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Activity dependent therapies modulate the spinal changes that motoneurons suffer after a peripheral nerve injury

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Abstract

Injury of a peripheral nerve leads to target denervation, but also induces massive stripping of spinal synapses on axotomized motoneurons, with disruption of spinal circuits. Even when regeneration is successful, inespecific reinnervation and the limited reconnection of the spinal circuits impair functional recovery. The aim of this study is to describe the changes that axotomized motoneurons suffer after peripheral nerve injury and how activity-dependent therapies can modulate these events. We observed a marked decrease in glutamatergic synapses, with a maximum peak at two weeks post-axotomy, which was only partially reverted with time. This decrease was accompanied by an increase in gephyrin immunoreactivity and a disintegration of perineuronal nets (PNN) surrounding the motoneurons. Application of neurotrophins at the proximal stump was not able to revert these effects. In contrast, activity dependent treatment, in the form of treadmill running, reduced the observed deseestructuration of perineuronal nets and the loss of glutamatergic synapses two weeks after injury. These changes were proportional to the intensity of the exercise protocol. Blockade of sensory inputs from the homolateral hindlimb also reduced PNN immunoreactivity around intact motoneurons, and in that case treadmill running did not revert that loss, suggesting that the effects of exercise on motoneuron PNN depend on increased sensory activity. Preservation of motoneuron PNN and reduction of synaptic stripping by exercise could facilitate the maintenance of the spinal circuitry and benefit functional recovery after peripheral nerve injury.

Key words: nerve injury, motoneurons, perineuronal nets, plasticity.

INTRODUCTION

Rehabilitation is one of the cornerstones for the treatment of injuries of the nervous system. It is assumed that repeated activity would reinforce the circuitry of the nervous system and facilitate functional recovery, by promoting structural plasticity and axonal growth. Therefore, either physical activity or exposure to enriched environment promote neurite outgrowth and functional plasticity (Ghiani et al., 2007; Rampon et al., 2000; Sale et al., 2007; Vaynman and Gomez-Pinilla, 2005a). The activity-dependent plasticity has been linked with changes in neurotrophin expression, neuronal growth genes and regulatory substances (Cobianchi et al., 2013; Molteni et al., 2004). However, other studies remark the importance of a specific rehabilitation therapy to improve functional recovery after neuronal damage (García-Alías and Fawcett, 2012; Wang et al., 2011).

Exercise and other activity-dependent therapies have been extensively used to improve functional recovery after spinal cord injuries (Hutchinson et al., 2004; Ying et al., 2008) and peripheral nerve lesions (Al-majed et al., 2000; Asensio-Pinilla et al., 2009; Meeteren et al., 1997; Sabatier et al., 2008). However, how these therapies can influence the plasticity of the central circuits where spinal motoneurons are involved is not clear. It is know that in the intact adult nervous system, the motoneurons in the ventral horn of the spinal cord are surrounded by perineuronal nets (PNNs) (Takahashi-Iwanaga et al., 1998), that restrict plasticity and play a key role in the maintenance of synapses (Kwok et al., 2011). It has been demonstrated that external stimulation, by increasing synaptic inputs, reduces PNN content, increasing the plastic abilities of cerebellar neurons and modulates the wiring of visual and somatosensory cerebral circuits (Corvetti and Rossi, 2005; Foscarin et al., 2011; McRae et al., 2007; Pizzorusso et al., 2002). Interestingly, task-specific rehabilitation increases the expression of PNN around decorticated spinal motoneurons (Wang et al., 2011), suggesting that PNN behaviour in the spinal cord can be differentially regulated by injury and activity when compared to brain neurons.

After a peripheral nerve injury, the interruption between the axons and their target organs is accompanied by important changes at the spinal cord and supraspinal levels (Navarro et al., 2007). Axotomized motoneurons suffer a massive stripping of their central synapses, related with the loss of trophic support from the muscle. When neurons regenerate and reinnervate targets organs, they partially recover their synaptic arbour, but in contrast to other excitatory and inhibitory inputs, muscle spindle Ia excitatory synapses, one of the most affected by the synaptic stripping, never recover basal levels, even when the muscle spindle and the muscle are

correctly reinnervated (Alvarez et al., 2011; Haftel et al., 2005). This reduced connectivity can explain the lack of recovery of a functional stretch reflex (Alvarez et al., 2011) . The stretch reflex is the simplest circuit but it plays a key role in neuromuscular self-control. It is a monosynaptic reflex where Ia afferents from the muscle spindle excite motoneurons innervating the same muscle. In fact, normalization of motor function requires both, specific reinnervation of peripheral target organs but also adequate reconnection of the central circuitry between sensory afferents and motoneurons (Alvarez et al., 2010). In contrast to the functional stretch reflex, its equivalent electrophysiological response, the H reflex, recovers after peripheral nerve injury and successful muscle reinnervation. In fact, there is a facilitation of this reflex, inversely correlated with the degree of reinnervation (Valero-Cabré and Navarro, 2001). Thus, the connection between motoneurons and sensory afferents measured by the H reflex does not guarantee a functional stretch reflex. The lack of correlation between the H reflex and the stretch reflex after injury suggests that peripheral axotomy favors an inadequate reorganization of the central circuitry, that can be detrimental for functional recovery. Interestingly, physical exercise and other activity dependent therapies reduce the facilitation of the H reflex observed after nerve injuries (Asensio-Pinilla et al., 2009; Udina et al., 2011b; Vivó et al., 2008), probably by modulating plasticity of spinal circuits.

Maintenance of the activity in the neural circuits after lesions can be a key-point to reduce the plastic changes that neurons suffer due to the loss of synaptic and neurotrophic inputs. Therefore, appropriate training and/or provision of afferent inputs to spinal neurons may help to prevent these changes. The aim of this study is to analyze the changes that axotomized motoneurons suffer after peripheral nerve injury and how activity-dependent strategies modulate these events. A better understanding of these changes would facilitate the knowledge of the best protocol to improve functional outcome.

MATERIAL AND METHODS

Experimental animals

Adult female Sprague Dawley rats (n=74, 8 weeks old; 250–300 g) were housed with free access to food and water at room temperature of 22 ± 2°C under a 12:12-h light–dark cycles. All experimental procedures were approved by the Ethics Committee of our institution, and followed the European Communities Council Directive 86/609/EEC. Animals were studied in four groups (see Table 1). For all the surgical interventions, rats were anesthetized by

intraperitoneal administration of ketamine (0.9 ml/kg; Imalgen 2000) supplemented with xylazine (0.5 ml/kg; Rompun 2%).

Retrograde labeling

To identify motoneuron pools from tibialis anterior (TA) and gastrocnemius medialis (GM) muscles, retrograde tracing was applied to the muscle at least 1 week before any intervention. Bilaterally, two retrotracers, True Blue Chloride (TB, Setareh Biotech) and Fluorogold (FG, Fluorochrome), were applied to identify in the same animal both motoneuron pools. Firstly, the muscle was exposed by making a small cut to the skin and two injections (2.5 µl/injection) were distributed throughout the body of the muscle by a hand-made glass pipette using a Picospritzer. In a first set of experiments, both tracers were used in the two muscles, and after corroborating that the results were similar, further experiments were performed applying FG in TA and TB in GM muscles.

Surgical procedure

Under anesthesia, the sciatic nerve was exposed at midthigh and cut by using microscisors. In a first group of animals (n=16), the transection was not repaired. In another group of animals (n=16), the proximal and the distal stump were immediately rejoined by two epineural sutures. Afterwards, muscles and skin were sutured in layers, iodine povidone was applied to the wound, and rats were allowed to recover in a warm environment under close observation. Animals were followed for 1, 2, 4 and 8 weeks after injury (n=4 for each time and each condition).

Neurotrophic factor application

In another group of animals (n=4 for each time and each condition) we applied brain-derived neurotrophic factor (BDNF), neutrotrophin-3 (NT3) or a mixture of the two to the injury site by repairing the transected nerve with a silicone tube filled with a collagen matrix enriched with these factors. The tubes were filled with a mix of 8.5 µl collagen type I (Corning), 2.5 µl MEM (GIBCO 10X) and 0.10 µl bicarbonate. 14 µl of phosphate-buffered saline (PBS 0.1M) were added in the tubes of the control group. The collagen matrix was enriched with 14 µl of PBS containing 2 ng/µl of BDNF, NT3 or a mixture of both (Ionova). The homogenous solution was carefully pipetted onto the mid zone of 8 mm long silicone tubes (2.0 mm i.d.), and the tubes incubated overnight at 37ºC. The sciatic nerve was exposed at the mid thigh and sectioned as described above. Then, the proximal and distal stumps were sutured at each end of the silicone

tube with microsutures, leaving a gap of 6 mm between stumps. The wound was sutured and disinfected. Animals were followed for 1 or 2 weeks.

Treadmill running

Treadmill running exercise consisted of two different protocols, each one carried out during 1 and 2 weeks, involving 4 different groups of animals (4 rats per group and condition). All animals were placed on the treadmill for 60 minutes twice a week prior to surgery in order to acclimatize them to a motor-driven rodent treadmill (Treadmill LE 8706 LEICA, Spain). During the training sessions, previous to surgery, shock grid intensity was set at 0.4 mA to provide a mild negative stimulus. The training protocol was started 3 days after surgery. The high-intensity treadmill running program (HTRP) consisted of 1 session of treadmill running daily for 5 days, with duration and intensity being progressively increased; running started at a locomotion speed of 10 cm/s that was increased 2 cm/s every 5 min, until a maximal speed of 30 cm/s for 60 min during the final training session (Cobianchi et al., 2013). The low-intensity treadmill running program (LTRP) was performed with a constant treadmill speed of 10 m/min in two sessions of 30 minutes each with 10 minutes resting period, daily during all the follow-up.

Suppression of homolateral sensory inputs

In order to evaluate the role of segmentary sensory inputs from the homolateral hindlimb, we performed unilateral L3 to L6 rhizotomy of preganglionar dorsal roots (n=6). After unilateral laminectomy, L3 to L6 DRG were exposed. The dorsal roots were grabbed with fine forceps and carefully transected 2–3 mm proximal to the DRG. Care was taken to avoid damage to the nearby ventral roots and the DRG. After surgery, all wounds were sutured in layers and animals were allowed to recover in a warm environment. To prevent infection, amoxicillin (500 mg/l, Normon) was given in the drinking water for one week. Postoperative analgesia was provided with buprenorphine (0.05mg/kg).

A subgroup of these animals (n=3) was followed for 2 weeks to evaluate the role of sensory inputs on the synaptic contacts on motoneurons and the PNN. Since motoneurons were not injured in this model, the changes observed can be exclusively attributed to the loss of the segmentary sensory inputs from the homolateral hindlimb. Another subgroup (n=3) was submitted to HTRP for 2 weeks, following the same protocol described above.

Immunohistochemical analysis of spinal changes

At the end of follow up, deeply anesthetized animals were transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline. The L3–L6 spinal cord segment was removed, post-fixed for 24h, cryoprotected in 30% sucrose and stored at 4ºC until used. Samples were embedded in TissueTek, serially cut (15 μm thickness) in the transverse plane with a cryostat, and collected onto gelatin-coated glass slides. All sections were first blocked with 2% normal bovine serum for 1h, followed by overnight incubation at 4ºC with combinations of primary antibodies (see Table 2). After washes, immnoreactive sites were revealed by using species-specific secondary antibodies conjugated to 488 Alexa Fluor (1:200, Invitorogen), 538 Alexa Fluor (1:500, Invitrogen), Cy3 (1:200 Millipore) or Streptavidin 488 Alexa Fluor (1:200, Invitrogen). After 2 h incubation at room temperature, the sections were thoroughly washed, mounted on slides and coverslipped with Fluoromount-G (SouthernBiotech). Labeled motoneurons were localized and images captured with a scanning confocal microscope (LSM 700 Axio Observer, Carl Zeiss 40x/1,3 Oil DIC M27***).***

Image analysis, processing and regression analysis was performed by means of in-house software implemented in Matlab R2012b (The Mathworks Inc, Natick, MA, USA). Firstly, motoneurons were automatically selected, and a constant threshold was used to segment and obtain an estimated average density for each labeling. Immunoreactivity was evaluated in two specific regions: at the soma of the motoneuron and in a perimeter of 5μm thickness surrounding the soma. This 5μm-thick perimeter covers the synaptic area surrounding the neuron and limits the overlapping with synapses of neighbour motoneurons (Fig. 1). Indeed, the maximum depletion in synapses after axotomy has been described close to the soma (Alvarez et al., 2011). For each animal, 10 to 15 motoneurons of each pool and each side were analyzed.

Statistical analysis

For quantitative variables, normality was assessed by Shapiro-Wilk test (Royston, 1993). For normal variables One-way ANOVA was used to test the significance of the difference between the lesion side and the contralateral side. For non-normal variables such analysis was performed by Kruskall-Wallis test. SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for this statistical analysis. A nested design ANOVA test was used in order to determine if the variability was due to the difference between the different motoneurons or because of the variability between animals in the exercise groups. A value of $P< 0.05$ was considered significant. Due to the

interindividual variability previously described for some of the parameters analyzed (F. Alvarez et al., 2011), the values for each marker are expressed as percentage of decreased/increased density versus the contralateral side of each animal. In the animals receiving exercise, since both hindlimbs are affected by the treatment, absolute values are also used.

RESULTS

Measurements of the size of motoneurons soma indicated that TA motoneurons had slightly lower area (mean 60±20 μm^2) than those of the GM muscle (mean 95±10 μm^2), independently of the retrotracer used (FG or TB). When comparing the size of intact motoneurons with axotomized ones, no statistically significant differences were found.

The mean synaptic content of intact motoneurons showed a relatively wide range of normal values. In the 5µm perimeter surrounding the motoneuron soma, the average density of synaptophysin ranged 130 to 160 μm^2 in TA and 170 to 200 μm^2 in GM motoneuron pool. Vglut1 density was also similar in all the neurons, ranging 100-130 μm^2 for TA and 140-170 μm^2 for GM motoneurons. In TA motoneurons, gephyrin density ranged from 35 to 45 μm^2 and from 50 to 70 μm^2 in GM ones. Regarding the PNN, intact motoneurons showed a well defined staining surrounding the soma, with a density of 130-160 μm^2 in TA and 180-210 μm^2 in GM motoneurons.

Time course of spinal synaptic changes induced by axotomy of motoneurons

Synaptophysin, Vglut1, Gephyrin

The time course of synaptic stripping was analyzed at 1, 2, 4 and 8 weeks after transection of the sciatic nerve without repair, by comparing the immunoreactivity against synaptophysin, VGlut1 and gephyrin between the injured and the contralateral sides. Motoneurons of both TA and GM pools showed a decrease of synaptophysin density, with a maximum reduction at 2 weeks, and progressive recovery at 4 and 8 weeks (Fig. 2.2.A). At 2 weeks, TA motoneurons had a 34±4% synaptic loss, whereas the reduction was 50±5% in GM motoneurons. At 8 weeks, values in the experimental side were similar the control ones. Vglut1 immunoreactivity was also decreased at all post-injury times, with a negative peak at 2 weeks (Fig. 2.3.A). Comparatively, the decrease in Vglut1 density was higher than for synaptophysin. At two weeks, the reduction was 68±3% in TA motoneurons, and 74±3% in GM motoneurons. This reduction

tended to recover at 4 and 8 weeks, but still Vglut 1 levels remained significantly lower (27±2%) than in the contralateral side (Fig. 2.3.A). In contrast to Synaptophysin and Vglut1 immunoreactivity, gephyrin labeling surrounding the axotomized motoneurons was significantly increased at all times postinjury ($P < 0.05$) compared to the contralateral intact motoneurons. Again, the maximum changes were observed at 2 weeks, with a peak increase of 42±6% in TA and of 45±5% in GM motoneurons. At 8 weeks, the gephyrin density remained slightly increased by about 10% (Fig. 3.2.A).

Perineuronal nets and glial cell reactivity

After peripheral nerve injury, the PNN surrounding axotomized neurons showed signs of desestructuration (Fig. 4). When assessing the immunoreactivity of Wisteria Floribunda, that specifically labels for PNN, in a 5µm wide-perimeter surrounding the motoneuron, we observed a significant decrease of immunostaining at all four times after injury ($P < 0.01$). Similar to the synaptic stripping observed for synaptophysin and Vglut1, the peak decrease in PNN immunoreactivity was 2 weeks after injury (about 74±5% in both motor pools; Fig. 4.2.A). Partial recovery was observed at 4 and 8 weeks, being of 7±1% in TA motoneurons and of 20±2% in GM motoneurons at 8weeks.

To evaluate the role of glial cells into the stripping process, we also immunolabeled against Iba1 to indentify microglia and against GFAP to label astrocytes. In non injured motoneurons, the presence of glial cells close to the soma is minimum, but 1 week after axotomy we observed a marked increase of microglia (280±20 Iba1/µm^2, $P < 0.01$), that decrease at 2 weeks postinjury (80±10 Iba1/µm^2, $P < 0.05$) (Fig. 4.1.A). In contrast, astrocyte reactivity showed a slower time course; astroglia immunolabeling around axotomized motoneurons was increased at 4 (170±20 GFAP/µm^2, $P < 0.01$) and most marked at 8 weeks postinjury (270±20 GFAP/µm^2, $P < 0.01$) (Fig.4.1.B).

Effects of nerve repair on spinal synaptic changes of axotomized motoneurons

In order to analyze if the presence of the distal segment could affect the changes observed after injury, we studied the time course of synaptic stripping and glial cell reactivity in animals in which the cut sciatic nerve was immediately repaired by suturing the proximal and distal stumps. There were no significant differences between animals with just axotomy and those with axotomy and repair (Figs. 2-4).

Influence of trophic support on the synaptic changes induced by axotomy of motoneurons

To evaluate if an additional trophic factor support could ameliorate the changes in synaptic inputs found after axotomy, neurotrophins BDNF and NT3 were applied at the proximal stump by means of a silicone tube containing a collagen matrix enriched with these factors. In the contralateral side, the nerve was also cut and repaired with the same tube containing the collagen matrix without addition of trophic factors.

One and 2 weeks after application of BDNF at the lesion site, we detected a lower decrease of synaptophysin and Vglut1 immunoreactivity surrounding motoneurons when compared with the contralateral side, where BDNF was not applied, although these differences reached statistical significance only in GM motoneurons 1 week postinjury (P=0.025). Regarding gephyrin immunoreactivity, a smaller non-significant increase was observed with BDNF application (Fig. 5). In contrast, NT3 application did not affect the changes induced by axotomy on synaptophysin, Vglut1 and gephyrin immunolabeling surrounding the motoneurons. Neither BDNF nor NT3 application affected the changes detected in PNN after axotomy (Fig. 5.4).

Influence of treadmill running exercise on the synaptic changes induced by axotomy of motoneurons

In order to know if physical exercise could modulate the plastic changes affecting motoneurons after axotomy, two different treadmill protocols were performed.

HTRP was able to partially prevent the decrease in synaptophysin immunoreactivity surrounding axotomized motoneurons 2 weeks post injury by about 12% (31% and 43% decrease in TA and GM versus 44% and 55% decrease, respectively in non trained animals) ($p<0.001$) (Fig. 10.1). Vglut1 decrease observed after axotomy was also partially reduced with HTRP already at 1week post lesion in both motoneuron pools, and was only of 50±5% at 2 weeks, in front of the 71±6% observed in non trained animals (Fig. 10.2). Gephyrin increase was partially reduced by HTRP, at 1 week for GM motoneurons and at 2 weeks for both motoneuron pools (Fig. 10.3). On the other hand, the low intensity treadmill exercise (LTRP) was not able to revert the synaptic stripping changes observed after axotomy. In GM motoneurons, a non significant lower increase in gephyrin immunoreactivity was observed at 2 weeks when compared to the contralateral side (Fig. 10.3).

When analysing PNN immunoreactivity surrounding the motoneurons, we found that both protocols of exercise reduced about 20% the decrease observed 2 weeks after axotomy. Moreover, the high intensity protocol (HTRP) prevented this decrease already at 1 week.

Unexpectedly, we observed that PNN immunoreactivity surrounding the contralateral intact motoneurons of intensively trained animals was higher than in non exercised rats (Fig. 11, Fig 13 $1B_2$ $2B_2$).

At 1 week, TA motoneurons in LTRP animals had a 35±4% PNN loss, whereas in GM motoneurons the reduction was of 42±1%. At 2 weeks, levels were 54±3 for both pools of motoneurons. In the HTRP group, 1 week after being axotomized, TA motoneurons had a 22±2% reduction in PNN, whereas this fall reached a 34±2% in GM motoneurons. At 2 weeks, the reduction was of 51±2% in TA motoneurons and of 61±2% in GM ones. In fact, GM motoneurons of animals receiving HTRP for 2 weeks showed a significant increase in PNN staining than motoneurons from untrained animals. Therefore, we expressed the amount of PNN immunoreactivity as absolute values (Fig. 11).

HTRP also modulated glial activation. Animals submitted to HTRP showed reduced microglia activation compared to non-trained animals. There was a lower immunoreactivity to Iba1 surrounding axotomized motoneurons in exercised rats (60±10 Iba1/μm^2, $p<0.05$) than in non-trained animals. On the contrary, there was an increase in astrocyte reactivity compared with non-trained animals, especially when the treadmill protocol was carried out during 2 weeks (280±15 GFAP/μm^2, $p<0.01$). In the contralateral side there was also increased astrogliosis but not significant compared to untrained animals (Fig. 7.1). For the low intensity protocol (LTRP) no changes in astroglia and microglia immunoreactivity between exercised and non-trained animals were observed.

Influence of homolateral hindlimb sensory inputs on PNN

In order to assess the influence of sensory inputs on motoneuron changes after axotomy, we sectioned the dorsal roots L3 to L6 at preganglionar level. To corroborate that the additional surgery did not affect motoneuron function, nerve conduction tests were performed 2 weeks after rhyzotomy. In all the animals, the amplitude of the M wave recorded from TA and GM msucles was similar to control values, whereas the H wave was abolished (data not shown).

As expected, disruption of peripheral sensory input induced a significant reduction, of about 60%, in Vglut1 staining surrounding motoneurons of both pools ($P>0.01$). We observed a slight reduction in synaptophysin staining (20±5%) that was not significant compared to contralateral motoneurons. Regarding gephyrin, no significant differences were observed surrounding the

axotomized motoneurons versus the intact ones. Animals exercised in the treadmill showed similar values than non-trained ones.

Two weeks after rhyzotomy, even when motoneurons were not axotomized, we found a 22±2% reduction in the density of PNN arounf motoneuron pools of TA and GM muscles. This reduction was also observed in animals receiving HTRP for 2 weeks; therefore, exercise was not able to revert the effects of sensory deprivation on PNN (Fig. 8).

Dorsal root lesion did not induce a significant microglia activation surrounding motoneurons in the ventral horn of the injured side (60±10 Iba1/μm^2) compared to the contralateral side. In contrast, we observed a significant increase of astroglia immunoreactivity (250±15 GFAP/μm^2) around motoneurons of the sensory denervated side. Values in trained animals were similar to non-trained ones but in the contralateral side a significant increase of astroglia reactivity around motoneurons was observed (100±10 GFAP/μm^2 $P>0.05$) (Fig. 8).

DISCUSSION

In this paper we have evaluated the central changes that motoneurons suffer after a peripheral nerve injury. As previously described (Alvarez et al., 2011; Rotterman et al., 2014) , we observed a marked decrease in proximal glutamatergic synapses, with a maximum peak at 2 weeks post-axotomy, that was partially reverted with time. This decrease was accompanied with an increase in gephyrin immunoreactivity and a desintegration of PNN surrounding the motoneurons. Application of neurotrophins at the proximal stump was not able to revert these effects. In contrast, activity dependent therapies, as treadmill running, reduced the desestructuration of PNN and the loss of glutamatergic synapses 2 weeks after injury.

Due to the high variability described in the synaptic content of motoneurons (Alvarez et al., 2011, 2010), we specifically evaluated two motoneuron pools, the extensor GM and the flexor TA, both innervated by the sciatic nerve and compared the changes induced by axotomy with the contralateral intact side. In fact, we observed a higher synaptic depletion in axotomized GM motoneurons.

The synaptic stripping that motoneurons suffer after axotomy is a well-known phenomenon that has been related with a shifting state of the neurons from one subserving distribution of information to one where mechanisms for survival and repair are warranted (Aldskogius, H. and Svensson, 1993). This synaptic detachment has been linked with the rapid activation of microglia, which withdraw their processes, proliferate, and migrate towards the axotomized

motoneuron cell body (Greaber et al., 1993). In our study, we found a marked increase of microglial processes surroundeing axotomized motoneurons already at one week. At this time point, we also observed some synaptic stripping, with reduction of Synaptophysin immunoreactivity around motoneurons. This loss reached its maximum low peak at 2 weeks. This reduction can be partially attributed to the loss of glutamatergic synapses, one of the synapses most affected by the stripping after injury (Alvarez et al., 2011; Novikova et al., 2000), Vglut1 is a specific marker for Ia sensory afferent from the muscle spindle (Todd et al., 2003) and, therefore, it is a key element in the circuit of the stretch reflex. The group of Cope (Alvarez et al., 2010) proposes that Vglut1 synapses never recovery after injury and, therefore, there is a permanent disconnection between Ia afferents and motoneurons (Alvarez et al., 2011) and a consequent loss of the functional stretch reflex (Haftel et al., 2005). We also found that Vglut1 immunoreactivity surrounding the motoneurons never recover basal levels after axotomy. Interestingly, we observed an increased immunoreactivity of Vglut1 into the soma of axotomized motoneurons at 4 and 8 weekss postinjury. In parallel to Vglut1 decrease, we observed a dramatic increase of gephyrin immunoreactivity around motoneurons. Gephyrin organizes the postsynaptic density of the inhibitory GABAergic and glycinergic receptors (Fritschy et al., 2008; Tretter et al., 2008; Yu and De Blas, 2008), and it is a key inhibitory scaffolding protein. Upregulation of gephyrin may be a protective action that tries to compensate the excitability observed in motoneurons after peripheral nerve injury.

Synaptic stripping have been related to loss of trophic support of the neurons after axotomy (Davis-López de Carrizosa et al., 2009; Novikova et al., 2000). In fact, in oculomotor neurons, addition of BDNF and NT3 was able to revert synaptic stripping. These neurotrophins differentially affected excitatory and inhibitory synapses (Davis-López de Carrizosa et al., 2009). Therefore, we wanted to test if addition of these neurotrophins in the proximal stump of the nerve could revert these stripping, by mimicking the trophic support received from the muscle. However, we just found marginal preservation of Vglut1 synapses after BDNF administration. Direct application of these factors into the spinal cord could be a more effective method to revert synaptic stripping (Davis-López de Carrizosa et al., 2009; Novikova et al., 2000) but application at the injury site is a more clinical strategy, since it can be performed in conjunction with the surgical repair and also favour axonal regeneration. However, continuous delivery of these factors, either by microparticules or by viral vector–mediated transfer of neurotrophins (Blits et al., 2003; Ruitenberg et al., 2002) could be a more effective strategy to guarantee trophic support for longer periods of time.

Accompanying the synaptic changes, we observed a desestructuration in the PNN surrounding axotomized motoneurons. A previous paper points that motoneurons do not loss PNN after nerve injury (Kalb and Hockfield, 1988), although they do not perform a quantitative analysis of these nets. In fact, we still can detect PNN staining surrounding these motoneurons, but there is a clear reduction of its immunoreactivity, with a maximum low peak at two weeks. It seems logical that these nets, that stabilize synapses, get affected with the process of synaptic stripping. It is interesting to note that in other neurons of the central nervous system, as those of the visual system (Guimarães et al., 1990; Lander et al., 1997; P. C. Kind, 1995; Sur et al., 1988), the somatosensory system (McRae et al., 2007) or the cerebellum (Carulli et al., 2013), PNN formation and maintenance is influenced by the activity of sensory afferents, with increased activity reducing PNN. After a peripheral nerve injury, there is a loss of sensory inputs from Ia afferents that are also injured and denervated from their target organ, the muscle spindle. But accompanying this sensory loss, motoneurons are also injured and switch to a proregenerative state that can affect their dendritic arbour. Therefore, to further understand the mechanism that can explain the reduction of PNN after injury, we also evaluated their fate when motoneurons were not axotomized but lost the sensory inputs from their limb. Interestingly, after transection of the dorsal roots inervating the lower limb (L3 to L6), PNN were significantly reduced, indicating that sensory inputs are key players in the maintenance of these nets. Therefore, our findings suggest that activity can differentially regulate PNN in spinal motoneurons when compared with cortical sensory or cerebellar neurons.

We indeed were interested in evaluating the effects of activity dependent therapies in the spinal changes observed after peripheral nerve injury. The effects of exercise on motor function after peripheral nerve injury are a bit controversial, with studies concluding that exercise has beneficial effects (Asensio-Pinilla et al., 2009; Marqueste et al., 2004; Meeteren et al., 1997; Sabatier et al., 2008; Udina et al., 2011a) and others indicating it is detrimental (Gutmann, E and Jokoubek, 1963; Herbison et al., 1080; Herbison, G.J., Jaweed, M.M., Ditunno, 1974; Meeteren et al., 1997; Soucy , M Seburn and Gardiner, 1996). Moreover, activity dependent therapies can also modulate the plastic changes observed after peripheral nerve injury, by means of modulating the H reflex (Asensio-Pinilla et al., 2009; Udina et al., 2011a; Vivó et al., 2008). Since synaptic stripping might be a source of poor functional recovery following peripheral nerve injury, these combined effects of treadmill training have potential for improved functional recovery after injury. However, the mechanism through which exercise is facilitating functional

recovery after injuries of the nervous system is not clear. It is proposed that modulates plasticity, probably by increasing BNDF (Vaynman and Gomez-Pinilla, 2005b).

Plasticity of the nervous system has been related with PNN reduction. Enzymatic degradation of PNNs reopened the critical period for experience-dependent plasticity in the visual cortex(Pizzorusso et al., 2006, 2002) and favours functional recovery after spinal cord injuries (Massey et al., 2006) by facilitating plasticity of the circuitry.

However, there are few studies that evaluate the fate of spinal motoneuron PNN when exercise or other rehabilitative therapies are applied. The group of Fawcett described that rehabilitation therapies increased motoneuron PNN in a spinal cord injury model. Our results agreed with their findings, since we also observed that treadmill exercise reduced the decrease in PNN content observed in axotomized motoneurons. Moreover, HTRP also increased PNN in the contralateral non-injured side. Therefore, the plastic changes attributed to exercise are probably not linked to reduction in PNN in the spinal circuitry. In contrast to what happens in other circuitry in the brain, disruption of the segmentary circuitry that regulates motoneuron control could be detrimental for functional recovery. Exercise, by reinforcing the wiring of the original circuits can partially compensate the changes observed after injury and improve functional recovery.

Interestingly, the 20% preservation of PNN immunoreactivity in motoneurons of exercised animals is similar to the 20% loss observed after disruption of synaptic inputs in non injured motoneurons. In fact, if animals that suffered disruption of the sensory inputs of the hind limb were also trained in the treadmill, PNN loss was not reverted, suggesting that the effects of exercise on motoneuron PNN depend on increased activity mediated by sensory afferents. We performed a wide dorsal ryzothomy to supress most of the sensory inputs of the hind limb, not just the sciatic afferents. It is proposed that exercise can influence injured neurons by stimulating sensory afferents (Molteni et al., 2004). Even in the denervated period, exercise can stimulate afferents of proximal non-injured muscles (Koerber et al., 2006).

Exercise was also modulating glial activation surrounding axotomized motoneurons. The same protocol that we used was able to reduce microglia activation after chronic constriction injury in mice (Cobianchi et al., 2010). In agreement, we observed a significant reduction of microglia processes around motoneurons already at one week. It seems that the intensity of exercise would

correlate with the amount of microglia reactivity attenuation, since low intensity treadmill has no effect on microglia activity. Although it is proposed that reduction in microglia reactivity can prevent neuronal loss (Milligan and Watkins, 2009), others suggest that activated microglia have a predominantly protective role (Cullheim and Thams, 2007). In our study, reduction in microglia activation at early time points can explain the less severe synaptic stripping observed in the animals that were running at high intensity.

Since inflammatory mediators released by activated microglia may activate astrocytes (JI, 2007), we expected that reduced reactivity of microglia induced by exercise would be accompanied with a reduction in astrocyte reactivity. Nevertheless, we observed a marked increase of astrocytes 2 weeks post injury in the trained animals, fact that suggests that astrocyte activation was independent of microglia activation. In not trained animals we observed an increase of astroglia surrounding motoneurons 8 weeks post injury, the same time point when there was a recovery of PNN immunoreactivity to basal levels. A previous work shows that the formation of synapses promoted by astrocytes is paralled to the emergence of PNNs in embryonic hippocampus (Pyka et al., 2011). Therefore, the increased astrocyte reactivity induced by exercise can be related to the preservation of PNN in axotomized motoneurons of trained animals.

Future studies analysing the functional recovery of the central circuits after treadmill running and its relation with motoneuron PNNs changes would help elucidate the complex effects of activity dependent therapies on the nervous system.

ACKNOWLEDGMENTS

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FIGURE / FIGURE LEGENDS

Experimental group	Injury	Lesion/treatment	Follow up
1. Time course of spinal synaptic changes (n=32)	Sciatic nerve section	No repair	1 week 2 weeks
	Sciatic nerve section	Direct suture	4 weeks 8 weeks
2. Influence of trophic support on the synaptic changes (n=20)	Sciatic nerve section	Silicone tube with BDNF	1 week 2 weeks
	Sciatic nerve section	Silicone tube with NT3	
	Sciatic nerve section	Silicone tube with BDNF+NT3	
3. Influence of treadmill running exercise on the synaptic changes (n=16)	Sciatic nerve section	Direct suture/ LTRP	1 week 2 weeks
	Sciatic nerve section	Direct suture/ HTRP	
4. Suppression of homolateral sensory inputs (n=6)	L3-L6 Rhizotomy (intact sciatic nerve)	Untrained	2 weeks
	L3-L6 Rhizotomy (intact sciatic nerve)	HTRP	

Table 1 Experimental design. Groups and treatments applied. The animals were divided into 4 experimental groups: **1.** Time course of spinal synaptic changes. Sciatic nerve was cut and left unrepaired or reapired by direct suture. Animals were sacrificed at 4 different times postinjury. **2**. Influence of trophic suport on the synaptic changes. BDNF, NT3 or a mixture of the two neutrophic factors were applied at the injury site by using a silicone tube **3**. Influence of treadmill running exercise on the synaptic changes. Two days after nerve repair animals were submitted to LTRP or HTRP, for 1 or 2 weeks. 4. Suppression of homolateral sensory inputs. After L3 to L6 rhizotomy, half of the animals were submitted to HTRP and the other half was left untrained.

Antigen	Immunogen	Host type	Working dilution	Manufacture
VGlut1	*Synthetic peptid from rat VGlutI*	*Guinea pig polyclonal*	*1:300*	*Millipore Ref AB5905*
Perineuronal nets	*Lectin from Wisteria floribunda, Biotin conjugated*		*1:200*	*Sigma*
Gephyrin	*Rat Gephyrin aa. 569-726*	*Mouse*	*1:200*	*BD*
GFAP	*Purified GFAP from porcine spinal cord*	*Mouse*	*1:1000*	*Millipore Ref AG230*
Iba1	*C-terminus of Iba-1 synthetic peptide*	*Rabbit polyclonal*	*1:500*	*Wako*
Synaptophysin	*C-terminus of human synaptophysin*	*Rabbit polyclonal*	*1:500*	*Invitrogen*

Table 2 **Primary antibodies used in this study.**

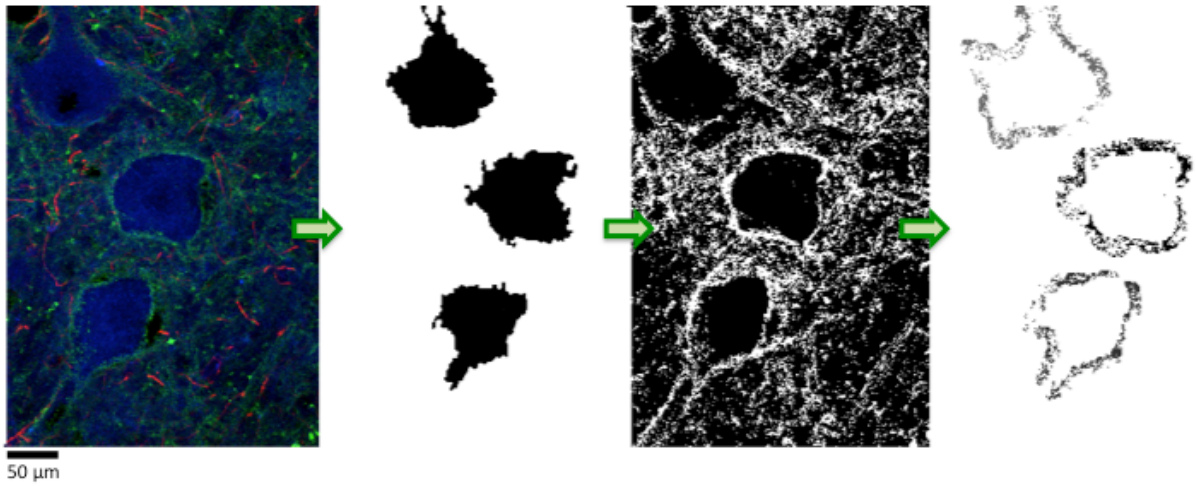

Figure 1 **Analysis of the amount of immunostaining surrounding motoneurons**. Automatic selection of the somata of backlabeled motoneurons, detection of the immunostaining using a fix threshold for every staining, determination of a circular perimeter area surrounding the soma of a 5µm thickness and measurement of the staining contained in that perimeter or into the soma.

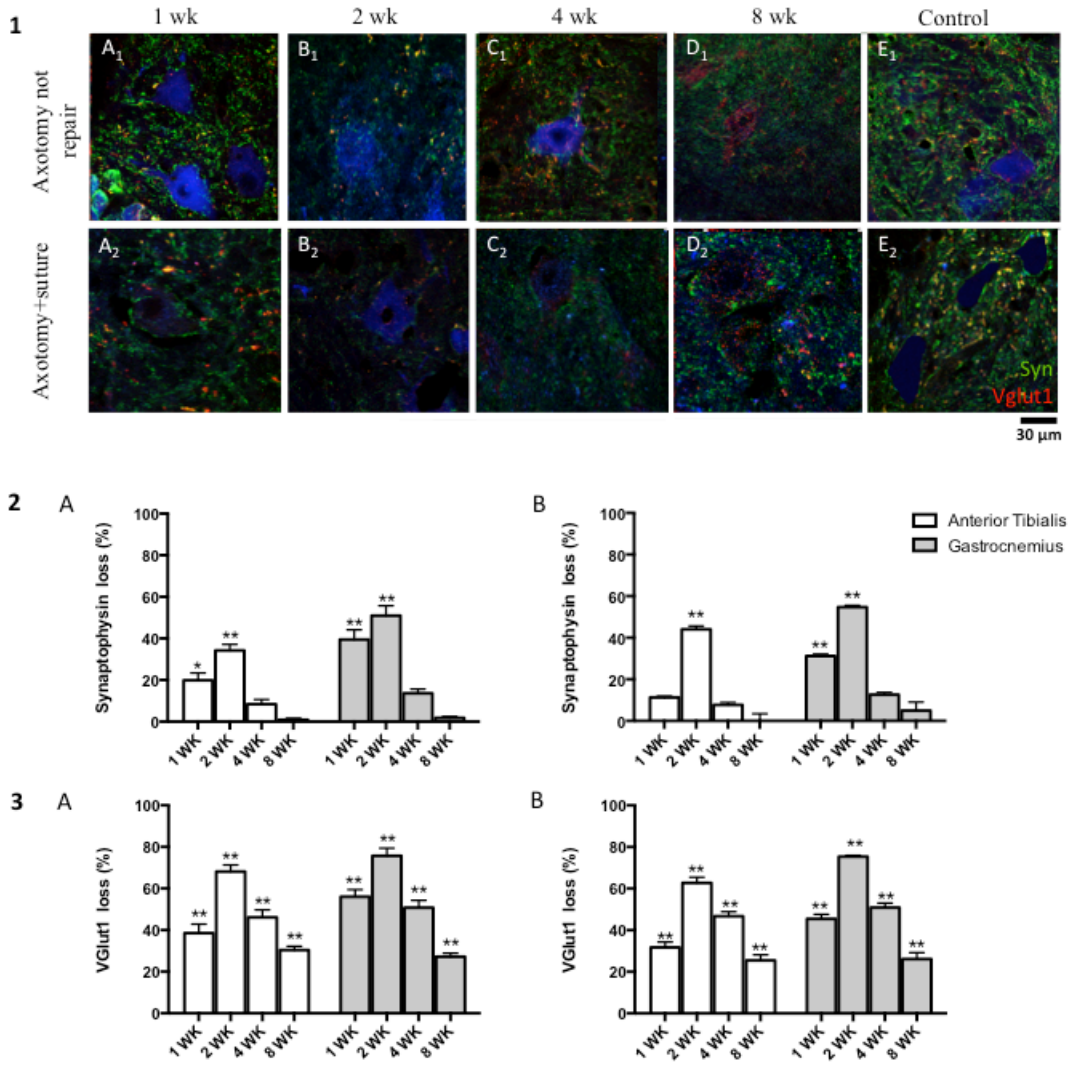

Figure 2 Evaluation of synaptic stripping after axotomy. 1. Synaptophysin (green) and Vglut1 (red) immunostaing were evaluated in confocal images of spinal cord regions containing bakclabeled motoneurons (blue) from TA and GM at 1 week (A), 2 weeks (B), 4 weeks (C) and 8 weeks (D) after nerve cut (axotomy not repaired) or nerve cut repaired with direct suture (axotomy+ suture). Control motoneurons (E_1, E_2) show strong density of all labelings. Evaluation of Synaptophysin (**2**) and Vglut1(**3**) staining at 1 week , 2 weeks, 4 weeks and 8 weeks after axotomy (A) or axotomy repaired with direct suture (B) in backlabeled motoneurons from TA (white bars) and GM (grey bars). Vglut1 and synaptophysin reach a maximum low peak of depletion 2 weeks after injury in both motor pools, that recovers with time. In contrast to synaptophysin, Vglut1 never returns to basal levels. Changes are slightly more marked in motoneurons from GM than from TA (Data are expressed as mean ± SEM, **P*<0.05, ***P*<0.01).

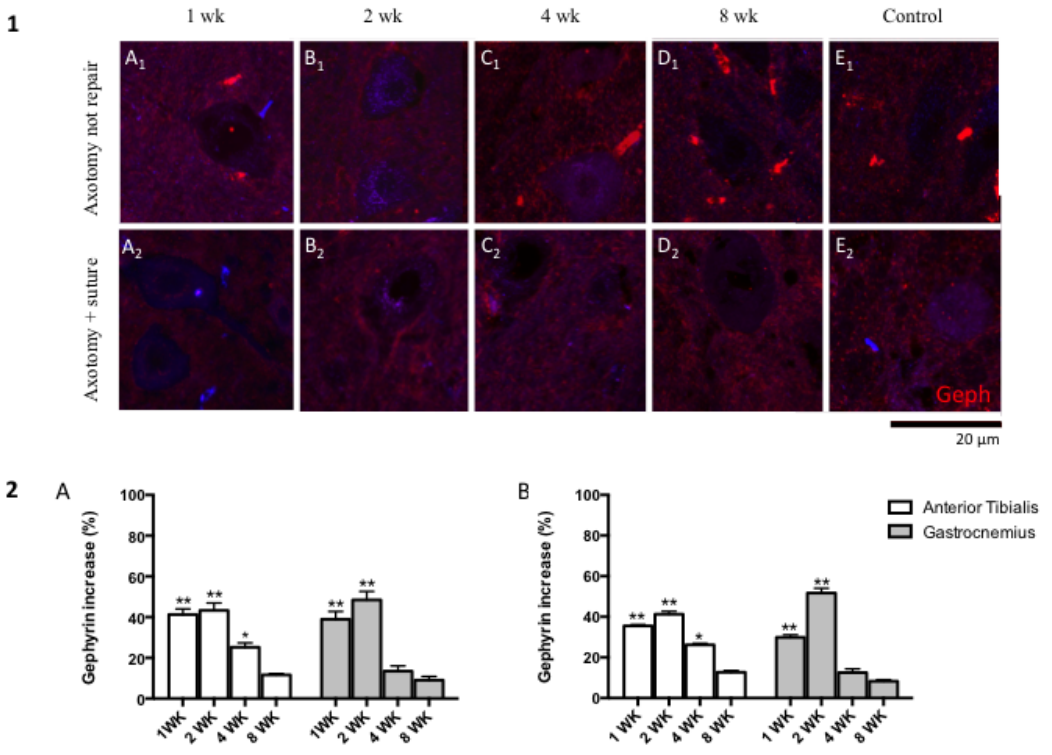

Figure 3. Gephyrin staining in axotomized motoneurons. 1. Confocal images of spinal cord regions stained against gephyrin at 1 week (A), 2 weeks (B), 4 weeks (C) and 8 weeks (D). Backlabeled motoneurons from TA and GM in blue, gephyrin in red. Control motoneurons (E_1, E_2) show less immunoreactivity to Gephyrin, whereas after axotomy, there is an increase with maximum peak at 2 weeks. **2.** Evaluation of Gephyrin staining at 1 week, 2 weeks, 4 weeks and 8 weeks after axotomy (A) or axotomy repaired with direct suture (B) in backlabeled motoneurons from TA (white bars) and GM (grey bars). Repair of the nerve does not change the evolution of synaptic stripping, with a maximum increase of gephyrin at 2 weeks. Changes are slightly more marked in motoneurons from GM than from TA (Data are expressed as mean ± SEM, **P*<0.05, ***P*<0.01).

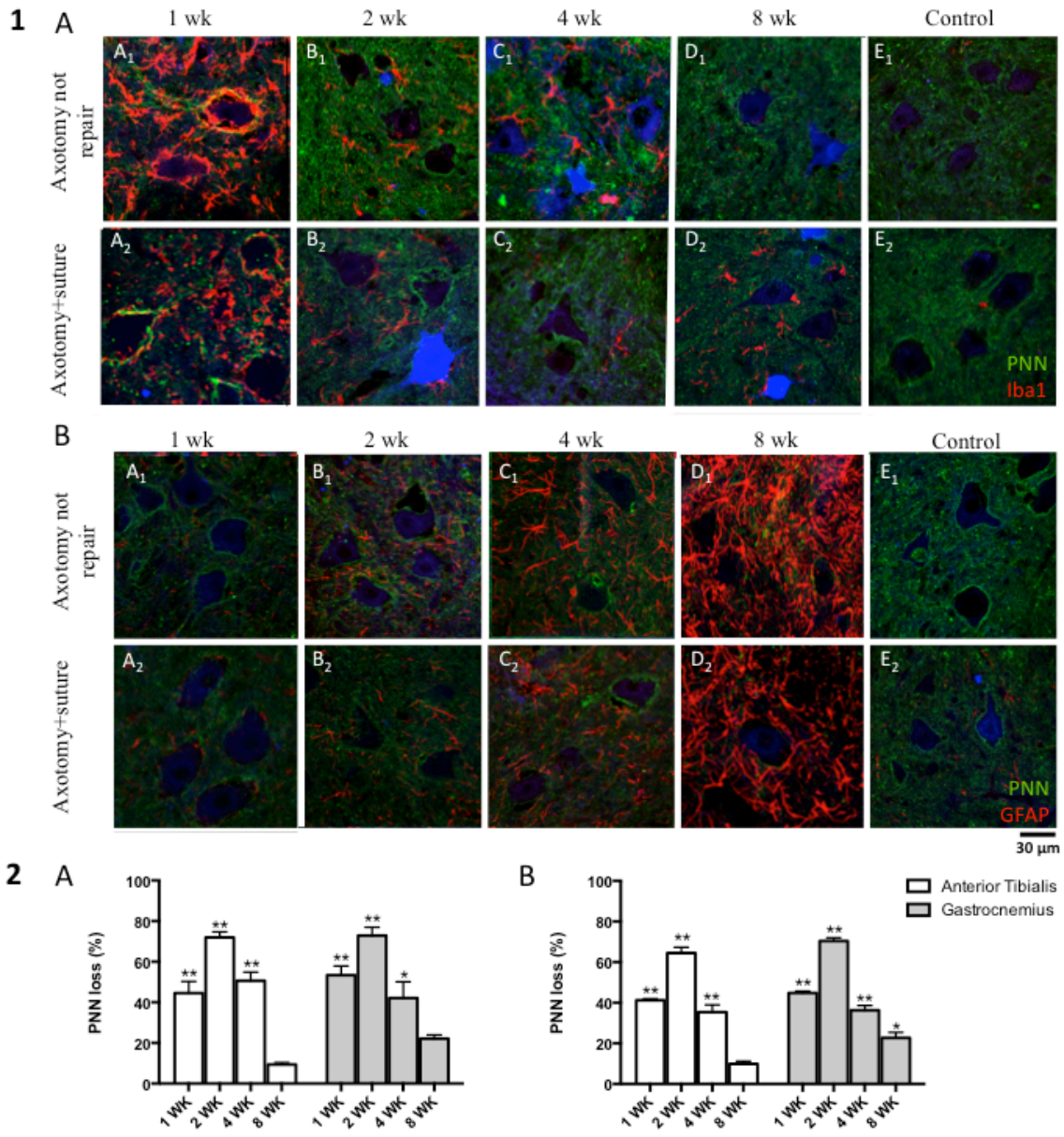

Figure 4 Glia and PNNs surrounding axotomized motoneurons. 1. Confocal images of spinal cord regions stained for microglia or astroglia and PNNs 1 week (A), 2 weeks (B), 4 weeks (C) and 8 weeks (D) after injury 1A. Backlabeled motoneurons from TA and GM in blue, , PNNs (Wisteria Floribunda) in green and microglia (Iba1, panel A) or astrocytes (GFAP, panel B) in red. Control motoneurons (E_1, E_2) have no glial processes around their soma, and a strong density of PNN surrounding them. **2.** Quantitative analysis of motoneuron PNN after axotomy. Evaluation of Wisteria Floribunda staining at 1 week, 2 weeks, 4 weeks and 8 weeks after axotomy (A) or axotomy repaired with direct suture (B) in backlabeled motoneurons from TA (white bars) and GM (grey bars). Repair of the nerve does not change the evolution of PNN desestructuration, with a maximum loss of staining at 2 weeks. There is a marked increase of microglial processes around axotomized motoneurons 1 week after injury, which decreases with time; whereas astroglia process around axotomized motoneurons increased at 8 weeks after injury, concomitant with the recovery of PNN staining. (Data are expressed as mean ± SEM, **P*<0.05, ***P*<0.01).

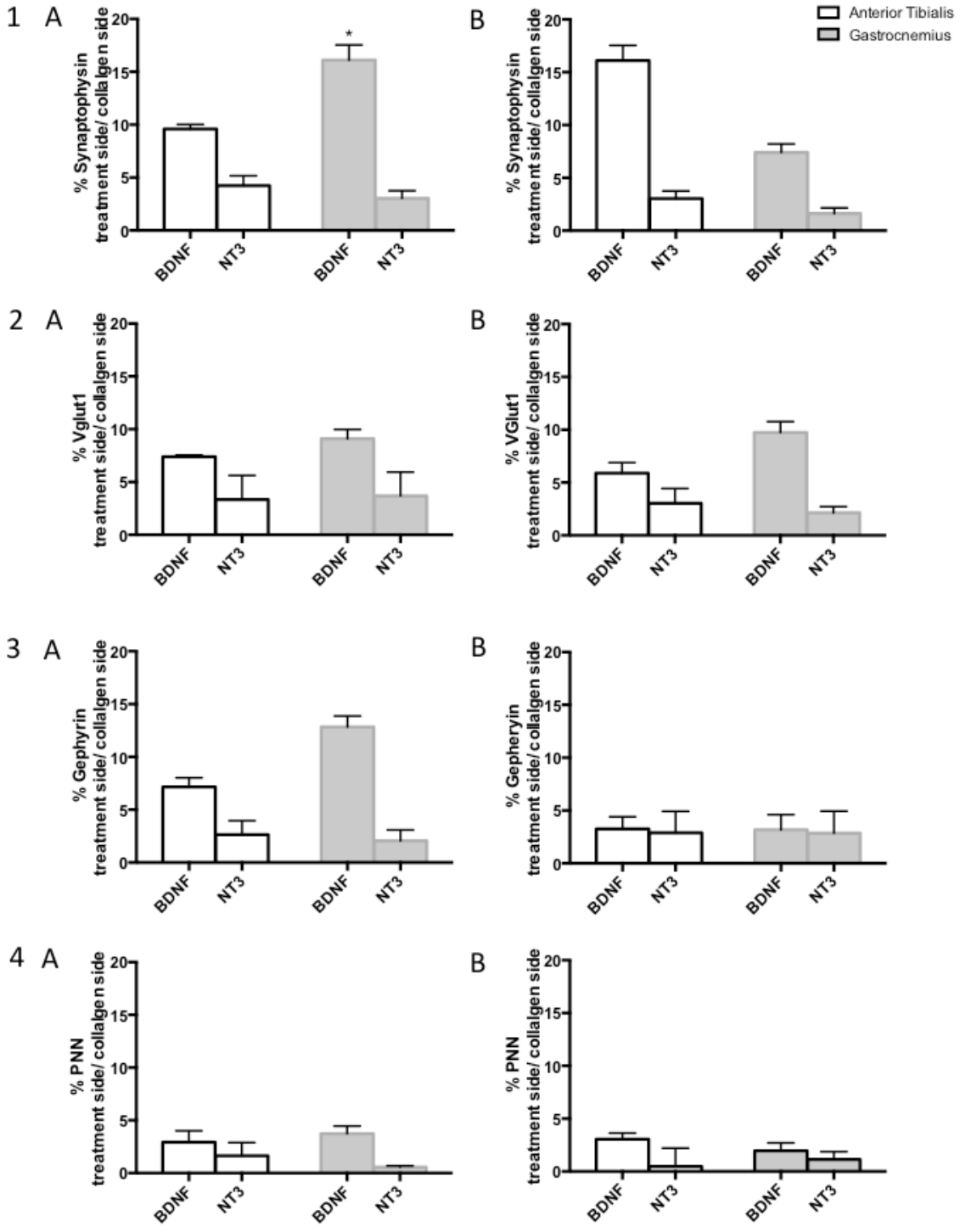

Figure 5 Quantitative analysis of synaptic stripping after axotomy and application of neurotrophic factors. Evaluation of Synaptophysin (**1**), Vglut1 (**2**), Gephyrin (**3**) and PNN (**4**) stainings 1 week (A) and 2 weeks (B) after repairing the sciatic nerve with a tube containing a collagen matrix with BDNF or NT3, expressed as percentage versus de contralateral side, that was repaired with a tube with collagen matrix. BDNF application shows a trend to reduce synaptic stripping, although it is not significant. NT3 application has no effect. (Data are expressed as mean ± SEM, **P*<0.05, ***P*<0.01).

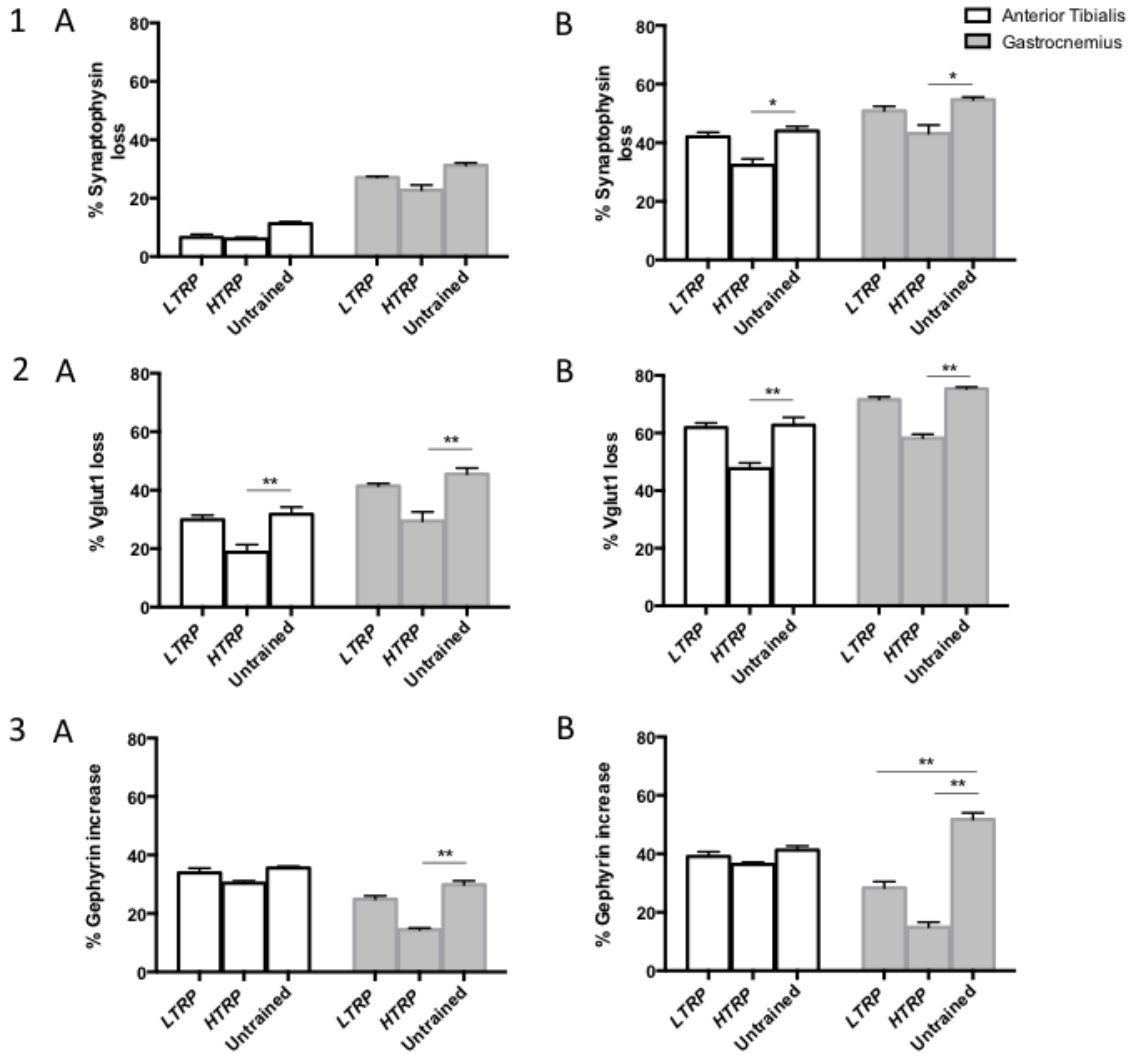

Figure 6 Quantitative analysis of synaptic stripping after axotomy in animals submitted to different protocols of treadmill. Two protocols were used, a low intensity treadmill running program (LTPR) and a high intensity program (HTRP) and compared to untrained animals. Evaluation of Synaptophysin (**1**), Vglut1 (**2**) and Gephyrin (**3**) staining at 1 week (A) and 2 weeks (B). Percentage of loss of staining is expressed versus the contralateral non-injured side (Data are expressed as mean ± SEM, **P*<0.05, ***P*<0.01). The high intensity protocol was able to reduce synaptic stripping, partially protecting Vglut1 reduction in both motoneuron pools, and synaptophysin loss 2 weeks after injury. The low intensity protocol was not able to influence synaptic stripping.

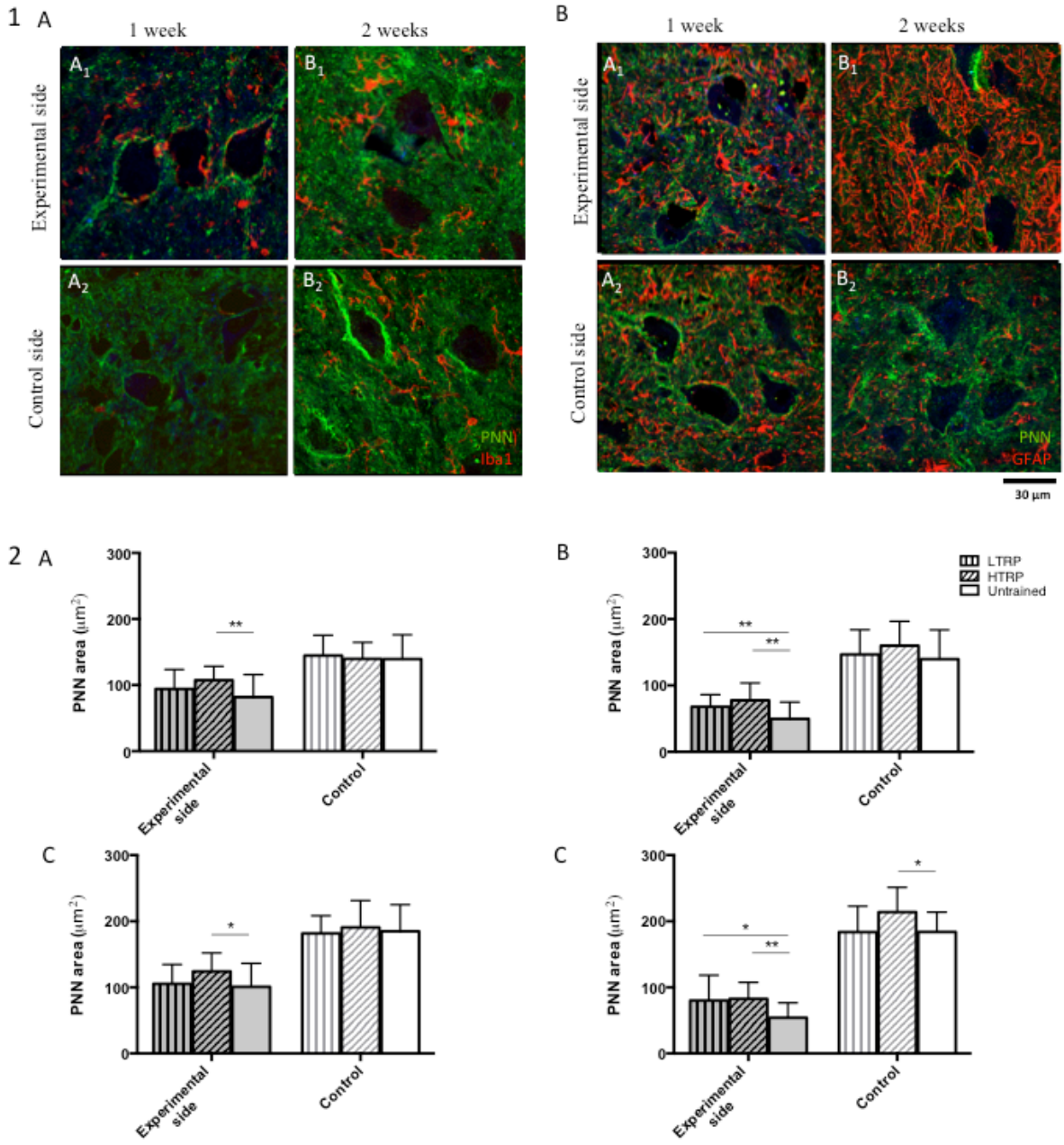

Figure 7 Glia and PNNs surrounding axotomized motoneurons on animals submitted to different treadmill protocols 1. Confocal images of spinal cord regions stained for microglia (A), astrocytes (B) and PNNs (A, B) 1 week and 2 weeks after injury in HTRP animals. Backlabeled motoneurons from TA and GM muscle in blue, microglia (Iba1) and astrocytes (GFAP) in red, PNNs (Wisteria Floribunda) in green. **2.** Evaluation of PNNs staining in animals submitted to low intensity (LTPR, vertical dashed bar), high intensity (HTRP, dashed bar) treadmill or untrained (solid bar) in TA (1) and GM (2) motoneurons axotomized for 1 week (2A, 2B) or 2 weeks (2C, 2B) of cut and suture of the sciatic nerve. Data are expressed in absolute values, since the contralateral non-injured side is also submitted to the treatment. Exercise reduced the loss of motoneuron PNN observed after injury, being the effect more marked with the more intense protocol. At 2 weeks, animals submitted to HTRP also showed an increased PNN immunostainig surrounding MN of the contralateral non-injured side. (Data are expressed as mean ± SEM, **P*<0.05, ***P*<0.01).

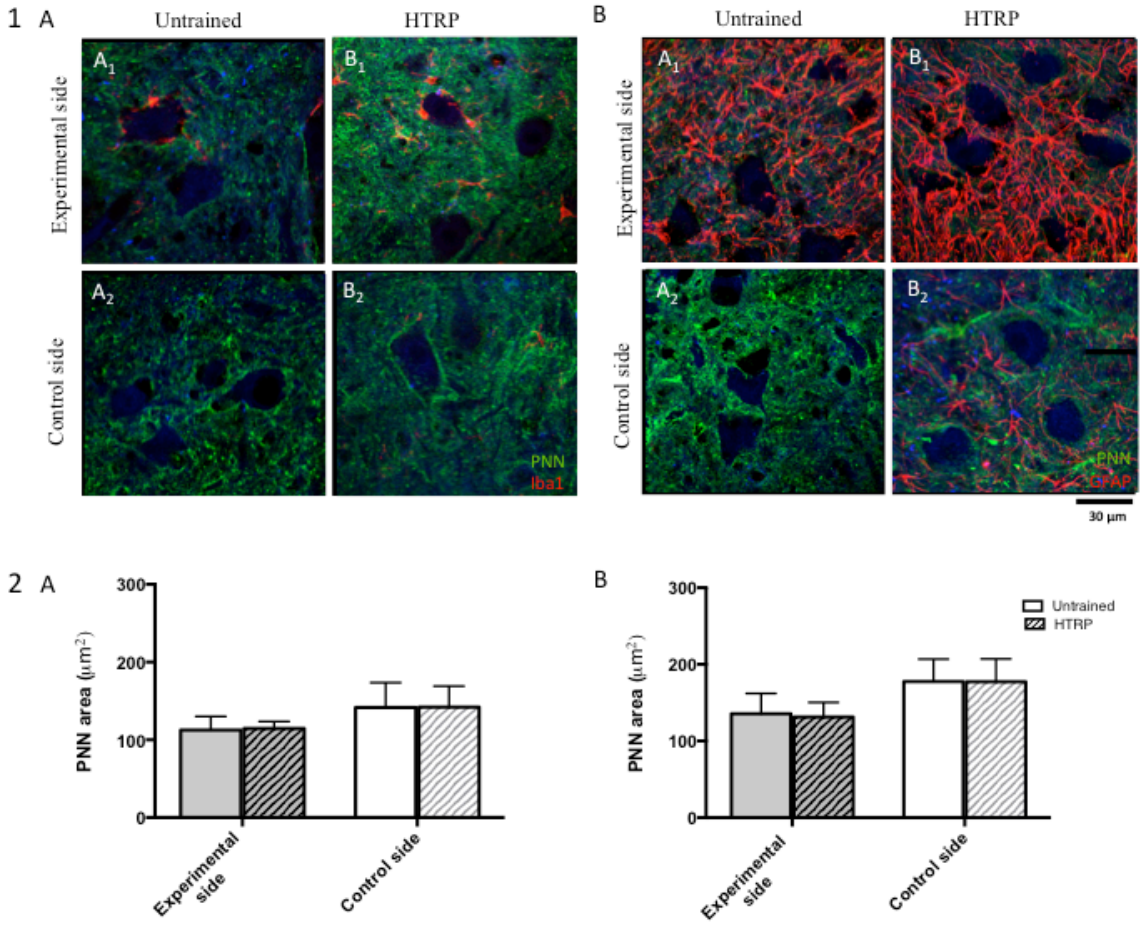

Figure 8 Glia and PNNs surrounding intact motoneurons that lost the segmentary sensory inputs of the homolateral hindlimb. 1.Confocal images of spinal cord regions stained for PNNs (Wisteria Floribunda, green) and microglia (Iba1, red, A) or astroglia (GFAP, red, B) 2 weeks postinjury in untrained animals (A) or animals submitted to HTRP. **2.** Quantitative analysis of motoneuron PNN of animals submitted to a dorsal ryzothomy (L3 to L6) to disrupt the homolateral sensory inputs of the limb. TA (2A) and GM (2B) motoneurons of untrained animals (solid bars) or animals submitted to HTRP (dashed bars) 2 weeks after surgery. Disruption of sensory inputs reduced PNN around intact motoneurons, and exercise was not able to revert that loss. In the control no injured side of trained animals there was reduction in microglia reactivity, a mild increase of astroglia and PNN were similar to controls. (Data are expressed as mean ± SEM, **P*<0.05, ***P*<0.01).