3). Caudal to the site of injury, we observed a 60.8%, 54.9% and 65.1% reduction in the number of positive profiles at 1, 4 and 10 wpl, respectively, as compared to control un-lesioned animals (ANOVA, $p = .0006$; Fig. 6B, F-I; Table 3).

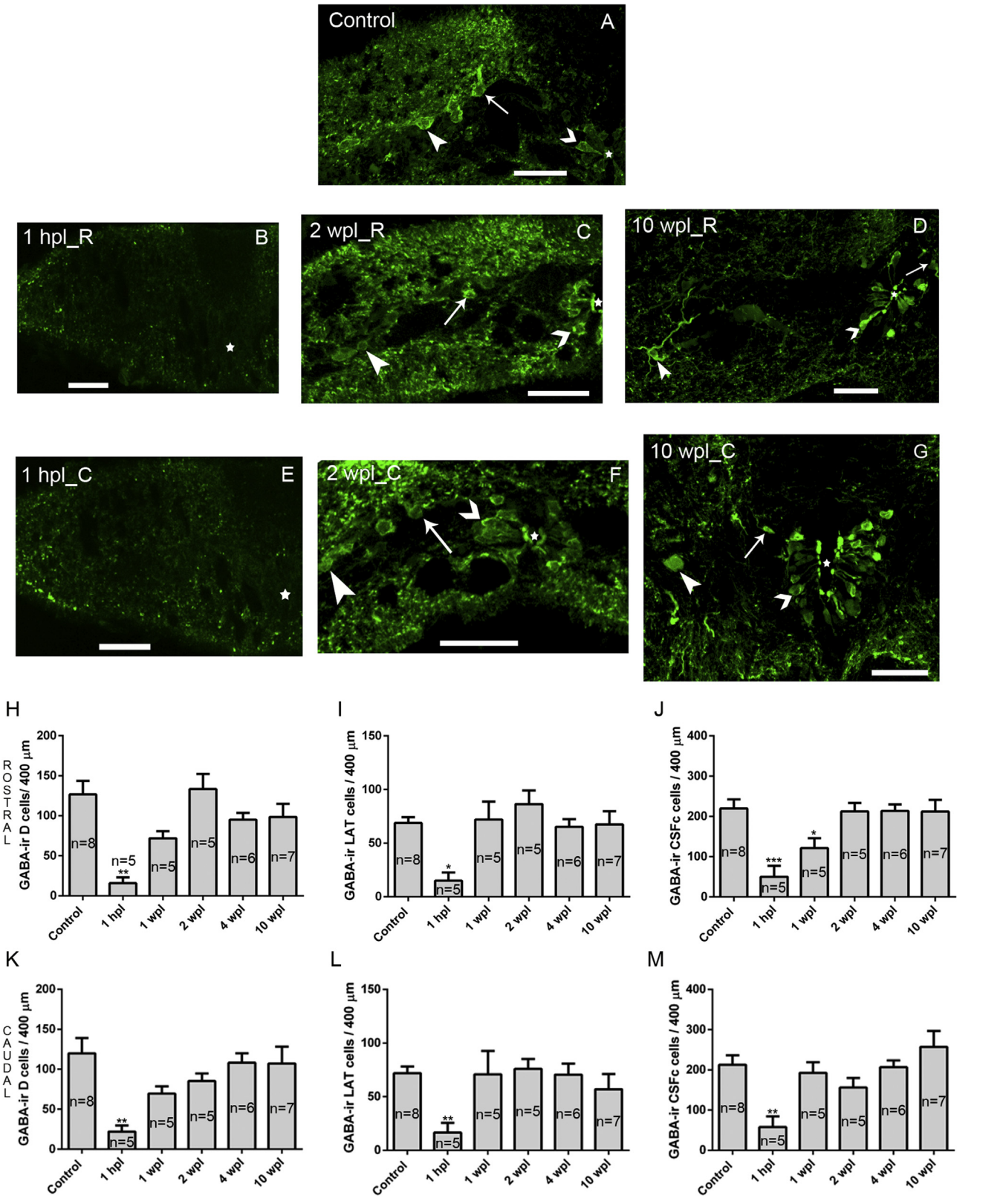

Fig. 2. A-G: Confocal photomicrographs of transverse sections of the spinal cord showing GABA-ir neurons in control animals (A) and in the rostral (B-D) and caudal (E-G) stumps of the cord of 1 hpl (B, E), 2 wpl (C, F) and 10 wpl (D, G) larvae. **H-M**: Graphs showing changes in the number of GABA-ir dorsal interneurons (H and K), lateral interneurons (I and L) and CSF-c cells (J and M) rostral (H-J) and caudal (K-M) to the site of injury. The mean ± SEM values are provided in Table 1. Arrows indicate GABA-ir dorsal cells; arrowheads indicate GABA-ir lateral cells; barbed arrowheads indicate CSF-c cells; the star indicates the central canal. Dorsal is to the top and the midline to the right. Abbreviations: R, rostral; C, caudal. Scale bars: 40 µm.

Table 1
Mean ± SEM values of the number of GABA-ir cells/400 μm in each of the spinal cord populations of control and injured animals (rostral and caudal). Refers to Fig. 2.

GABA-ir neurons/ 400 μm		Control	1 hpl	1 wpl	2 wpl	4 wpl	10 wpl
D	*Rostral*	126.7 ± 16.8	15.7 ± 7.3	71.9 ± 8.7	133.5 ± 18.9	95.1 ± 8.6	98.6 ± 16.4
	Caudal	120.0 ± 19.1	22.1 ± 7.7	69.6 ± 9.1	85.3 ± 9.5	108.3 ± 11.8	107.2 ± 21.0
LAT	*Rostral*	68.8 ± 5.4	14.9 ± 7.7	72.07 ± 16.5	86.4 ± 12.7	65.2 ± 7.0	67.4 ± 12.2
	Caudal	71.9 ± 6.2	16.5 ± 9.2	70.8 ± 21.7	75.9 ± 9.2	70.6 ± 10.3	57.0 ± 14.1
CSF-c	*Rostral*	219.8 ± 22.3	50.1 ± 27.1	121.5 ± 24.3	212.7 ± 21.0	213.5 ± 16.5	212.2 ± 28.9
	Caudal	212.8 ± 23.4	57.9 ± 26.7	192.4 ± 26.5	156.1 ± 24.0	206.9 ± 16.4	257.2 ± 39.5

4. Discussion

Our study shows that following a complete SCI in lampreys, and after the initial loss of GABA immunoreactivity (Fernández-López et al., 2014b; quantified here), there is a complete recovery of the number of GABAergic neurons and processes in the spinal cord. This is accompanied by a decreased expression of both subunits of the gabab receptor, even at 10 wpl when the animals recovered normal appearing locomotion (stage 6 in the Ayers' scale of locomotor recovery).

The number of GABAergic cells and processes is quickly recovered rostral and caudal to the site of injury after the initial loss of GABA immunoreactivity. The dopaminergic system also shows a full recovery after a complete SCI in lampreys (Fernández-López et al., 2015), although the dopaminergic system is not fully restored until approximately 4 wpl. This contrasts with the glutamatergic (Fernández-López et al., 2016) and serotonergic (Cohen et al., 2005) systems, which show a good but incomplete restoration even in the presence of functional recovery. Regarding changes in the expression of neurotransmitter receptors, different responses have been also observed for different receptors following a complete SCI in the sea lamprey. Here, we observed a decrease in the expression of the gabab subunits after the complete SCI, which differs with the response of other neurotransmitter receptors. Following a complete SCI in lampreys, there is an acute increase in the expression of the serotonin 1a receptor (Cornide-Petronio et al., 2014) and there are no changes in the expression of the dopamine d2 receptor (Fernández-López et al., 2015). Plasticity is also seen at the functional level in lampreys. Excitability is increased below the site of lesion in lampreys recovered from a complete SCI (Cooke and Parker, 2009; Hoffman and Parker, 2011) and the cellular and synaptic modulatory effect of serotonin differs in lesioned and unlesioned animals (Becker and Parker, 2015). Also, good recovery of locomotor function in lampreys following SCI is associated with stronger tonic GABAergic inhibition (Svensson et al., 2013). These studies stress the importance of studying each neurotransmitter system in lesioned animals (below and above the site of injury) to understand the mechanisms that lead to functional recovery in lampreys. This shows that the circuits underlying locomotion in lampreys are highly plastic after SCI, both at the anatomical and functional level.

The fast and full recovery of the number of GABAergic cells and processes after the initial loss of GABA immunoreactivity agrees with a previous physiological study in lampreys looking at the proprioceptive system, which showed that good recovery of function is associated with raised endogenous GABA levels (Svensson et al., 2013). Bicuculline (a GABAA receptor antagonist) only potentiates the bending-evoked responses in lesioned animals, which reflects that there is a stronger tonic GABAergic inhibition in lesioned animals through the GABAA receptor (Svensson et al., 2013). These results suggest that there is a need of high GABA levels for recovery following SCI in lampreys. Our study also shows that functional recovery is associated with lower levels of expression of gabab transcripts. The decrease in the expression of GABAB receptors could explain the increased inhibition through GABAA receptors in lesioned animals. First, a lower expression of post-synaptic GABAB receptors in the presence of a completely recovered GABAergic system means that more GABA is available to act on GABAA receptors. Second, GABAB receptors address second messenger systems through the binding and activation of guanine nucleotide-binding proteins (G-protein-coupled receptors), which inhibit adenylyl cyclase decreasing cAMP levels (Padgett and Slesinger, 2010). Lower levels of expression of gabab transcripts following SCI could lead to an increased availability of cAMP, which subsequently leads to increased protein kinase A (PKA) activity. Phosphorylation by PKA is a key factor to maintain a stable surface expression of GABAA receptors (Mele et al., 2016). So, decreased levels of gabab transcripts could lead to the anchoring of GABAA receptors in the cell surface in lesioned animals. Finally, a decrease in GABAB receptor expression in GABAergic cells could also potentiate GABA release, since GABAB receptors acting presynaptically are known to suppress GABA release (Bowery et al., 2002; Kobayashi et al., 2012). It would be of interest to analyse the possible changes in the expression of GABAA receptors in response to injury in future studies. This would require first to identify the GABAA subunits in the sea lamprey, which have not been properly annotated in the sea lamprey genome yet.

Our results also open the question of how GABA immunoreactivity is lost and how it is recovered following a complete SCI in lampreys. The immediate loss of GABA immunoreactivity (Fernández-López et al., 2014b; present results) following a complete SCI in lampreys probably occurs due to GABA release from GABAergic neurons. However, a minor contribution of cell death causing a loss of GABAergic cells cannot be excluded since some TUNEL positive nuclei are observed in the region between 150 and 450 μm rostral and caudal to the site of injury 1 day post-lesion (Fernández-López et al., 2016). The disappearance of GABA immunoreactivity precludes us from performing double TUNEL and GABA staining to confirm whether some GABAergic neurons die following the complete SCI in the sea lamprey. On the other hand, the fast recovery in the number of GABAergic cells and processes suggests that most of the GABA-ir cells survive the injury and recover their GABA-ir phenotype after the acute and massive GABA release. New neurons are generated following a complete SCI in lampreys (Zhang et al., 2014). However, the production of new GABAergic neurons after the injury probably is a minor contributor to the recovery of GABAergic cell numbers; because the peak in cell proliferation is seen 5 wpl and most of the new neurons do not migrate away from the periventricular region (Zhang et al., 2014). The different fixative methods required for GABA immunohistochemistry and for the available proliferation markers in lampreys preclude us from confirming whether new GABAergic neurons are generated after SCI. Other processes could also take a part in the early recovery of the GABAergic populations, as the occurrence of changes in neurotransmitter phenotype in non-GABAergic neurons that survive the injury. Transitory changes in neurotransmitter phenotype

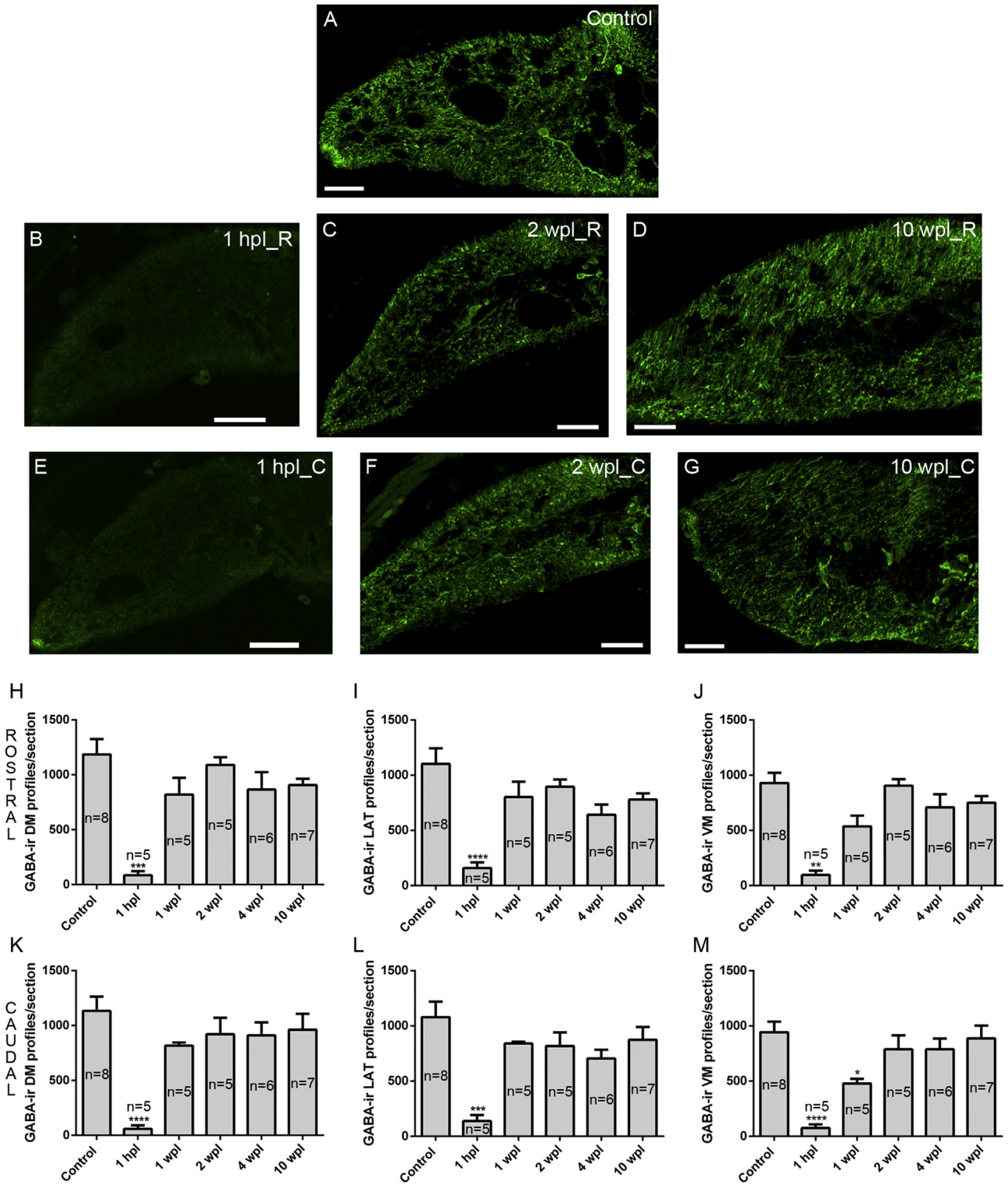

Fig. 3. A-G: Confocal photomicrographs of transverse sections of the spinal cord showing GABA-ir processes in control animals (A) and in the rostral (B-D) and caudal (E-G) stumps of the cord of 1 hpl (B, E), 2 wpl (C, F) and 10 wpl (D, G) larvae. **H-M**: Graphs showing the changes in the number of GABA-ir dorsomedial (H and K), lateral (I and L) and ventromedial processes (J and M) rostral (H-J) and caudal (K-M) to the site of injury. The mean ± SEM values are provided in Table 2. The stars indicate the central canal. Dorsal is to the top and the midline to the right. Abbreviations: R, rostral; C, caudal. Scale bars: 40 μm.

following an injury have been recently reported in *Caenorhabditis elegans* (Alam et al., 2016). In this invertebrate, axotomy in a type of GABAergic neuron induces the temporary expression of tryptophan hydroxylase leading to serotonin production, while maintaining the expression of the vesicular GABA transporter. In any case, we have not found studies reporting a similar process in lamprey cells or in other vertebrate species.

It should be noted that the complete recovery in the number of

Table 2
Mean ± SEM values of the number of GABA-ir profiles/section in each of the spinal cord regions of control and injured animals (rostral and caudal). Refers to Fig. 3.

GABA-ir profiles/ section		Control	1 hpl	1 wpl	2 wpl	4 wpl	10 wpl
DM	*Rostral*	1186.0 ± 139.6	85.9 ± 37.6	820.6 ± 151.1	1091.0 ± 68.6	865.9 ± 158.1	906.9 ± 58.0
	Caudal	1133.0 ± 129.5	59.6 ± 31.3	817.5 ± 27.6	920.8 ± 149.7	910.6 ± 117.6	962.0 ± 145.1
LAT	*Rostral*	1103.0 ± 140.5	159.2 ± 51.3	802.0 ± 140.2	895.0 ± 65.6	642.4 ± 92.0	778.9 ± 57.0
	Caudal	1080.0 ± 140.3	138.0 ± 54.0	840.9 ± 15.5	818.1 ± 123.6	704.0 ± 81.0	874.4 ± 116.5
VM	*Rostral*	929.6 ± 92.4	97.2 ± 38.4	537.2 ± 95.6	905.5 ± 57.8	708.8 ± 118.2	750.8 ± 60.2
	Caudal	943.1 ± 96.5	75.7 ± 32.6	478.0 ± 42.9	788.4 ± 127.8	788.3 ± 97.5	886.5 ± 116.6

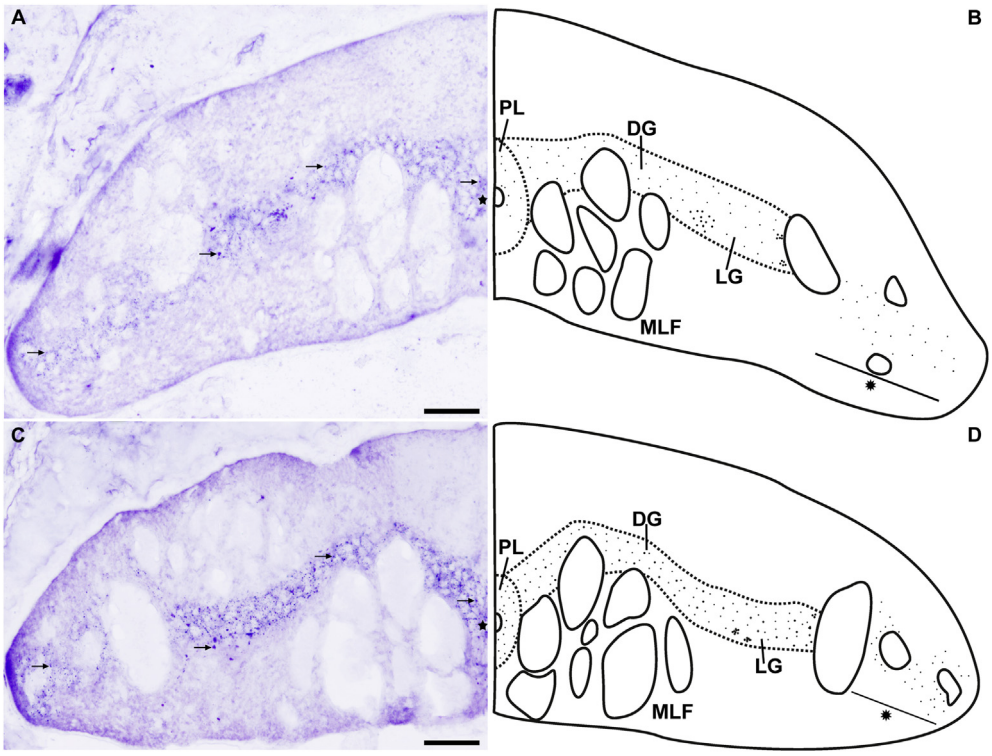

Fig. 4. A-D: Photomicrographs (A, C) and schematic drawings (B, D) of a transverse section of the spinal cord of a sea lamprey larva showing the expression of the gabab1 (A, B) and gabab2 (C, D) transcripts. Please note in panels **B** and **D** the presence of both gabab transcripts in a band of cells lateral and outside the grey matter in a region where mainly glial cells are present (asterisks) (Retzius, 1893). Stars indicate the central canal. Arrows indicate examples of positive gabab *in situ* profiles. Dorsal is to the top. Abbreviations: DG, dorsal grey; LG, lateral grey; MLF, medial longitudinal fasciculus; PL, periventricular layer. Scale bars: 50 μm.

GABA-ir processes does not imply a return to the pre-injury situation. In lampreys, most of the GABAergic innervation of the spinal cord comes from local GABAergic interneurons, which restrict their arborisations to the neighbouring segments (Brodin et al., 1990). But, there is also an extrinsic component due to the presence of a few GABA-ir descending cells located in the rhombencephalon (Valle-Maroto et al., 2011). We can assume that the recovery in the number of GABA-ir processes between 1 wpl and 2 wpl is due to the recovery of local GABA-ir cells and not because of the regeneration of descending GABAergic projections. In lampreys, at 2 wpl, axonal retraction predominates and axotomized reticulospinal descending axons do not start to regrow until 4 wpl (Jin et al., 2009). This suggests that some sprouting might be happening in the first 2 wpl from local GABAergic cells, at least caudally, to compensate the loss of descending GABAergic projections. Sprouting has been also proposed to occur in the spinal glutamatergic system (Fernández-López et al., 2016) and in the brain of lampreys (Lau et al., 2013) following a complete SCI. Compensatory sprouting in propriospinal circuits has been observed in other vertebrates (Duffy et al., 1990; Stelzner and Cullen, 1991; Bareyre et al., 2004; Courtine et al., 2008), including humans (Grasso et al., 2004). Our study establishes an anatomical basis to study the molecular mechanisms underlying the plastic changes in the GABAergic system following SCI.

4.1. Comparison to other animal models

In mammals, the dysfunction of GABAergic interneurons located in the spinal dorsal horn has been studied after a spinal injury. Such dysfunction is responsible of CNP (Zhang et al., 1994; Drew et al., 2004; Liu et al., 2004; Gwak et al., 2006). These GABAergic cells in the mammalian dorsal horn can be compared to the GABAergic cells of the dorsal population of lampreys due to their origin and location (Meléndez-Ferro et al., 2003; Villar-Cerviño et al., 2008c). The loss of GABA expression in the first weeks following SCI has been reported in rodents (Gwak et al., 2008; Meisner et al., 2010), which can be associated to cell death (Meisner et al., 2010). Tillakaratne et al. (2000) observed an increase in the expression of GAD67, but not in GAD65, protein and mRNA from 3 to 9 months after a spinal cord transection in cats. An initial loss of GABA

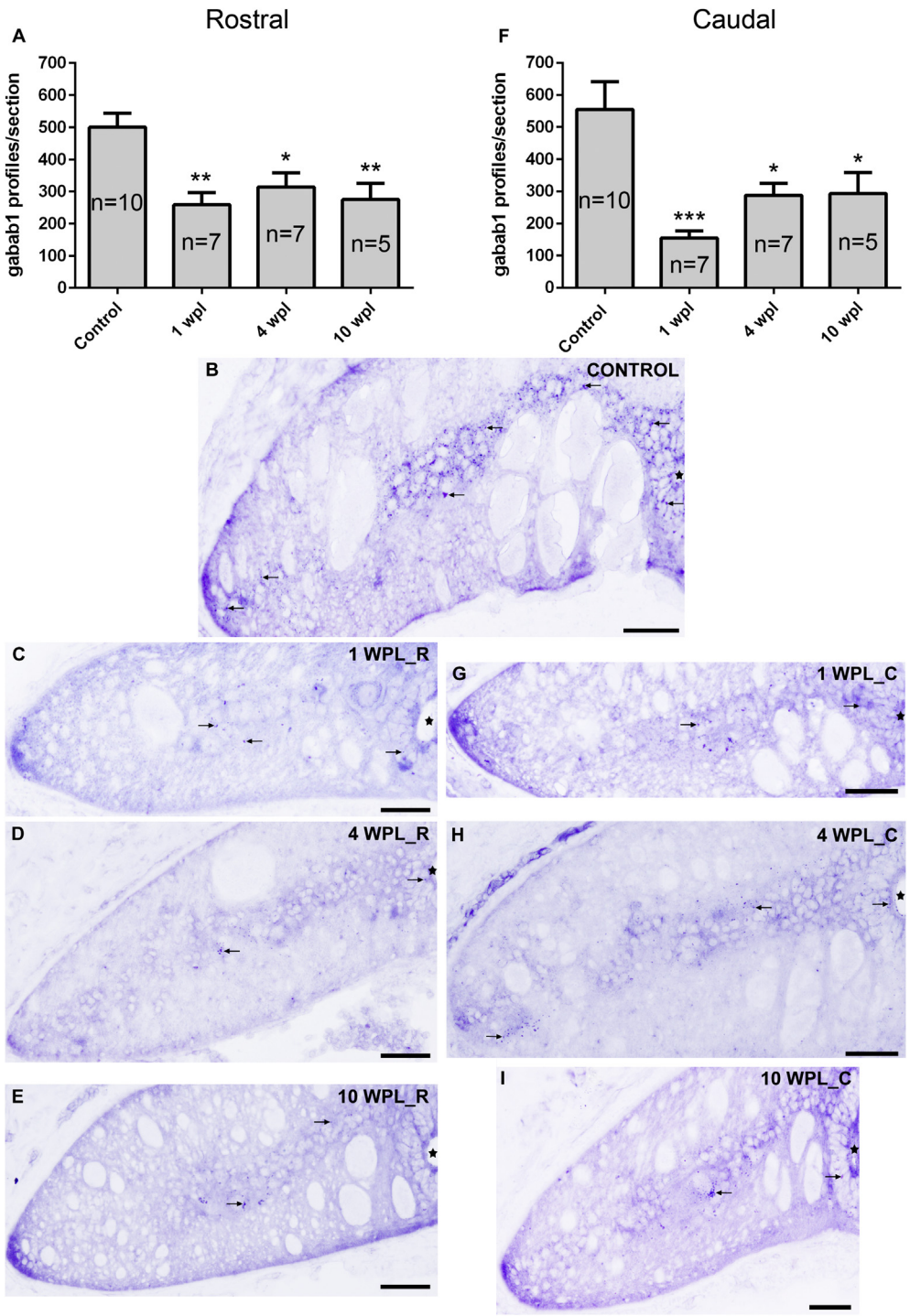

Fig. 5. Quantitative changes in the expression of the gabab1 subunit rostral (R) and caudal (C) to the site of injury after a complete spinal cord transection. **A**: Graph showing significant changes (*asterisks*) in the number of gabab1 positive profiles in the spinal cord rostral to the lesion site. The mean ± SEM values are provided in Table 3. **B:** Photomicrograph of a transverse section of the spinal cord showing the high level of expression of the gabab1 transcript in the spinal cord at the level of the fifth gill in control animals. **C-E**: Photomicrographs of transverse sections of the spinal cord rostral to the site of injury showing the decreased expression of the gabab1 transcript in lesioned animals at 1 wpl (C), 4 wpl (D) and 10 wpl (E). **F**: Graph showing significant changes (*asterisks*) in the number of gabab1 positive profiles in the spinal cord caudal to the lesion site. The mean ± SEM values are provided in Table 3. **G-I**: Photomicrographs of transverse sections of the spinal cord caudal to the site of injury showing the decreased expression of the gabab1 transcript in lesioned animals at 1 wpl (G), 4 wpl (H) and 10 wpl (I). Stars indicate the central canal. Arrows indicate examples of positive gabab1 *in situ* profiles. Dorsal is to the top and the midline to the right. Scale bars: 50 μm.

following SCI appears as a common feature of lampreys and mammals, but lampreys recover the GABAergic system better and faster than mammals.

As far as we are aware, this is the first study demonstrating a reduction in the expression of the gabab receptor following a traumatic injury to the spinal cord in vertebrates. Previous studies have reported changes in rodent GABAB receptors after other types of spinal or brain injuries. The expression of GABAB receptors is

Table 3
Mean ± SEM values of the number of gabab positive profiles/section in the spinal cord of control animals and injured animals (rostral and caudal). Refers to Figs. 5 and 6.

gabab profiles/section		Control	1 wpl	4 wpl	10 wpl
gabab1	*Rostral*	500.3 ± 42.89	258.7 ± 38.1	314.1 ± 44.2	275.3 ± 50.6
	Caudal	554.8 ± 85.77	154.4 ± 22.6	287.1 ± 37.9	293.4 ± 64.9
gabab2	*Rostral*	1324 ± 176.2	411.6 ± 97.4	568.2 ± 110.5	309.2 ± 74.5
	Caudal	1047 ± 119.6	410.2 ± 75.8	472.3 ± 149.9	364.9 ± 58.7

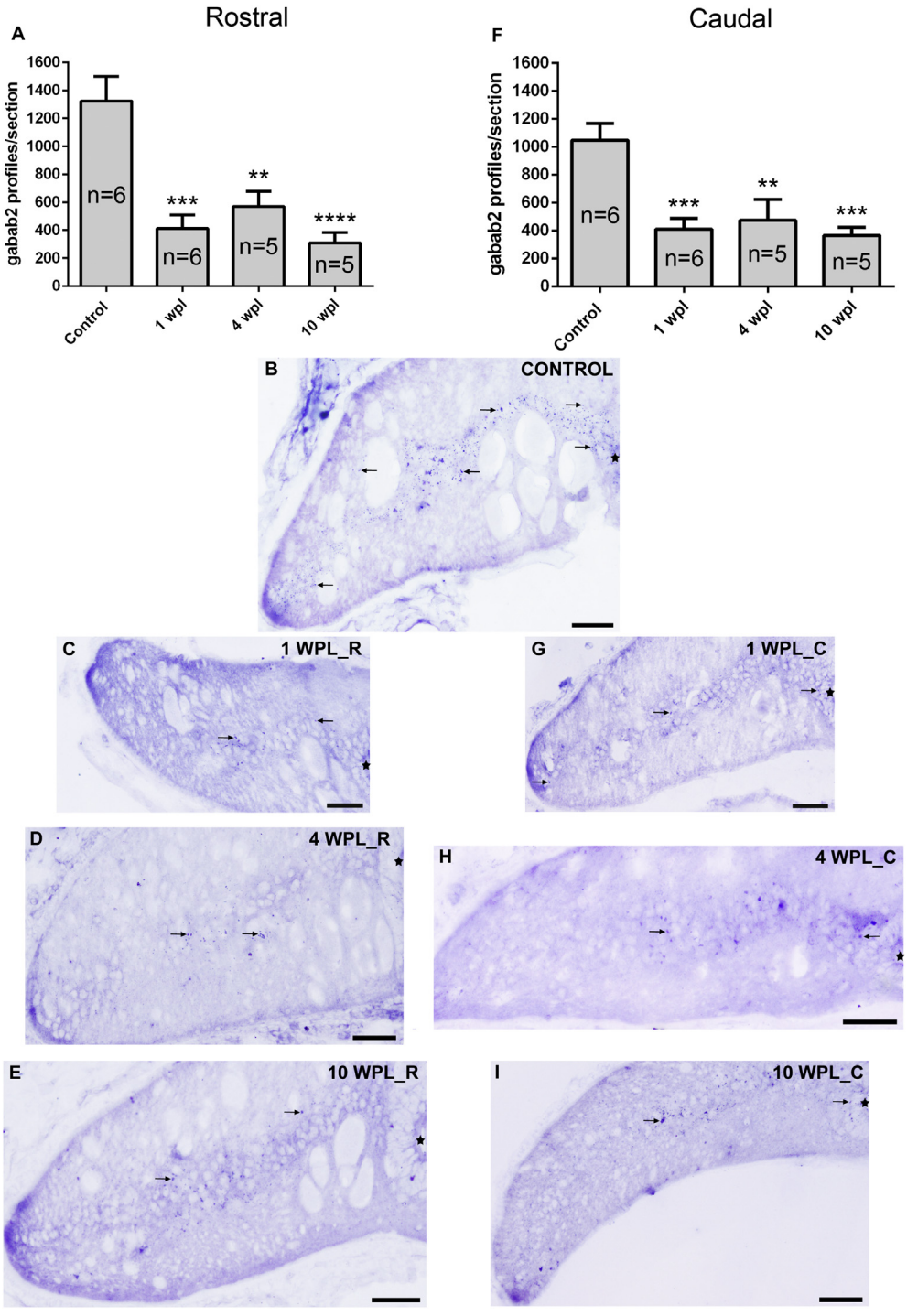

Fig. 6. Quantitative changes in the expression of the gabab2 subunit rostral (R) and caudal (C) to the lesion site after a complete spinal cord transection. **A**: Graph showing significant changes (*asterisks*) in the number of gabab2 positive profiles in the spinal cord rostral to the lesion site. The mean ± SEM values are provided in Table 3. **B:** Photomicrograph of a transverse section of the spinal cord showing the high level of expression of the gabab2 transcript in the spinal cord at the level of the fifth gill in control animals. **C-E**: Photomicrographs of transverse sections of the spinal cord rostral to the lesion site showing the decreased expression of the gabab2 transcript in lesioned animals at 1 wpl (C), 4 wpl (D) and 10 wpl (E). **F**: Graph showing significant changes (*asterisks*) in the number of gabab2 positive profiles in the spinal cord caudal to the site of injury. The mean ± SEM values are provided in Table 3. **G-I**: Photomicrographs of transverse sections of the spinal cord caudal to the lesion site showing the decreased expression of the gabab2 transcript in lesioned animals at 1 wpl (G), 4 wpl (H) and 10 wpl (I). Stars indicate the central canal. Arrows indicate examples of positive gabab2 *in situ* profiles. Dorsal is to the top and the midline to the right. Scale bars: 50 μm.

decreased in the thalamus of rats after a traumatic brain injury (Drexel et al., 2015). The expression of post-synaptic GABAB receptors is also decreased in area 3b of the cortex and in the cuneate nucleus of the adult squirrel monkeys 1–5 years after median and ulnar nerve transection (Mowery et al., 2015). Castro-Lopes et al. (1995) observed a significant reduction in the expression of GABAB receptors 2-4 weeks after sciatic nerve ligation and in rats with chronic peripheral inflammation induced by injections of complete Freund's adjuvant. Diabetic neuropathy also decreases protein and mRNA levels of GABAB receptors in the spinal dorsal horn of rats (Wang et al., 2011). It seems that a decrease in the expression of the GABAB receptor is a common characteristic after nervous system injuries in different vertebrate species.

Acknowledgements

Grant sponsors: Spanish Ministry of Science and Innovation and the European Regional Development Fund 2007-2013, Grant number: BFU2010-17174; Spanish Ministry of Economy and Competitiveness and the European Regional Development Fund 2007-2013, Grant number: BFU2014-56300-P and Xunta de Galicia, Grant number: GPC2014/030. ABI was supported by a grant from the Xunta de Galicia (Grant number: 2016-PG008). The authors thank the staff of Ximonde Biological Station for providing the lampreys used in this study, and the Microscopy Service (University of Santiago de Compostela) and Dr. Mercedes Rivas Cascallar for confocal microscope facilities and help.

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