Universitat Autònoma de Barcelona

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INc Institut de Neurociències

BCFI

... i via fora!
Que tot està per fer i tot és possible.
Miquel Martí i Pol

Als meus pares

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After a peripheral nerve injury, axons are able to regenerate but functional recovery is usually limited, mainly due to unspecific reinnervation of target organs and also to maladaptive plastic changes in the spinal circuitry. In this thesis we wanted to modulate the stretch reflex arc to improve functional recovery after peripheral nerve lesions in animal model.

Firstly, we carried out an immunohistochemical characterization of the changes surrounding spinal motoneurons after sciatic nerve injury; these changes were studied in postnatal and adult animals. In postnatal animals, we also studied VGlut1 contacts along dendrites, observing a high loss of excitatory synapses that were not recovered at 2 months. Regarding adult motoneurons, we found that the maximum loss of glutamatergic synapses and perineuronal nets (PNN) took place two weeks after injury, with a progressive recovery at 4 weeks.

To try to ameliorate this loss of synapses and PNN, we studied the effect of electrical stimulation and different trophic factors (applied directly to the injury with a collagen matrix in a silicone tube). No significant differences were observed in none of them. We also evaluated different exercise protocols, specifically forced, voluntary and passive exercise. A high intensity protocol was able to partially prevent the synaptic and PNN loss that suffer axotomized motoneurons, whereas low intensity programs did not show significant differences compared to untrained ones. We also observed an increase of astrogliosis surrounding axotomized motoneurons and a decrease of microglia activation in exercised animals, except for those receiving low intensity voluntary and passive exercise, where there was a significant increase of microglia.

Due to the positive effects induced by physical exercise on central plastic changes, we evaluate potential mechanisms involved in these effects. Although it is known that exercise increase neurotrophins, it is unknown how exercise modulates these neurotrophins and their specific actions. To evaluate the role of BDNF in the effects of exercise on axotomized motoneurons, we systemically administered a TrkB agonist and antagonist. We observed that the maintenance of synapses mediated by

exercise was partially dependent of TrkB activation, but pharmacological activation of this receptor did not mimic exercise effects.

As after physical exercise there was an increase of neural activity, we studied the role of noradrenergic descending projections from brainstem in spinal cord motoneurons after exercise. These descending pathways modulate excitability of the spinal motoneurons and are activated by stress situations, such as forced exercise. By DSP-4 administration, we provoked the desestructuration of the Locus Coeruleus and thus, loss of noradrenergic descending projections, observing a reduction of PNN and a marked reactivity of microglia. In animals submitted to exercise, the loss of these projections prevented the preservation of synapses and PNN around injured motoneurons, although microglial reactivity was also decreased. These findings suggest that modulation of spinal changes induced by physical exercise would be partially dependent on the activation of noradrenergic projections, whereas the modulation of microglia is independent of the exercise.

Després d'una lesió del sistema nerviós perifèric, aquest té la capacitat de regenerar però la seva recuperació funcional sol ser limitada, principalment degut a la inespecificitat de la reinnervació dels òrgans perifèrics i als canvis plàstics maladaptatius de la circuiteria espinal. En aquesta tesi hem volgut modular els canvis a la circuiteria espinal del reflex d'estirament per poder millorar la recuperació funcional després de lesions del nervi perifèric en un model animal.

En primer lloc, es va dur a terme una caracterització immunohistoquímica dels canvis que patien les motoneurones espinals després d'una lesió del nervi ciàtic; aquests canvis es van estudiar en animal postnatal i en l'adult. En els animals postnatals, també es va estudiar la composició sinàptica de l'arbre dendrític de les neurones, i es va observar una elevada pèrdua de sinapsis excitatòries que no s'arriben a recuperar després de 2 mesos. Pel que fa als canvis produïts en l'adult, vam observar que la màxima pèrdua de sinapsis glutamatèrgiques i de xarxes perineurals (PNN) es produïa dues setmanes després de la lesió, amb una progressiva recuperació a partir de la quarta setmana.

Per intentar atenuar aquesta pèrdua sinàptica i de PNN, vam estudiar l'efecte de l'estimulació elèctrica i de la capacitat de diferents factors tròfics (aplicats a la zona de lesió mitjançant una matriu de col·lagen en un tub de silicona). No es van obtenir diferències significatives en cap de les dues teràpies. També vam avaluar diferents protocols d'exercici físic, concretament l'exercici forçat, voluntari i passiu. Si es feia servir un protocol d'alta intensitat, la pèrdua sinàptica i de PNN que patien les motoneurones era menor que la pèrdua observada en les motoneurones d'animals que no havien estat sotmesos a exercici, mentre que en aquells que corrien menys no presentaven millores respecte als animals no exercitats A més, en els animals exercitats també vam observar un augment de l'astrogliosis al voltant de les motoneurones axotomitzades i una disminució de l'activació de la microglia, excepte en l'exercici voluntari i passiu a baixa intensitat, on vam observar un augment de l'activació de microglia.

Degut als efectes positius induïts per l'exercici físic sobre els canvis plàstics en el nostre model, vam valorar potencials mecanismes implicats en aquestes efectes.

Tot i que se sap que l'exercici incrementa l'expressió de neurotrofines, es desconeix com l'exercici modula aquestes neurotrofines i quines són les seves accions específiques. Per valorar el paper del BDNF en els efectes de l'exercici observats en el nostre model, vam administrar sistèmicament un agonista i un antagonista del TrkB. Vam observar que el manteniment de les sinapsis mediat per l'exercici físic depenia parcialment de l'activació del TrkB, però l'activació farmacològica d'aquest receptor no mimetitzava els efectes de l'exercici.

Com que l'exercici físic provoca un augment de l'activitat neural, també vam valorar el paper de les projeccions descendents noradrenèrgiques del tronc de l'encèfal en els efectes moduladors de l'exercici sobre les motoneurones. Aquestes vies descendents modulen l'excitabilitat de les motoneurones espinals i s'activen en situació d'estrès, com l'exercici forçat. Mitjançant l'administració de DSP-4, que destrueix el locus coeruleus i per tan, provoca la pèrdua de les projeccions noradrenergiques descendents, vam veure una reducció de PNN i una marcada reactivitat de la microglia. En els animals sotmesos a exercici, la pèrdua d'aquestes projeccions impedia la preservació de sinapsis i de PNN al voltant de les motoneurones lesionades, si bé la reactivitat microglial es veia igualment disminuïda. Aquestes troballes suggereixen que la modulació dels canvis espinals induïts per l'exercici físic seria parcialment dependent de l'activació d'aquestes projeccions noradrenèrgiques, mentre que la modulació de la micròglia per l'exercici en seria independent.

Most common abbreviation in the manuscript:

BDNF	Brain-derived neurotrophic factor
DSP-4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
ES	Electrical stimulation
EMG	Electromyography
GM	Gastrocnemius medialis mucle
HBP	HIgh intensity bicycle program
HTRP	High intensity treadmill running protocol
LBP	Low intensity bicycle program
LC	Locus coeruleus
LTRP	Low intensity treadmill running protocol
LW	Long distance wheel runners
MN	Motoneuron
MW	Medium distance wheel runners
NA	Noradrenaline
TA	Tibial anterior muscle
TR	Treadmill running
TrK	Tropomyosin-receptor kinase
PNI	Peripheral nerve injury
PNN	Perineuronal nets
SC	Spinal cord
SW	Short distance wheel runners
VGat	Vesicular GABA transporter
VGlut	Vesicular glutamate transporter
WFA	Wisteria floribunda agglutinin

Published manuscripts from the work of this thesis:

Peer reviewed publications

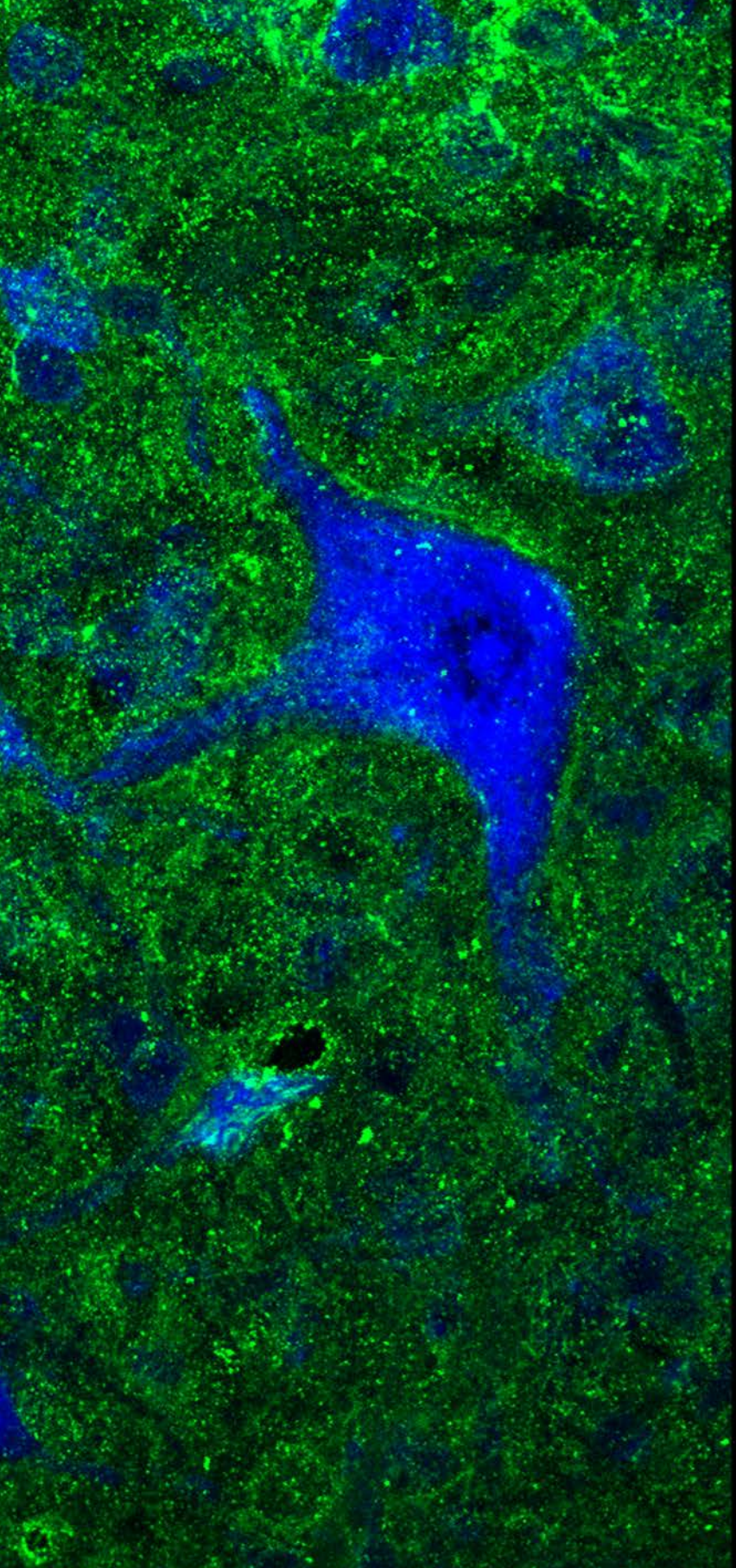
1. Arbat-Plana A, Torres-Espín A, Navarro X, Udina E. 2015. **Activity dependent therapies modulate the spinal changes that motoneurons suffer after a peripheral nerve injury.** Experimental neurology 263, 293-305.

Under revision

2. Arbat-Plana A, Cobianchi S, Herrando-Grabulosa M, Navarro X, Udina E. **Endogenous modulation of TrkB signaling by treadmill exercise after peripheral nerve injury.**
3. Arbat-Plana A, Navarro X, Udina E. **Effects of physical exercise and noradrenergic descending inputs on changes in spinal motoneurons after peripheral nerve injur**y.

In preparation:

4. **Effects of passive, active and voluntary exercise in the spinal circuitry after a peripheral nerve injury.**
5. **Characterization of the dendritic arbor and the spinal changes that postnatal motoneuron suffer after peripheral nerve injury.** *Study in collaboration with Dr. Alvarez (Alvarez group), partially realized in Emory University School of Medicine, Atlanta (EUA) during a 4 month stage.*



Introduction

PERIPHERAL NERVOUS SYSTEM

The peripheral nervous system (PNS) is the part of the nervous system external to the vertebrae and the cranium where the central nervous system (CNS), brain and spinal cord (SC) is contained. It includes the cranial nerves, spinal nerves and their roots and branches, peripheral nerves, and neuromuscular junctions. In the PNS, bundles of nerve fibers or axons conduct information to and from the CNS.

In the CNS, a bundle of axons is called a tract. In contrast, nerves are bundles of axons in the PNS. Nerves have connective tissues invested in their structure, as well as blood vessels supplying the tissue. The epineurium is the outer surface of the nerve and is formed by layer of fibrous connective tissue; within the nerve, axons are further bundled into fascicles, which are each surrounded by their own layer of fibrous connective tissue named perineurium. Finally, the endoneurium is a connective tissue that is enveloping individual axons.

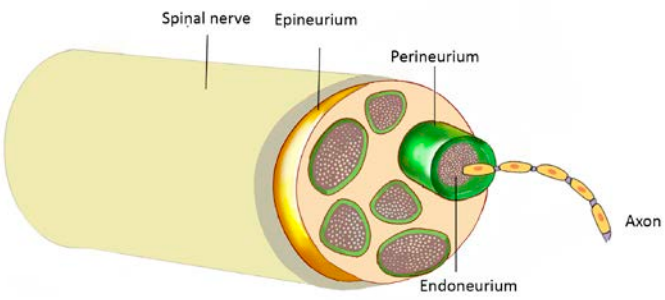


Fig 1. **Peripheral nerve.** Normal architecture of a peripheral nerve with the different layers of connective tissue.

Peripheral nerve fibers are grouped based on their myelination, their diameter and their propagation speeds (Erlanger and Gasser, 1930). A-fibers are myelinated, have large diameter, high conduction velocity and can be subdivided in 4 different groups based on the carried information: A-α, A-β, A-γ and A-δ fibers. A-α fibers include the motoneurons that innervate skeletal muscles (α-MN) and the fastest sensory neurons, related with proprioceptive sensibility: some sensory neurons from the muscle spindle (also classified as Ia type fibers) and Golgi tendon organ fibers (also classified as type Ib fibers). A- β fibers carry information from specialized cutaneous

mechanoreceptors and also from secondary muscle spindles terminals (also called type II fibers). A-γ fibers are fibers from MNs that innervate intrafusal fibers and modulate the activation of the muscle spindle. A- δ fibers are free nerve endings that conduct painful stimuli related to mechanical and thermal stimulus. On the other hand, B group fibers are myelinated, have a small diameter, with low conduction velocity and transmit autonomic information. Finally, C group fibers are unmyelinated, are the smallest ones and have the lowest conduction velocity. These transmit autonomic information and also mechanical, thermal and chemical painful sensibility, as well as protopatic touch and itch.

Classically, sensory information innervating muscular branches is classified with roman numbers, being type I equivalent to Aa, type II to Ab, type III to Ad and type IV to Ad. This is the reason that the fibers innervating muscle spindles are usually called type Ia and type II afferents (Hunt, 1990). The Ia fibers form the primary sensory nerve endings (Banks, 1986) and are the faster ones, whereas secondary sensory nerve endings (Banks et al., 1982) are slower type II afferents (30-70 m/s conduction velocity).

The spinal cord and its relation with the PNS

A SC transverse section shows white matter in the periphery and gray matter in the medial part. White matter is composed by axonal fibers travelling along the spinal cord, whereas the gray matter is mainly occupied by neuron cell bodies and forms 3 pairs of horns throughout most of the SC: the dorsal horns, with a component mainly sensorial, the lateral horns, well defined in thoracic segments and containing visceral neurons, and the ventral horns, where MN are localized.

The gray matter of the SC can be histologically organized in several regions called laminae. The dorsal horn of the SC has been subdivided into laminae I through to VI, with laminae VII to X comprising the ventral horn (Rexed, 1952).

The somas of MNs are situated in the ventral horn of the SC, and through the ventral roots, their axons emerge to the PNS. By means of descending motor tracts, the motor cortex and some motor nucleus into the brain stem send projections to

these motoneurons, directly or indirectly through interneurons, to modulate their actions and, eventually, their effectors the skeletal muscles. On the other hand, primary sensory neurons from the DRG are pseudo-bipolar, with an axon that through the dorsal root emerges to the PNS and an axon that enters the spinal cord. to project to superior centers or to relay in different laminae of the dorsal horn.

The laminae in the dorsal horn comprises laminae I to IV, and are mainly related with exteroceptive sensation, being superficial lamina I and II more related with nociception, whereas laminae V and VI are concerned primarily with proprioceptive sensations; in this laminaes MNs dendrites could also reach dorsally. Laminea VII is the intermediate zone and acts as a relay between proprioceptive neurons to midbrain and cerebellum. The ventral horn includes laminae VIII to IX, that are predominantly comprised of MNs and occupy the ventral horn. The axons of these MNs innervate mainly skeletal muscles and control the muscle movements. The central branches of proprioceptors project to the SC and form synaptic connections with interneurons in the intermediate zone of the SC (group Ia, Ib, and II afferents) or directly with MNs in the ventral horn (group Ia and group II afferents) (Brown, 1981). These connections are the basis of different spinal circuits, being the stretch reflex arc the simplest one. The stretch reflex circuit consist in the direct synapses between α -MNs and Ia afferents from the muscle spindle of the same muscle, providing the motor pool with monosynaptic proprioceptive feedback from the muscle their innervate.

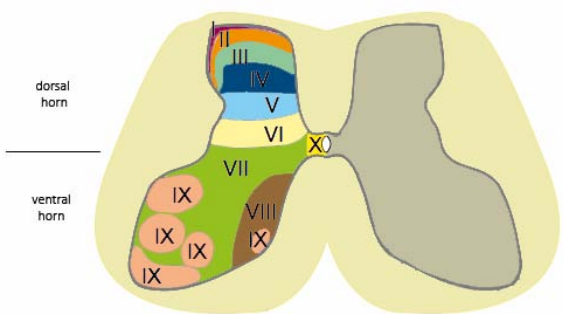


Fig 2. **Schematic representation of spinal cord laminae organization.**

Spinal reflexes are involuntary and stereotyped movements based on sensory inputs that are computed by the gray matter of the SC. As stated before, one of the most well-known reflexes is the stretch reflex, a monosynaptic segmentary reflex that evokes a muscle contraction in response to the stretch of the muscle. The Golgi tendon reflex is a synaptic segmentary reflex that operates as a feedback mechanism to control muscle tension by causing muscle relaxation before muscle force becomes so great that tendons might be torn. There are also more complex reflexes, polysynaptic, that include different spinal segments, like the withdrawal reflexes, intended to protect the body from damaging stimuli. In front of a painful stimulus in one extremity, there is a flexion of the muscles of the affected limb, combined with an extension of the contralateral limb. When the damaging stimulus is intense, this response can be accompanied with a crossed response in the other two limbs.

Being the stretch reflex the simplest one of the spinal circuits and implying just a MN and a proprioceptive neuron that innervates the same muscle, this reflex can be a useful tool evaluate the effects of peripheral nerve injuries on the spinal cord, and to provide valuable insight about the reorganization of the spinal circuits after injury.

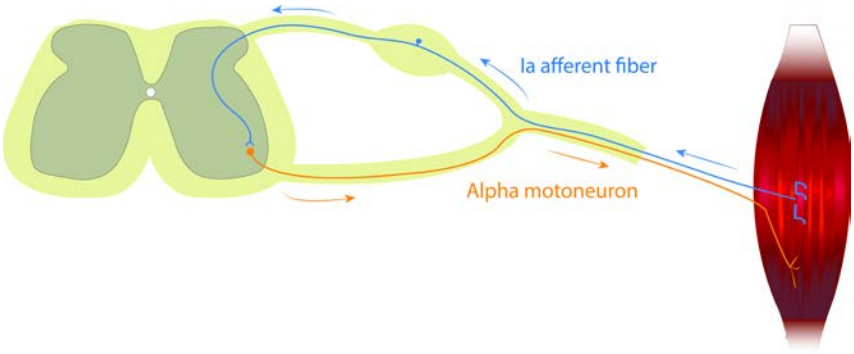


Fig 3. **Monosynaptic stretch reflex**. Muscle stretch is sensed by muscle spindles. This reflex is a monosynaptic circuit that provides automatic regulation of skeletal muscle length. The stretch reflex arc is formed by the primary Ia afferent of the muscle spindle organ that synapses directly to a α-MN of the same muscle at the ventral horn of SC. Thus, when the muscle spindle organ is activated (either by stretch of the muscle or by contraction of the intrafusal fibers), the sensory afferent delivers glutamate to the central synapses. A sufficient depolarization on the MNs causes a reflex contraction in the muscle.

Hoffman reflex

The electrical analog of the stretch reflex is the Hoffmann reflex or H-reflex. Hoffmann demonstrated that when an electrical stimulation is applied on a mixed nerve to evoke short latency responses of the muscle, two responses at different latencies can be observed (Hoffman, 1918). This double response is due to the capability of the nerve impulses to travel in both directions when an axon is electrically stimulated. The first response, which is called M-wave, is a direct motor response from orthodromic stimulation of the motor axons, and the second response, known as H-wave, (Magladery and McDougal, 1950) is induced by the antidromic activation of Ia afferents, that directly synapses with MN in the SC. Depolarization of these MNs at the soma level would evoke a second response in the muscle, of longer latency and lower amplitude than the M-wave (Fig 4).

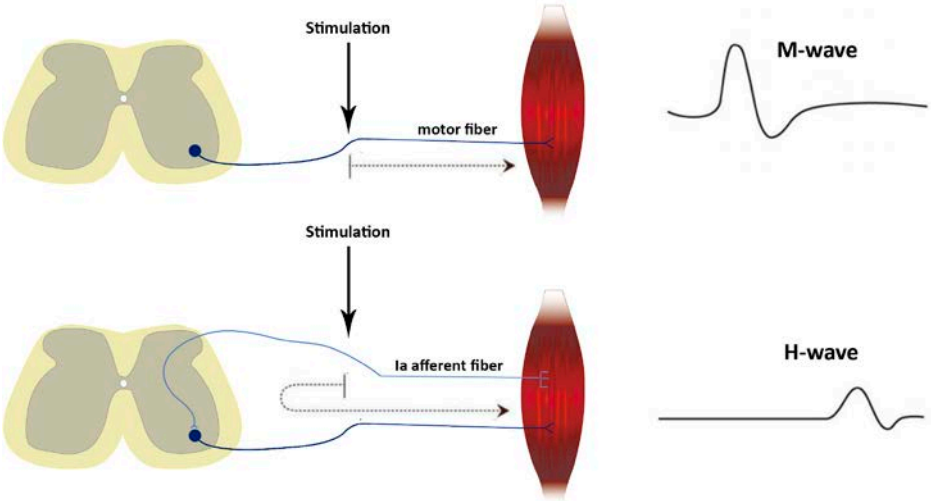


Fig 4. Representative recording of evoked M and H waves. The M-wave is evoked by excitement of efferent, motor, axons whereas the H-wave, is due to activation of the stretch reflex by excitement of Ia afferent that monosynapse with motoneurons.

The characteristics of both waves have been described in detail previously (Pierrot-Deseilligny and Burke, 2005; Schieppati, 1987). As the stimulus intensity is increased through repeated stimulations, the M-wave amplitude increases until a maximum is reached (MMax), while the H-wave amplitude increases to a maximum

(HMax) and then decreases. When the stimulus intensity is low, only a few motor axons and Ia afferents are stimulated. At intermediate intensities, most Ia afferents are stimulated whereas still not all motor axons are recruited, and thus the H-reflex response peaks.

After electrical stimulation, it is also important to differentiate the H-reflex from other waves, like the F-wave. The F-wave results from backfiring of MNs due to antidromic conductions of action potentials (Kimura, 2001).

It is extensively accepted that the H-reflex is monosynaptic, however it is possible that oligosynaptic inputs as well as other afferent input could influence the H-reflex. Ia afferents are able to excite MNs directly, but their input can also be inhibitory via interneurons (Hultborn et al., 1975). Since there are relatively short distances and close conduction velocities, it is possible that non-Ia fibers influence the H-reflex (Schieppati, 1987). However, since the rise time of the H-reflex excitatory post-synaptic potential last only a few milliseconds, the effects of postsynaptic temporal summation from these other inputs is probably limited. On the other hand, Electrical nerve stimulation activates the Ia afferents almost simultaneously and therefore elicits a volley that is only little dispersed in time on its arrival at the spinal cord (Burke et al., 1983). Thus, H-reflex is due to monosynaptic connections from Ia afferents and is minimally influenced by other synaptic inputs.

The H-reflex amplitude gives us a value of the excitability of MNs, and consequently can change depending of task or time as a function of segmental and supraspinal influences (Schieppati, 1987). In addition, it is not a simple reflection of the excitability of MNs, but also a measure of synaptic efficacy dependent on presynaptic inhibition and neurotransmitter levels (Capaday and Stein, 1987). Thus the H-reflex amplitude depends on different factors and consequently, it can be quite variable.

Perineuronal nets

Perineuronal nets (PNNs) are specialized compounds of extracellular matrix (ECM), and in the spinal cord, encapsulate about 70–80% of spinal neurons, particularly projection neurons and MNs (Galtrey and Fawcett, 2007). These PNN are localized around the soma and proximal dendrites of MNs in the ventral horn of the SC (Takahashi-Iwanaga et al., 1998) and also around inhibitory GABAergic neurons, specifically those interneurons that express the calcium binding protein parvalbumin (Dityatev et al., 2010). During CNS development the formation of PNNs parallels synaptic maturation and the closure of critical periods (Carulli et al., 2013), such as proliferation, migration, synaptogenesis, synaptic stability and cell signaling (Dityatev and Schachner, 2003; McRae and Porter, 2012). In the mature ECM these molecules can either be diffusely dispersed or can form tight net-like structures (Deepa et al., 2006). The net like structures surround cell bodies, dendrites and axon initial segments leaving open "holes" at sites of synaptic contact (Celio and Blümcke, 1994; Wang and Fawcett, 2012; Zaremba et al., 1989). In the adult CNS, their repellent properties against approaching axons/dendrites and their highly negatively charged content support the role of these nets in synaptic stabilization (Geissler et al., 2013; Karetko and Skangiel-kramska, 2009; Kwok et al., 2011). Thus, PNNs plays a key role in spontaneous anatomical and functional reorganization of spinal circuits at multiple levels, and its involvement in neuronal plasticity has been extensively studied over recent years, but mainly in the brain cortex and the cerebellum.

PNNs are mainly comprised of hyaluronan, link proteins, tenacin-R and proteoglycans (Deepa et al., 2006; Wang and Fawcett, 2012). Hyaluronan synthase anchors PNNs to the cell membrane and is responsible for synthesizing and secreting hyaluronan to the pericellular environment. The N-terminal of proteoglycans binds to the hyaluronan polymer chains and link protein appear to stabilize these interactions (McRae and Porter, 2012). The C-terminal of proteoglycans in turn binds to tenacin-R allowing for cross-linking and the formation of large aggregates on cell membrane surfaces (Wang and Fawcett, 2012). Proteoglycans contain a core protein with covalently linked glycosaminoglycan side chains of varying number and length. These

chains can be made of heparin, keratin or chondroitin sulfate (Karetko and Skangiel-kramska, 2009). Chondroitin sulfate proteoglycans (CSPGs) are also known as lecticans and are the most abundant protein of the ECM in the CNS. The family of lecticans includes: aggrecan, versican, neurocan and brevican, differentiated mainly by their number of glycosaminoglycan side chains (Yamaguchi, 2000). Aggrecan appears to be an important lectican because it is present in almost all PNNs, where other lecticans are only found in subpopulations of PNNs (Galtrey et al., 2008; Wang and Fawcett, 2012).

The exact function of PNNs in the adult CNS has yet to be determined. Different conditions that cause functional plasticity, such as physical exercise or exposure to enriched environment, are accompanied by a reduction of PNNs in brain and cerebellum (Carulli et al., 2013). PNNs temporal formation corresponds to the end of the critical period, when CNS is particularly plastic and when a specific repertoire of synaptic connections is selected. Lack of essential experiences during early development, such as dark rearing, has been shown to cause inappropriate synaptic stabilization and a reduced the number of PNNs in the primary visual and somatosensory cortex respectively (Guimarães et al., 1990; McRae et al., 2007; Pizzorusso et al., 2002). Therefore, increasing synaptic inputs could reduce PNN content, enhancing plastic abilities of cerebellar neurons (Nithianantharajah and Hannan, 2006) and visual and somatosensory cerebral circuits (Corvetti and Rossi, 2005; Foscarin et al., 2011; McRae et al., 2007). In addition, different studies showed that the degradation of PNNs could reactivate synaptic plasticity (Corvetti and Rossi, 2005; Gogolla et al., 2009; Pizzorusso et al., 2002; Wang and Fawcett, 2012) giving further evidence for the role of PNNs in synapse stabilization and plasticity prevention.

Therefore, in the mature CNS, presence of PNN limits plasticity and stabilizes circuits. However, an insult can affect these PNN and therefore, facilitate the increased plasticity that the CNS shows after injuries.

PERIPHERAL NERVE INJURY

In contrast to central axons, peripheral axons have the capacity to regenerate after an injury (Fu and Gordon, 1997). Therefore, these are able to reconnect with their target organs and eventually the lost functions induced by the injury can be reversed.

However, functional recovery is usually limited, even when regeneration of injured axons can be quite successful. Firstly, an incorrect reinnervation of targets organs causes a misdirection of sensory and motor signals between the PNS and CNS (Maki, 2002) and markedly impairs functionality. Furthermore, maladaptive plastic changes in the SC and the uncomplete restitution of the spinal circuitry after PNI can also interfere with the recovery (Navarro et al., 2007).

Sensory and motor axons have to regenerate and reconnect with their target organ after PNI. A large number of factors can affect regeneration (Brushart, 2011; Navarro et al., 2007), like the type of lesion, the location of trauma, differences in species, type of injured neurons and age of the animals (Koliatsos and Price, 1996). For example, in newborn animals, avulsion or crush of the sciatic nerve produces a 73-80% loss of lumbar MNs (Koliatsos and Price, 1996), whereas axotomy of the same nerve in adult animals generates no visible loss of MNs (Schmalbruch, 1984).

The type of nerve affected is also important for the functional outcome: smaller and distal nerves have better functional recovery than larger mixed nerves (Sulaiman and Gordon, 2013). The distance over which the axons must grow and the moment at which surgical repair is done after injury are also factors to be taken into account. On the other hand, the type of lesion and the surgical repair used is decisive. After axonotmesis, such as a crush, there is a disruption of the axons but endoneurium, perinuerium, and epineurium remain intact; in that case, surgical repair is not needed and the endoneurial tubes will guide the axons towards the right target organ, favoring functional recovery. In contrast, after a neurotmesis, such as a complete nerve transection, epineurial, perineurial and endoneurial sheaths are disrupted, and there is a discontinuity of the two nerve stumps. In that situations, surgical repair is mandatory either with direct suture or interposing a nerve graft, so

injured axons will be able to extend along the distal degenerating nerve; however, functional recovery will be quite poor due to misrouting of axons to erroneous distal endoneurial tubes.

After injury, a complex molecular response is activated in the neuron. The biochemical changes develop within hours after axotomy and induce a decreased synthesis of products for neurotransmission, increased synthesis of growth associated proteins and components of the membrane and also changes in transcription factors gene expression, thus neurons switch from a neurotransmitter to a pro-regenerative state (Leah et al., 1991). Signaling through the axotomy-activated kinases lead to up-regulation or activation of several transcription factors, such as c-Jun, CREB, STAT-3, Akt and Nuclear Factor kB. This up-regulation leads to changes in gene expression in a large number of genes, many of which involves changes in transcription factors, cytoskeletal proteins, cell adhesion and guidance molecules, trophic factors and receptors, cytokines, neuropeptides and neurotransmitter synthesizing enzymes, ion channels and membrane transporters (Navarro et al., 2007).

There are also morphological changes in the nucleus of injured neurons; there are signs of chromatolysis, swelling of the cell body, nucleolar enlargement, displacement of the nucleus to the periphery and Nissl bodies dissolution (Kreutzberg, 1995). Chromatolysis is a change in the neuron metabolism aimed to increase their regenerative potential but may also be a sign of severe trauma with loss of a large cytoplasm within of the axon (axoplasm) volume. All of these actions could result in both neuronal survival and regeneration or neuronal death (Lundborg, 2000). The main determinant of the extent of neuronal death after axotomy seems to be the loss of target-derived neurotrophic factors (Terenghi, 1999). Evidently, survival of neurons is of great importance for the final outcome (Fu and Gordon, 1997).

On the other hand, the distal stump undergoes Wallerian degeneration. In this process, Schwann cells switch from a differentiate to a proliferating pro-regenerative state. Schwann cells together with resident macrophages or monocytes from the vascular network are responsible for the phagocytosis of myelin and axons debris. Schwann cells will line up in columns called bands of Bungner to provide a

guide for regenerating axons, and will secrete growth factors such as fibroblast growth factor (FGF), nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF) (Mirsky and Jessen, 1999) to create a permissive environment for regeneration.

From functional point of view, the goal of regeneration is to replace the distal nerve segment lost after injury, to reinnervate the target organs and to restitute their function. However, as stated above, the type of injury, the nerve affected and the level of the lesion will markedly affect the final functional outcome. The disparities in observed regeneration rates are also largely dependent on the animal model and methods for measuring regeneration. Moreover, the rate of regeneration can depend on the duration of denervation, survival of central neurons, the degree of vascularization and the condition of the peripheral tissues (Carlson, 2014). On the other hand, aging has also been shown to slow down the rate of axonal regrowth (Krekoski et al., 2002). However, functional recovery depends on the correct reinnervation of peripheral target organs. Following a PNI, there are alterations in motor performance that are recovered to different extents after reinnervation. Peripheral reinnervation restored voluntary control over muscle contraction. Studies in the cat on self-reinnervated GM nerves conclude that normal recruitment patterns are recovered when MNs successfully reinnervate the same muscles (Cope and Clark, 1993); despite this, when there is an unspecific reinnervation, coordinated movement remained impaired, suggesting the importance of correct reinnervation for ameliorate movement (Thomas et al., 1987). After nerve transection, axons are often misdirected and reinnervate incorrect target organs although there has been repaired (Allodi et al., 2012; Bodine-Fowler et al., 1997; Valero-Cabré and Navarro, 2002). The disruption of endoneurial tubes favors misdirection of axons to inappropriate targets. Thus, although the amount of axonal regeneration can be considerably high, the lack of selectivity of axon-target reconnection leads to a poor functional recovery (Allodi et al., 2012).

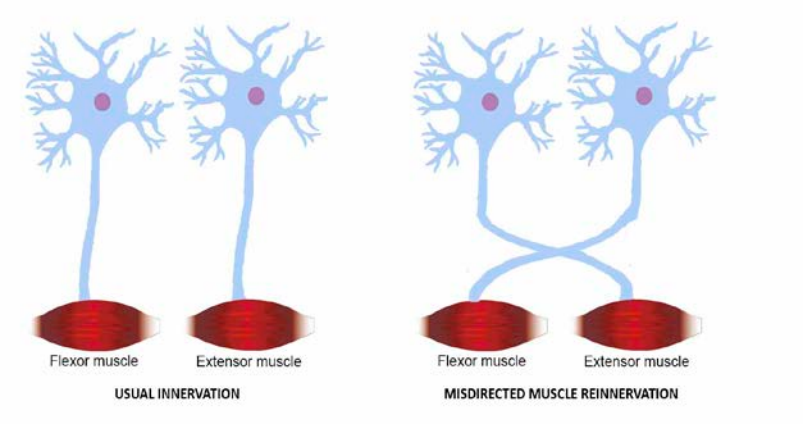


Fig 5. Example of an incorrect reinnervation after peripheral nerve injury. There is a misdirected regeneration of axons to functionally inappropriate muscular targets.

EFFECTS OF PERIPHERAL NERVE INJURIES ON THE SPINAL CIRCUITRY

After axotomy, the interruption between the axons and their target organs is accompanied by maladaptive changes in the neural circuit in the SC and at supraspinal levels (Navarro et al., 2007); these have been related with neuropathic pain and a disruption of the spinal circuitry. The disruption of the spinal circuitry has been correlated with central synaptic stripping, a massive loss of synapses, probably linked to microglia activation. Microglia can remove damaged cells as well as dysfunctional synapses, facilitating regrowth and remapping of damaged neuronal circuitry (Gehrmann et al., 1995).

Synaptic stripping of motoneurons

After axotomy, spinal MNs suffer important changes in the soma, as well as in its surrounding environment. MNs suffer an important loss of synapses at the dendritic level, that was firstly described in the rat facial nucleus (Blinzinger and Kreutzberg, 1968; Eccles et al., 1958) and then in the rat hypoglossal nucleus (B. Sumner, 1975; Sumner and Sutherland, 1973), and later in cat spinal MNs (Chen, 1978). The stripping of synaptic buttons is considered to be part of the global retrograde reaction in response to the loss of connectivity with the peripheral target

because synaptic inputs are largely reestablished once muscle reinnervation is achieved (Brannstrom and Kellerth, 1999; Sumner and Sutherland, 1973). However, not all synapses show the same capacities for recovery; it is know that restitution of excitatory synaptic inputs from primary Ia afferents in lumbar MNs after successful reinnervation of the muscle is incomplete after injury (Alvarez et al., 2011; Rotterman et al., 2014).

Dendritic content of spinal motoneurons

MNs receive excitatory and inhibitory synaptic inputs from sensory afferents and from pathways of supraspinal origin. Different studies suggested than there are different ratios of excitatory and inhibitory synapses in control MNs, under different neurotrophic influences or following PNI (Brannstrom and Kellerth, 1999; Davis-López de Carrizosa et al., 2009; Novikova et al., 2000).

Excitatory synapses

All primary afferents are excitatory and express glutamate, which is one of the main excitatory neurotransmitter. Glutamate is involved in the perception of acute and chronic pain as well as mechanical, thermal and chemical stimuli (Dickenson et al., 1997; Dougherty and Willis, 1992; Garry and Fleetwood-Walker, 2004; Haley et al., 1990). Glutamate is accumulated in the synaptic vesicles by means of vesicular glutamate transporters, which are divided in different subpopulations (VGlut 1,2,3) (Takamori et al., 2000).

VGlut1 is associated with primary muscle afferents in lamina IX (Alvarez et al., 2004; Oliveira et al., 2003; Todd et al., 2003). The descending axons from the pyramidal cells of neocortex and corticospinal tract also express VGlut1 (Du Beau et al., 2012; Fremeau et al., 2001). After injury, there is an important loss of VGlut1 afferent synapses, as part of the process of synaptic stripping on axotomized MNs (Alvarez et al., 2004; Blinzinger and Kreutzberg, 1968; Brannstrom and Kellerth, 1999; Novikova et al., 2000); interestingly, loss of VGlut1 synapses, is disproportionally higher than loss of other synapses during the synaptic stripping induced by PNI. In fact,

there is a permanent reduction of VGlut1 contacts on the cell body in the proximal 100 µm segments of dendrites in axotomized MN (Alvarez et al., 2011). The time course of VGlut1 synaptic loss is prolonged compared with other synaptic inputs and does not recover independently of the regenerative process of sensory and motor axons. Ia afferent contacts are reduced by at least 75% on the soma and by approximately 50% on the proximal dendrites of regenerated MNs that successfully reinnervate target organs.

Inhibitory synapses

In the adult CNS, the main inhibitory neurotransmitter is GABA. GABA is released from these inhibitory vesicles in response to an appropriate stimulus (Chaudhry et al., 1998) .

In the spinal cord, the modulation of excitatory and inhibitory balance is crucial to prevent homeostasis bias and eventually neuronal loss (Meisner et al., 2010). After peripheral nerve injury, the excitatory and inhibitory balance shifts, with a net increase of inhibitory synapses (De Luca et al., 2015).

Effects of PNI on the stretch reflex

The sensory component of the stretch reflex has a good recovery after axotomy. Over 75% of muscle spindles receive reinnervation, and about half of these endings in the equatorial region (where Ia afferents endings are normally located) recover their ability to encode changes in both muscle length and velocity (Haftel et al., 2005). Recordings in regenerated dorsal root axons shows that these afferents respond to muscle stretch (Banks and Barker, 1989; Lewin and McMahon, 1991). Even when muscles afferents innervate incorrect targets, these afferents tend to achieve characteristics of the end organ they innervate (Banks and Barker, 1989). However, after PNI functional recovery of this reflex is not adequate. In fact, 90% of patients who have undergone surgical repair fails to restore compete normal function after peripheral nerve injury (Brushart, 2011), and stretch of the muscle fails to produce a reflex contraction (Cope and Clark, 1993; Cope et al., 1994). Consequently, this

deficiency may contribute to motor disability, which is typically attributed to stripping of central synapses and to poor muscle spindle reinnervation (Abelew et al., 2000; Cope and Clark, 1993; Cope et al., 1994; Verdu and Navarro, 1997).

Neuropathic pain after axotomy

Neuropathic pain, resulting from a lesion or dysfunction of the peripheral or central nervous system, is a particularly debilitating chronic pain condition that is very difficult to treat. Neuropathic pain is characterized by an increased sensitivity to noxious stimuli (hyperalgesia), to nociceptive sensation in front of non-noxious mechanical stimuli (allodynia) and aberrant pain sensations (spontaneous pain). After a PNI, the increased afferent discharge associated with the injury can lead to the development of hyperexcitability of dorsal horn neurons (Bridges et al., 2006) and thus, to the development of neuropathic pain.

PNI can also lead to changes in inhibitory pathways, such as reduction in the inhibitory control over dorsal horn neurons (Sugimoto et al., 1990; Woolf and Mannion, 1999). Dorsal horn neurons receive a strong inhibitory input from GABA interneurons. PNI promotes a selective apoptotic loss of GABA-releasing inhibitory neurons in the superficial dorsal horn of the SC (Moore et al., 2002), a mechanism that further increases central sensitization. Dorsal horn neurons receive a powerful descending modulating control from supraspinal brainstem centers, which have inhibitory as well as facilitatory effects (Vanegas and Schaible, 2004). It has been hypothesized that a loss of function in descending inhibitory serotonin and noradrenalin pathways contributes to central sensitization during neuropathic pain (M H Ossipov et al., 2000). In animals, mechanical allodynia after PNI depends on tonic activation of descending pathways that facilitate pain transmission, indicating that structures in the mesencephalic reticular formation, possibly the nucleus cuneiformis and the periaqueductal gray matter (PAG) are involved in central sensitization during neuropathic pain (Michael H Ossipov et al., 2000). It has also been proposed an alternative mechanism of intraspinal disinhibition following PNI. This mechanism involves a trans-synaptic reduction in the expression of the potassium-chloride

cotransporter 2 (KCC2) in lamina I neurons, which disrupts anion homeostasis in these neurons. Loss of inhibitory tone in lamina I neurons is important in the initiation and maintenance of neuropathic pain. BDNF released by activated microglial cells after PNI decreases expression of KCC2 in Lamina I neurons. This decreases Cl- concentration gradient and reduces inhibition in these cells (Coull et al., 2003). Moreover, the blockade of glycine or GABA receptors by intrathecal injection of strychnine and bicuculline induces allodynia due to the removal of both inhibitory systems (Loomis et al., 2001). The contribution of GABA receptors to fast inhibitory synaptic transmission is greater than glycine receptors in the superficial dorsal horn whereas glycinergic inhibition appears to be more important as almost all deep dorsal horn neurons receive glycinergic drive and glycinergic mEPSCs have much larger amplitude and faster kinetics (Anderson et al., 2009).

Although the symptoms associated with neuropathic pain are well known, the molecular mechanisms that participate in its development are not completely identified and understood. It is know that there are some key molecules involved in the generation and maintenance of neuropathic pain; like neurotrophin nerve growth factor (NGF), that influence the phenotype of primary sensory neurons and regulates inflammatory and homeostatic pain states (Lewin et al., 1993; Ramer et al., 1998; Theodosiou et al., 1999; Woolf et al., 1994). In contrast, neurotrophin NT-3 antagonize the proinflammatory NGF pathway and its algesy-associated phenotype (Gratto and Verge, 2003; Wilson-Gerwing and Verge, 2006).

Glial reaction in the spinal cord after PNI

Plasticity of synaptic input around cell bodies of neurons occurs rapidly following injury to peripheral axons. Proximal to the lesion, this adaptive mechanism may both affect neuronal function into the circuitry and enhance neuronal viability (Hardingham, 2009). A critical but unresolved issue is the role of astrocytes and microglia reactivity in this process.

Astrocytes are the most abundant cell type in the CNS; it is well know that there are 5 times more astrocytes than neurons (Sofroniew and Vinters, 2010). As all

glial cells, the general view has been that astrocytes are a supportive component of neural tissue and not active contributors to synaptic events and plasticity. Astrocytes act or interact into/on synaptic regions, controlling and regulating ions, pH, and homeostasis. Furthermore, astrocytes contribute to transmitter homeostasis by expressing high levels of transporters for neurotransmitters such are glutamate, GABA and glycine (Sattler and Rothstein, 2006). It has also been demonstrated that astrocytes also play an active role in synaptic transmission and actually release molecules like glutamate, ATP, GABA in response to neuronal activity (Araque and Navarrete, 2010).

After injury, reactive astrocytes have been shown to express a number of molecules that inhibit plasticity (Silver and Miller, 2004). However, recent studies argue that they can also be beneficial for repair (Sofroniew and Vinters, 2010). It is known that there is an up-regulation of the intermediate filament GFAP following PNI. After that, astrocytic processes are then interposed between synaptic terminals and the postsynaptic membrane and also phagocytize degenerating nerve terminals (Aldskogius et al., 1999).

On the other hand, microglia is resident macrophage of the CNS. In uninjured CNS, microglia is branched and their branches are highly motile displaying continuous extension and retraction (Davalos et al., 2005). Microglia is activated following small changes in their microenvironment (Streit et al., 1999), and produces free oxygen radicals, nitric oxide, proteases and cytokines all of which can exert neurotoxic effects (Moore and Thanos, 1996; Théry et al., 1991). However, reactive microglia can also exert neuroprotective effects and enhance nerve repair (Streit et al., 2005).

In the nineteenth century, it was proposed that perineuronal microglia actively participated in the physical detachment and displacement of afferent axonal endings during the synaptic stripping process (Blinzinger and Kreutzberg, 1968). However, following studies suggested that reactive astrocytes, but not microglia, might correlate better with this phenomena (Aldskogius et al., 1999; Reisert et al., 1984; Yamada et al., 2011). Moreover, other studies have correlated the extent of synaptic loss with the degree of astroglial reactivity within a lesioned motor nucleus

(Zanon and Oliveira, 2006). Nevertheless, how glial cells are involved in this process and which are their specific roles still remains unknown. 2-4 days after injury, microglia forms overlapping sheets of processes between neurons and synaptic terminals (Blinzinger and Kreutzberg, 1968; Kerns and Hinsman, 1973a, 1973b). In contrast, astrocytic processes increase, replacing microglia at 2 weeks (Chen, 1978; B. Sumner, 1975) and peaking in number at 5 weeks (Sumner and Sutherland, 1973). At the same time the number and size of boutons decreases on the soma and dendrites (Chen, 1978; Sumner and Sutherland, 1973; Sumner, 1975).

STRATEGIES TO EVALUATE/MODULATE THE PLASTIC CHANGES THAT MN SUFFER AFTER PNI IN EXPERIMENTAL MODELS

Stretch reflex as a simple one

Since there are important changes in the connections of the spinal circuitry after PNI, the study of the stretch reflex arc can be a useful tool to evaluate the limitations in the recovery of the spinal circuit after nerve injury. Firstly, is one of the best-characterized circuits in the nervous system and has been extensively used as a model for neural plasticity after axotomy. Furthermore, it is a simple circuit, which allows us to observe how a direct input (muscle stretch) has a direct output (muscle contraction); for this simplicity, it is easy to see how a given input, in this case muscle stretch, results in a direct output, muscle contraction. It is possible to assess the status of the neural circuitry underlying the stretch reflex by studying the muscle force output in response to controlled stretch. The force response to stretch is representative of the net operation of a MNs pool, where MN activation necessarily causes muscle fiber contraction. If the circuitry is working normally, force response to stretch remains unchanged. However, disruptions of any portion of the circuitry will interfere with the ability of stretch to elicit force responses, resulting in decreased or absent responses (Cope et al., 1994).

IHQ evaluation

After PNI, there are many changes in both PNS and CNS. The central circuitry changes following PNI can be studied using unspecific immunolabelings.

Synaptic content of injured motoneurons can be evaluated by means of antibodies against Synaptophysin, a synaptic vesicle glycoprotein with four transmembrane domains that participates in synaptic transmission in brain and SC; consequently, it is involved in the regulation of short-term and long-term synaptic plasticity.

On the other hand, it is know that synaptic varicosities of physiologically identified normal Ia afferents always contain high amounts of VGlut1 (Alvarez et al., 2011), confirming that VGlut1 is indeed associated with Ia afferents. In sharp contrast to this normally high level, decreases in the expression of VGlut1 after PNI suggest that Ia synapses with MN are seriously decreased (Alvarez et al., 2011; Brumovsky et al., 2007; Hughes et al., 2004). VGlut1 mediates the uptake of glutamate into synaptic vesicles at presynaptic nerve terminals of excitatory neural cells. Therefore, VGlut1 immunoreactivity can be used as a marker of proprioceptive contacts on the soma and dendritic arbor of MN.

Evaluation of inhibitory synapses can be done by means of the antibody against Gephyrin. This scaffold protein is the main component of glycinergic and GABAergic postsynaptic structures (Fritschy et al., 2008). The Gephyrin scaffold itself is dynamically regulated by synaptic activity, emphasizing the role of receptor-scaffold interactions in providing a rapid control of receptor number at synapses (Hanus et al., 2006; Maas et al., 2006). VGat (Vesicular GABA transporter) antibody has also been extensively used to identify inhibitory axon terminals (del Cid-Pellitero and Jones, 2012; Liguz-Lecznar et al., 2014; Todd et al., 2003). VGat stores GABA in axon terminals.

To evaluate glial activity around injured motoneurons, the most widely used markers of astroglial cells is Glial Fibrillary Acidic Protein (GFAP). GFAP is an intermediate filament that forms a network to provide support and strength to cells. On the other hand, Iba1 could be used to detect microglia reactivity. Iba1 is an actin-

binding protein than plays a role in phagocytosis and also in macrophage activation and function.

As we mentioned above, PNNs play a crucial role in the maturation of synapses and closure of critical periods by limiting synaptic plasticity and stabilizing synapses (Carulli et al., 2013; Foscarin et al., 2011; Pyka et al., 2011). Therefore, after nerve injury, spinal PNN may contribute to the plasticity observed in the SC. The most common labeling to identify PNN is Lectin from Wisteria floribunda agglutinin (WFA) antibody, which has an affinity for N-acetylgalatosamine.

Electrophysiological studies

In experimental models, electrophysiological tests are used to evaluate muscle reinnervation after peripheral nerve injury (Lago and Navarro, 2006; Marsala et al., 2005; Navarro et al., 2007). Motor function can be evaluated by compound muscle action potentials (CMAPs), evoked after electrically stimulating the nerve. The activity recorded in the muscle includes the M-wave and the H-wave. These CMAPs reflects muscle function in normal and reinnervated muscles. Therefore, CMPAs are an indirect measure of the conduction capacity of the nerve (Frykman et al., 1988), but limited to study the recovery of motor axons and not suitable for the early evaluation of nerve regeneration. Spinal reflexes can also be recorded by means of electrophysiology techniques. In fact, the H-wave is the electrophysiological equivalent of the stretch reflex. It is know that H wave reappears early after the muscle is reinnervated following PNI, and its highly facilitated during the early phase of regeneration, facilitation that is reverted as muscle reinnervation advances (Valero-Cabré and Navarro, 2001; Vivó et al., 2008). However, recent studies show that the stretch reflex does not recover after axotomy (Haftel et al., 2005). Interestingly, this dysfunction does not correlate with the absence or reduction of H wave.

Alvarez group uses the STA technique (Mendell and Henneman, 1971) to assess the functional impact of partial synaptic disconnection and directly examine the

capacity of individual afferents that responded to muscle stretch. Briefly, they perform intracellular records of membrane potential from MNs with simultaneous records of action potentials from individual primary afferents. The presence or absence of STA EPSPs enables the assessment of the functional synaptic linkage, such as connectivity of a single afferent with its homonymous MNs (Alvarez et al., 2011; Bullinger et al., 2011).

Evaluation of neuropathic pain

The degree of sensitivity to a painful stimulus could be measured by algesimetry tests. These tests allow us to evaluate the ability of an animal to detect a noxious stimulus such as the feeling of pain, caused by stimulation of nociceptors. Mechanical nociceptive thresholds can be determined using electronic Von Frey. In this test, the animal is in on an elevated platform, and the Von Frey hairs are inserted through the mesh to push the animal hindpaw (Mogil, 2009). Normal reactions for the animal include withdrawing or licking or shaking the paw, and possible vocalization, but these can depend on variability within the experiment.

Moreover, cation-chloride cotransporter activity 2 (KCC2) up or down regulation could also be useful to evaluate neuropathic pain in experimental models. Normal GABAergic function is critically dependent on KCC2. In normal conditions, KCC2 is a protein that regulates intracellular Cl^- concentration in adult neurons. Elevation of intracellular Cl^- can lead to GABAergic hypersensitivity by reversing both the Cl^- equilibrium potential and the normal inhibitory action of GABA. After axotomy, there is a suppression of KCC2 expression and the efficacy of GABA signaling is affected (Coull et al., 2003). Different studies suggest that changes of the transporter expression play a role in inflammatory or neuropathic pain (Granados-Soto et al., 2005; Vinay and Jean-Xavier, 2008) as well as induced hypersensitivity (Beggs et al., 2012).

On the other hand, the plantar test is used to evaluate thermal allodynia. It measures hind paw withdrawal latency in response to a radiant heat.

STRATEGIES TO PROMOTE FUNCTIONAL RECOVERY AFTER PNI: MODULATION OF THE SPINAL CHANGES

Different strategies have been used to improve functional recovery after PNI, mainly in experimental models. Addition of trophic factors to enhance axonal regeneration has been extensively tested, although the delivery system, the dosage and the mixture used and contradictory results in different studies limit their potential clinical application. In contrast, activity dependent therapies could be a much better alternative. In fact, physical rehabilitation is one of the cornestones for the treatment of multiple pathologies of the nervous system. Although these therapies have shown the capacity to improve functional recovery after PNI, their effects on the spinal circuitry after injury have not been fully addressed in the literature. Moreover, their mechanism of action on the injured nervous system is not well understood.

Application of neurotrophins

Neurotrophins are important for the survival, maintenance, and regeneration of specific neuronal populations in the SNC. The neurotrophins that were identified as neuronal survival-promoting proteins in mammals include NGF, BDNF, NT-3 and NT-4/5 (Kim et al., 2011; Konar et al., 2011). These neurotrophic factors bind to specific high-affinity tropomyosin-receptor kinase (Trk) receptors and to the low affinity p75 receptor. TrkA is the high affinity receptor for NGF, TrkB is the receptor for BDNF and NT-4/5, and TrkC is the receptor for NT-3. Trk and p75 receptors trigger different downstream intracellular pathways and different cell responses. Low affinity p75 receptors are usually upregulated after injury and seem to hamper axonal regeneration (Allodi et al., 2012). Moreover, it is know that an up regulation of neurotrophin is directly related with a reduction of synaptic stripping suffered after axotomy (Krakowiak et al., 2015).

It is well known that neural function depends on the proper numbers, structural integrity, and connections of neurons and their axons. Neurotrophins are critical for the survival of neurons and the morphological development of axons, such as extension and branching (Lentz et al., 1999; Patel et al., 2003; Tucker et al., 2001).

Neurotrophic factors produced by Schwann cells are an essential for axonal regeneration: at distal nerve stumps, proliferating Schwann cells form tubes (bands of Büngner), through which they guide regenerating axons via the release of neurotrophic factors (Sofroniew et al., 2001; Stoll et al., 2002; Griffin and Thompson, 2008; Jessen and Mirsky, 2008; Bosse, 2012).

In uninjured condition, BDNF is expressed at low levels but after injury, BDNF and their receptors are up-regulated in MNs and in denervated distal nerve stump (Funakoshi et al., 1993). There is a steeply increase in mRNA levels of BDNF and with a changing magnitude between different nerves. In MNs BDNF mRNA is rapidly upregulated by axotomy and returns to basal levels in few days. In the distal nerve stump, different studies point that Schwann cells are the ones overexpressing BDNF following injury (Funakoshi et al., 1993; Tonra et al., 1998; Zhang et al., 2000).

Furthermore, TrkB gene expression is also increased in facial and sciatic MNs after injury. The increase begins in the immediate days post injury, reaches a three-fold peak increase by the end of the first week, and remains elevated during a month (Boyd and Gordon, 2003). Moreover, the time course of p75 mRNA response is similar to that of TrkB mRNA but the magnitude is three to four-fold higher (Boyd and Gordon, 2001).

Although BDNF levels increase after injury, this neurotrophic factor does not always improve regeneration. At low doses, exogenous BDNF does not produce a clear effect on axonal regeneration of acutely injured axons, whereas enhances regeneration of chronic axotomized ones. On the other hand, high doses of exogenous BDNF impair axonal regeneration, probably by signaling through p75 receptors (Boyd and Gordon, 2002).

The role of the NGF, NT-3 and NT-4/5, in promoting axonal regeneration is less well studied. Following axotomy, expression of NT-3 and NT-4/5, as well as that of TrkC, is down-regulated in MNs and in the distal nerve stump, whereas NGF levels increase in the distal nerve stump (Boyd and Gordon, 2003). Despite being down-regulated, NT-3 treatment increases the number of MNs that successfully regenerate through nerve gaps and improves muscle reinnervation, specifically in fast contracting

muscles (Sterne et al., 1997). Moreover, NT-3 is able to stimulate spinal axon elongation, modulate the muscle spindle MN connection after PNI and also play a role in mediating synaptic transmission (Chen et al., 2002; Xie et al., 1997). Since the mRNA for NT-3 is expressed in both muscle and skin (Schecterson and Bothwell, 1992) and the afferent fibers express the TrkC receptor (McMahon et al. 1994), NT-3 has a key role to mediate recovery after PNI.

Activity dependent therapies

Electrical stimulation

Electrical stimulation (ES) has been used to stimulate denervated muscle to reduce muscle atrophy after PNI. In 1952, Hoffman reported that ES of the SC or directly to the nerve resulted in accelerated sprouting of intact axons and reinnervation of the partially denervated muscles (Hoffman, 1952). Further studies have confirmed a beneficial role of ES in PNI since it promotes nerve regeneration, axonal guidance and elongation (Al-majed et al., 2000; Brushart et al., 2002). Moreover, acute ES of an injured nerve for 1 hour causes a sharp but short-lived increase of BDNF mRNA expression in motoneurons (Al-majed et al., 2000; Sharma et al., 2010). Different intensity, duration and frequency paradigms were evaluated but ES at 20 Hz for 1 h immediately after injury shows the more consistent positive effects after peripheral nerve regeneration (Al-majed et al., 2000).

Physical exercise

Exercise can be neuroprotective and may be used as a treatment to protect from nerve damage and strokes, to promote regeneration of injured axons and to reduce neuropathic pain. Exercise would mediate this neuroprotection through a variety of mechanisms such promotion of neurogenesis, enhancement of metabolism, decrease of apoptosis and amelioration of inflammation (Cobianchi et al., 2016; Nishioka et al., 2016; Vaynman and Gomez-Pinilla, 2005).

It is well studied that exercise could improve motor function following SC injury both in animal models and in clinical studies (Edgerton et al., 1996; Hutchinson et al., 2004a; Skinner et al., 1996) . In contrast, the effect of exercise after PNI has received less attention. Several studies had shown that moderate daily treadmill training after PNI produced a substantial enhancement of axon regeneration in animal model (Asensio-Pinilla et al., 2009; Sabatier et al., 2008). Treadmill running (TR) is also able to enhance muscle reinnervation and modulate the hyperreflexia observed after nerve injury (Cobianchi et al., 2016; English et al., 2011; Udina et al., 2011b). A sex difference in the promotion of mouse peripheral nerve regeneration due to TR has been identified; continuous slow training enhances axon regeneration in male mice, but not in female mice. Conversely, a series of interrupted short sprints is effective in female mice, but not in male (Wood et al., 2013).

It remains unknown the real differences between passive, voluntary or forced exercise. Treadmill running increase physical activity, but stress-related changes could influence the outcome (Meeteren et al., 1997). Moreover, passive exercise, which do not induce cortical activation, preserves the structure of the end-plates of denervated muscles and enhances reinnervation (Pachter and Eberstein, 1989). In contrast, continuous passive motion after tibial nerve section in rabbits shows a slightly slow nerve conduction velocity and less fiber density compared to untrained animals (Kim et al., 1998). Udina et al (2011) showed that both passive and active exercise produced slight improvement in regeneration after axotomy, with an increased recovery of CMAPs, a higher number of regenerated axons in the distal nerve and a reduction in the hyperexcitability of spinal reflexes observed after the injury.

Regarding voluntary exercise, in animal models it has been mainly evaluated in running wheels. Voluntary exercise, as forced exercise, induces a cortical activation but it is not stressful for the animals. When rats are given access to a running wheel they can run up to 10-12 km/day. There is however a great individual variation in rats running distances in the wheels and therefore they are usually divided into groups of either short, medium, or long distance runners (Afonso and Eikelboom, 2003; Lambert et al., 1996; Narath et al., 2001; Rodnick et al., 1989; Sexton, 1995). Free access to

running wheels following tibial nerve transection and repair, caused a delay in reinnervation of 18-20 days, but at 4 weeks outcome was similar to non-exercised animals 4 weeks. Moreover, free running counteracted muscular atrophy due to denervation in mice (Badke et al., 1989). On the other hand, voluntary running produced a decreased weight and force in extensor digitorum longus muscles but an increased force and weight in soleus muscles after nerve injury compared to non-exercised animals (Irintchev et al., 1991, 1990). These changes are typical of endurance training and could be interpreted as beneficial. Consistent with these observations, van Meeteren et al. (1997) reported beneficial effects of exercise training, in the form of voluntary static standing on both hindpaws, on the recovery of motor and sensory functions during the early phase of reinnervation after sciatic nerve crush.

There is an open debate about the exercise protocol that should be applied. One possible contributing factor to these discrepancies may be due to variations in intensity and duration of exercise and time of administration following injury. Many findings suggest that better recovery may be achieved when exercise is applied at early times. The first week after injury seems to be a crucial time window, when different intensities of activity may stimulate or inhibit the first axonal regenerative responses, that is highly dependent on the production of neurotrophic factors. The activation of neurotrophin signal transduction pathways peaks around 7 days post-injury and during exercise is crucial in regulating the growth of sensory neurons (R. Molteni et al., 2004). The most frequently reported underlying mechanism of neuroprotection in experimental studies was an increase in neurotrophin levels, particularly BDNF, which is beneficial for survival and regeneration of injured axons (R. Molteni et al., 2004; Ying et al., 2008). However, intense and prolonged exercise may do not stimulate but reduce regeneration since it was found to inhibit collateral sprouting of motor axons for compensatory reinnervation of denervated muscle fibers (Love et al., 2003; Tam and Gordon, 2003). Moreover, it was previously found that prolonged ES or TR also was detrimental for peripheral nerve regeneration and neuropathic pain (Asensio-Pinilla et al., 2009; Cobianchi et al., 2010).

MECHANIMS OF ACTION OF EXERCISE ON THE NERVOUS SYSTEM

Up-regulation of neurotrophins and other factors is extensively accepted that physical exercise increases the expression of BDNF and its receptor TrkB in both transcriptional and translational level indifferent brain regions (Cobianchi et al., 2016; Neeper et al., 1996, 1995; Skup et al., 2000). On the other hand, a downregulation of these factors has been demonstrated when animals are deprived of regular physical activity (Widenfalk et al., 1999). At the same time, neural activity induced by exercise has been studied after SC injury (Gómez-Pinilla et al., 2002, 2001). Different studies have shown that voluntary exercise is able to induce or restore levels of neurotrophins both in intact or injured SC and promote neuroplasticity (Gómez-Pinilla et al., 2002; R. Molteni et al., 2004; Skup et al., 2000; Ying et al., 2008, 2005).

Exercise also can delay neurodegeneration in aged Alzheimer transgenic mice (Ke et al., 2011; Liu et al., 2011; Um et al., 2011) and humans (Abe et al., 2012; Liang et al., 2010). Although the detailed mechanisms remain unclear, exercise has been shown to reduce the levels of detrimental factors, for example oxidative stress and inflammation (Ke et al., 2011; Um et al., 2011) and increase the levels of neurotrophic factors, such as BDNF and IGF-1 (Cotman and Berchtold, 2002; Cotman et al., 2007).

After PNI, increased BDNF induced by exercise seems to mediate the enhancement of axonal elongation into peripheral nerve grafts (Sabatier et al., 2008) and the reduction of synaptic stripping suffered by axotomized MNs (Krakowiak et al., 2015). Although there is no direct evidence that physical exercise could increase BDNF levels in axotomized neurons or in the distal nerve stump following injury, the effect of TR exercise in promoting axonal regeneration requires BDNF expression by MNs (Wilhelm et al., 2012).

Both voluntary exercise (Gómez-Pinilla et al., 2002) and treadmill running (Ying et al., 2005) have the ability to increase neurotrophins, and also up regulate BDNF receptor TrkB (MacIas et al., 2007). Specifically, it seems that neurotrophin overexpression induced by exercise in the SC its restricted to the lumbar region, probably due to the fact that this region receives most of the neural activity that takes place during exercise (Gómez-Pinilla et al., 2001).

However, some studies show a decrease of neurotrophic factors in the SC following exercise (Engesser-Cesar et al., 2007; Siamilis et al., 2009). One possible contributing factor to these discrepancies may be to variations in intensity and duration of exercise. BDNF and NT-3 in the lumbar SC are known to increase following short-term exercise (Gómez-Pinilla et al., 2002, 2001; Neeper et al., 1996). For instance, moderate-intensity exercise increases BDNF, but high intensity exercise decreases BDNF levels in the brain (Aguiar et al., 2007), suggesting that low to moderate intensities of exercise may be a more potent stimulus for increasing neurotrophic factor levels. A single day of exercise increases plasma and serum BDNF levels, probably proportionally to intensity (Ferris et al., 2007), as does a short intense bout of exercise (Tang et al., 2008). Basal BDNF levels also increase after training, although ambiguity exists regarding the duration, type and intensity of training required (Zoladz et al., 2008). The brain appears to be the main source of circulating BDNF during exercise, while peripheral blood mononuclear cells and endothelial cells may contribute approximately 20–30% of peripheral levels (Nakahashi et al., 2000). Despite this, there is a little bit of controversial about BDNF. It is known that short-term exercise training period (3, 7, 15 days running on treadmill) resulted in an equal BDNF in the hippocampus and basal forebrain in both control and exercise training groups, respectively (Albeck et al., 2006; Ferreira et al., 2011). On the other hand, a recently study shows that both high intensity interval training and continuous training increased rat brain BDNF and GDNF (Afzalpour et al., 2015). Moreover, different studies have shown that long-term exercise trainings at moderate intensity increases GDNF concentration in striatum, sciatic nerve and SC (Groover et al., 2014; Lau et al., 2011), while short-term exercise training at low to moderate intensity does not influence the GDNF concentration in the striatum and substantia nigra (Wu et al., 2011).

On the other hand, differences have been observed depending on the type of exercise (forced, voluntary or passive) applied. Voluntary exercise is able to increase the expression of several molecules associated with the action of BDNF on synaptic function and neurite outgrowth in the lumbar region of the SC and the soleus muscle

(Gómez-Pinilla et al., 2002). Moreover, Cotman and Berchtold has reported that 28 days of daily and interval voluntary wheel running increased BDNF concentration in the hippocampus as a result of increasing the conversion of pro-BDNF to the mature form (Cotman and Berchtold, 2002). Chronic and acute aerobic exercise leads to elevated peripheral BDNF levels, while strength training does not appear to influence peripheral BDNF (Goekint et al., 2010).

Neural activation of injured motoneurons

It is obvious that physical exercise increases the activity of the nervous system, and can also facilitate the activation of the circuitry that has been affected by the injury. In fact, the activation of these circuitry could be fundamental for most of the beneficial effects of exercise on the injured nervous system.

Exercise is facilitating activation of spinal motoneurons. In fact, in normal conditions, these neurons adapt to training by increasing their dendritic arbor and their protein synthesis, suffering also alterations in ion conductance. It seems that motoneurons adapt to endurance exercise by increasing their capacity to axonal transport and increasing neurotransmitter efficacy (Gardiner et al., 2006).

After nerve injuries, where motoneurons are axotomized, facilitation of activity of these neurons by physical exercise could be important to avoid some of the maladaptive changes observed after the injury and to facilitate regeneration and functional recovery. In fact, exercise of the limbs also stimulates muscle afferents of proximal innervated muscles, which may influence the axotomized motoneurons by spinal synaptic connections normally silent (Koerber et al., 2006).

Activation of descending projections

Active exercise activates motor descending projections. Among them, the noradrenergic descendent projections from the locus coeruleus (LC), a tract that has been implicated in potentiation of motor activity in stressful situations and

modulation of pain sensibility (Millan, 2002; Pertovaara, 2006) and modulation of excitability of motoneurons (Heckman et al., 2003) in the spinal cord.

The noradrenergic system and specifically, noradrenaline, is a key mediator of the stress response. Noradrenaline (NA) is synthesized by the enzyme dopamine beta-hydroxylase, which converts dopamine to noradrenaline. NA is converted to adrenaline, and it is released into the bloodstream by the adrenal medulla where it can modulate peripheral stress responses. It is released in response to activation of the sympathetic nervous system and is an important part of the "flight or fight" response. Thus, adrenaline released into the periphery prepares the animal to respond to a stressful situation (Kandel et al., 2000).

Noradrenaline descendent pathways

There are two sources of NA in the brain, the LC and the nucleus tractus solitarus. The locus coeruleus, located in the posterior area of the rostral pons in the lateral floor of the fourth ventricle in the brain, is the largest noradrenergic nucleus in the human and rat brain, containing approximately 15.000 NA neurons per hemisphere in humans and 1.600 neurons per hemisphere in rats (Coull, 1994; Rogawski et al., 1985).

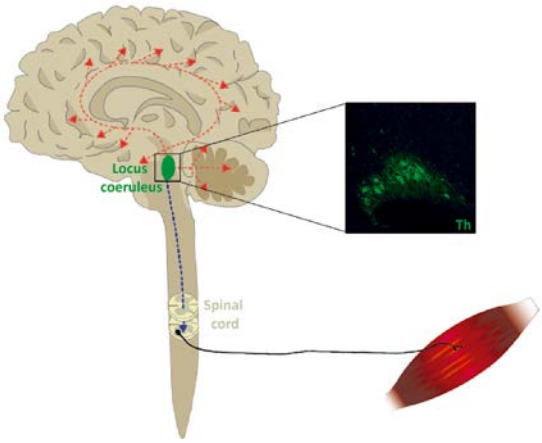
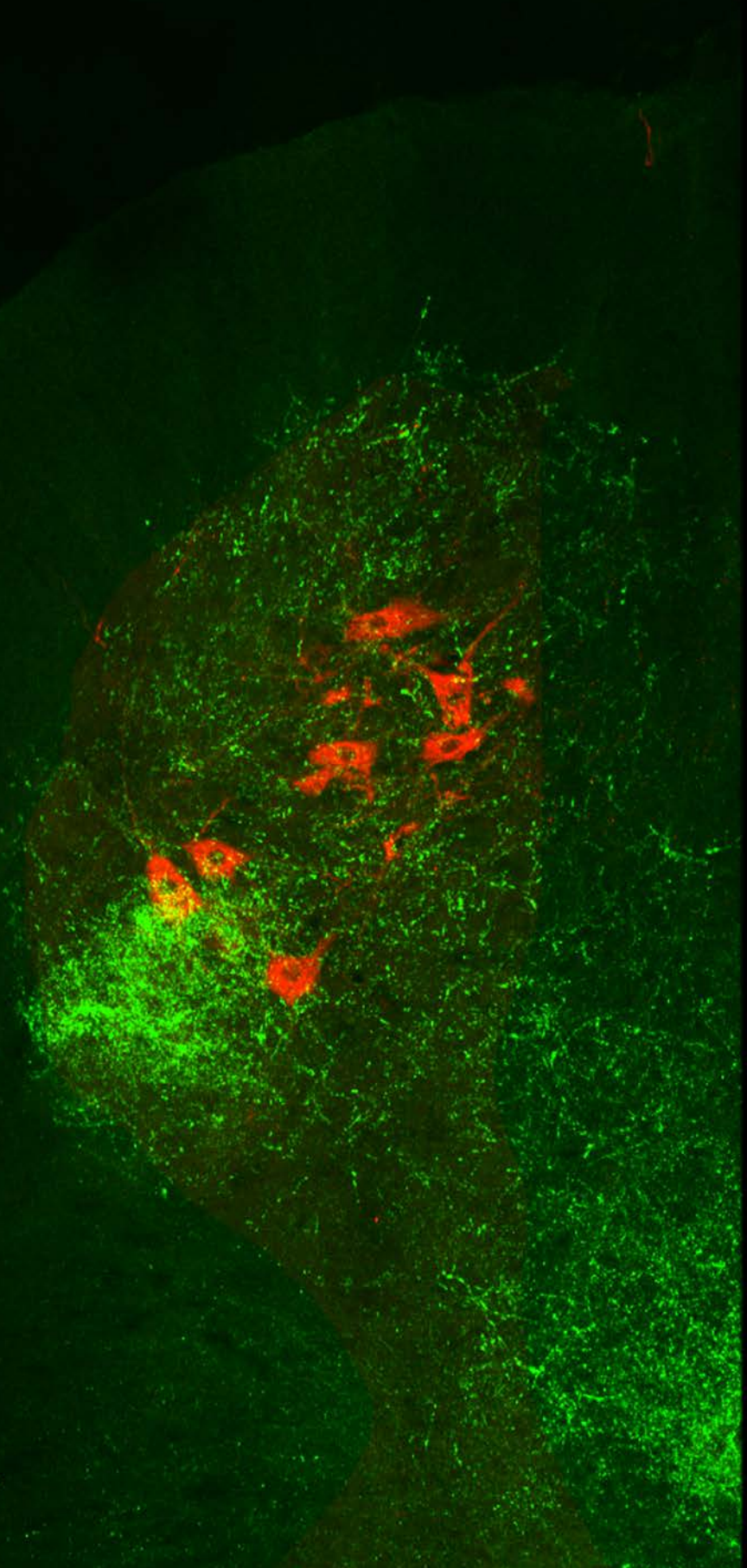


Fig 6. Noradrenaline projections. The output of the noradrenergic locus coeruleus cells extends broadly throughout the cerebrum (red lines). The output of these cells also projects prominently to the SC became to noradrenaline descendant projections (blue line). Rat locus coeruleus amplification (X20) using Tyrosine Hydroxylase (TH) immunolabeling.

The LC send descending projections to the spinal cord. At the ventral horn level, this projections are important in the modulation of motoneuron excitability (Shope et al., 1993; Wohlberg et al., 1986), facilitating their activation when receiving other synaptic inputs (Heckman et al., 2008). Projections to the dorsal horn will be key elements in the modulation of pain sensation.

LC is connected with important pain control centers like the PAG, in the midbrain (Bajic and Proudfit, 1999), the amygdala and some nucleus in the hypothalamus (Cedarbaum and Aghajanian, 1978). Due to their anatomical connections to multiple forebrain areas, the descending noradrenergic systems provide a putative subcortical relay for descending antinociceptive actions from some forebrain areas (Jasmin et al., 2004). Moreover, the descending analgesic influence triggered by PAG stimulation is partially mediated by recruitment of the descending noradrenergic system (Bajic and Proudfit, 1999; Sim and Joseph, 1992).



Objectives

The general objective of this thesis is to modulate the changes that the spinal circuit of the stretch reflex and suffers after peripheral nerve injury to improve functional recovery. Therefore, it is essential to have a better knowledge of the changes that these circuits and the axotomized motoneurons suffer after injury. On the other hand, it is also fundamental to evaluate potential strategies to modulate these reflex and the changes at the spinal level induced after peripheral nerve injury.

With this aim, we established the following general objectives that would be subdivided in specific objectives:

1. To characterize the spinal changes that axotomized motoneurons suffer after peripheral nerve injury:
   1.1. Evaluate the changes in the spinal circuitry in a postnatal animal model
   1.2. Evaluate the changes in the spinal circuitry in an adult animal model

2. To evaluate different therapeutic strategies to modulate the changes that axotomized motoneurons suffer after peripheral nerve injury:
   2.1. Evaluate the effects of direct application of neurotrophins, such as BDNF and NT-3, to axotomized motoneurons
   2.2. Evaluate different activity dependent therapies on the changes observed after PNI on axotomized motoneurons:
      2.2.1. Study the effects of electrical stimulation of the injured nerve
      2.2.2. Study the effects of forced exercise
      2.2.3. Study the effects of voluntary and passive exercise

3. Evaluate through which mechanism of action treadmill exercise is modulating the plastic changes observed after PNI
   3.1. Study the role of BDNF/ TrkB pathway in the effects of physical exercise
   3.2. Investigate the role of the noradrenergic descendent pathways in the effects of physical exercise

Summary of experimental design and animal groups to achieve the objectives:

1. To characterize of spinal changes that axotomized motoneurons suffer after peripheral nerve injury:

    1.1. Evaluate the changes in the spinal circuitry in a postnatal animal model ***(Chapter 2)***

Injury: sciatic nerve crush	
Study	**Follow up**
Changes surrounding motoneurons after axotomy	1 week (n=4)
	2 weeks (n=4)
	Control (n=4)
Distribution of VGLUT1 synapses on spinal motoneuron dendrites	1 week (n=5)
	2 weeks (n=5)
	8 weeks (n=5)
EMG evaluation	Control (n=5)

    1.2. Evaluate the changes in the spinal circuitry in an adult animal model ***(Chapter 1)***

Study /Injury	**Follow up**
Study of central changes surrounding motoneurons after axotomy (Sciatic nerve section no repaired)	1 week (n=4)
	2 weeks (n=4)
	4 weeks (n=4)
	8 weeks (n=4)
Study of central changes surrounding motoneurons after axotomy (Sciatic nerve section and repaired)	1 week (n=4)
	2 weeks (n=4)
	4 weeks (n=4)
	8 weeks (n=4)

2. To evaluate different therapeutic strategies to modulate the changes that axotomized motoneurons suffer after peripheral nerve injury:

    2.1. Evaluate the effects of direct application of neurotrophins, such as BDNF and NT-3, to axotomized motoneurons ***(Chapter 3)***

Injury: sciatic nerve section repaired with a direct suture	
Treatment	**Follow up**
Proximal and distal stumps sutured with silicone tube with BDNF	1 week (n=4)
	2 weeks (n=4)
Proximal and distal stumps sutured with silicone tube with NT-3	1 week (n=4)
	2 weeks (n=4)
Proximal and distal stumps sutured with silicone tube with NT-3+BDNF	1 week (n=4)

2.2. Evaluate different activity dependent therapies on the changes observed after PNI:

2.2.1. Study the effects of electrical stimulation of the injured sciatic nerve ***(Chapter 3)***

Injury: sciatic nerve section repaired with a direct suture	
Treatment	**Follow-up**
Transcutaneous electrical stimulation (20Hz, 0.1ms pulse duration, supramaximal intensity)	2 week (n=4)

2.2.2. Study the effects of forced exercise ***(Chapter 3)***

Injury: sciatic nerve section repaired with a direct suture	
Treadmill program	**Follow-up**
Low intensity treadmill protocol (LTRP): constant velocity of 10 cm/s	1 week (n=4)
	2 week (n=4)
High intensity treadmill protocol (HTRP): from 10 cm/s to 30 cm/s	1 week (n=4)
	2 week (n=4)
Suppression of homolateral sensory inputs and HTRP	2 weeks (n=9)

2.2.3. Study the effects of voluntary and passive exercise ***(Chapter 3)***

Injury: sciatic nerve section repaired with a direct suture			
Exercise protocol	**Exercise program**		**Follow-up**
Wheel	Low (LW):	4-5 km/day (n=4)	2 week
	Medium (MW):	5-6 km/day (n=4)	
	High (HW):	>10 km/day (n=4)	
Bicycle	Low (LBP):	constant velocity of45 rpm (n=4)	2 week
	High (HBP):	from 45 rpm to 75 rpm (n=4)	

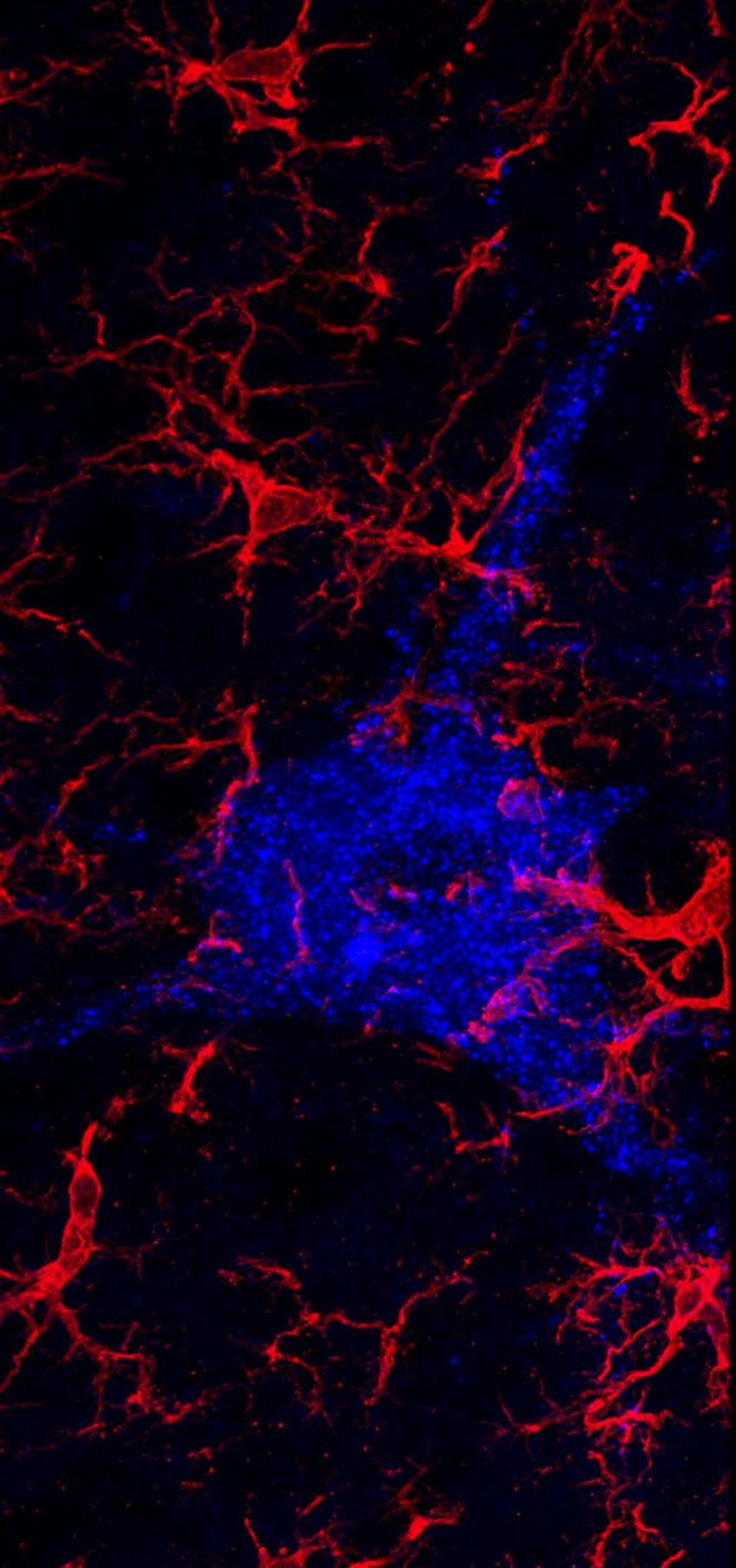
3. Evaluate through which mechanism of action treadmill exercise is modulating the plastic changes observed after PNI:

   3.1. Study the role of BDNF/ TrkB pathway in the effects of physical exercise after PNI ***(Chapter 4)***

Injury: sciatic nerve section repaired with a direct suture			
Group	**Drug treatment**	**Exercise program**	**Follow-up**
TrkB activation	7,8-DHF (5 mg/kg once a day)	No exercise (n=5)	2 weeks
	7,8-DHF (10 mg/kg twice a day)	No exercise (n=5)	
	7,8-DHF (5 mg/kg once a day)	No exercise (n=4)	1 week (WB)
TrkB inactivation	ANA-12 (0.5 mg/kg twice a day)	HTRP (n=5)	2 weeks
		No exercise (n=5)	
Control group	DMSO 17% (twice a day)	No exercise (n=4)	2 weeks
	No drug treatment	HTRP (n=5)	
		No exercise (n=5)	
	No drug treatment	HTRP (n=4)	1 week (WB)
		No exercise (n=4)	
Naïve group	No drug treatment	No exercise (n=4)	1 week (WB)

   3.2. Investigate the role of the noradrenergic descendent pathways in the effects of physical exercise after PNI ***(Chapter 5)***

Injury: sciatic nerve section repaired with a direct suture		
Drug treatment	**Exercise program**	**Follow-up**
DSP-4 administration (50 mg/kg)	HTRP	2 weeks (n=4)
		EMG evaluation: 75 days (n=4)
	No exercise	2 weeks (n=4)
		EMG evaluation : 75 days (n=4)
Control group	HTRP	2 weeks (n=4)
		EMG evaluation: 75 days (n=4)
	No exercise	2 weeks (n=4)
		EMG evaluation: 75 days (n=4)



Chapter 1

Characteritzacion of the spinal changes that adult motoneurons suffer after peripheral nerve injury

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, unspecific reinnervation and the limited reconnection of the spinal circuits impair functional recovery. The aim of this study was to describe the changes that axotomized motoneurons suffer after peripheral nerve injury. We observed a marked decrease in glutamatergic synapses, with a maximum peak at two weeks post-axotomy, which was only partially reversed with time. This decrease was accompanied by an increase in Gephyrin immunoreactivity and a disintegration of perineuronal nets (PNN) surrounding the motoneurons.

INTRODUCTION

Axons in the peripheral nerves are able to regenerate after injury. This capacity constitutes the main basis for the possibility of functional recovery after peripheral nerve lesions. The reinnervation of target organs following nerve injuries is often incomplete, resulting in abnormal motor, sensory, and autonomic functions. When neurons regenerate and reinnervate target organs, they partially recover their synaptic arbor, but in contrast to other excitatory and inhibitory inputs, muscle spindle Ia excitatory synapses, among the most affected by synaptic stripping, never recover baseline levels, even when the muscle spindle and the muscle are correctly reinnervated (Alvarez et al., 2011; Haftel et al., 2005). This reduced connectivity may 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 not only 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, which can be detrimental to functional recovery.

Motoneurons of ventral horn spinal cord are surrounded by extracellular matrix, known as perineuronal nets (PNNs) (Takahashi-Iwanaga et al., 1998). In recent years, several other functions of the PNNs have been revealed, including restriction of

neuronal plasticity, neuronal protection maintenance of synapse (Carulli et al., 2013; Kwok et al., 2011).

The aim of this study was to analyze the changes that axotomized motoneurons suffer after peripheral nerve injury. A better understanding of these changes would make it easier to determine the best protocol to improve functional outcome.

MATERIAL AND METHODS

Experimental animals

Adult female Sprague Dawley rats (n=19, 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 cycle. All experimental procedures were approved by the ethics committee of our institution and followed the European Commission on Animal Care (Directive 2010/63/EEC). 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 1 week before any intervention. Bilaterally, two retrotracers, True Blue Chloride (TB, Setareh Biotech) and Fluorogold (FG, Fluorochrome), were applied to identify both motoneuron pools in the same animal. Firstly, the muscle was exposed, and two injections (2.5 µl/injection) were distributed throughout the body of the muscle with a 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 the mid-thigh and cut by using micro scissors. In a first group of animals (n=16), the transection was not repaired. In another group of animals (n=16), the proximal and distal stumps were re-joined with two epineural sutures. Afterwards, muscles and skin were sutured in layers, iodine povidone was applied to the wound, and the 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).

Immunohistochemical analysis of spinal changes

At the end of follow-up, deeply anesthetized animals were transcardially perfused with 4% paraformaldehyde in PBS. The L3–L6 spinal cord segment was removed, post-fixed for 24h, cryoprotected in 30% sucrose, and stored at 4ºC until use. 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 1). After washes, immunoreactive sites were revealed by using species-specific secondary antibodies conjugated to 488 Alexa Fluor (1:200, Invitrogen), 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***).***

Antigen	Immunogen	Host type	Working dilution	Manufacture
VGlut1	Synthetic peptide from rat VGlut!	Guinea pig polyclonal	1:300	Millipore
Perineuronal nets	Lectin from Wisteria floribunda	----	1:200	Sigma
Gephyrin	Rat Gephyrin aa. 569-726	Mouse	1:200	BD
GFAP	Purified GFAP from porcine spinal cord	Mouse	1:1000	Millipore
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 1. Primary antibodies used in this study.

Image analysis and processing, and regression analysis were performed by means of in-house software implemented in Matlab R2012b (The MathworksInc, 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 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 neighboring 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.

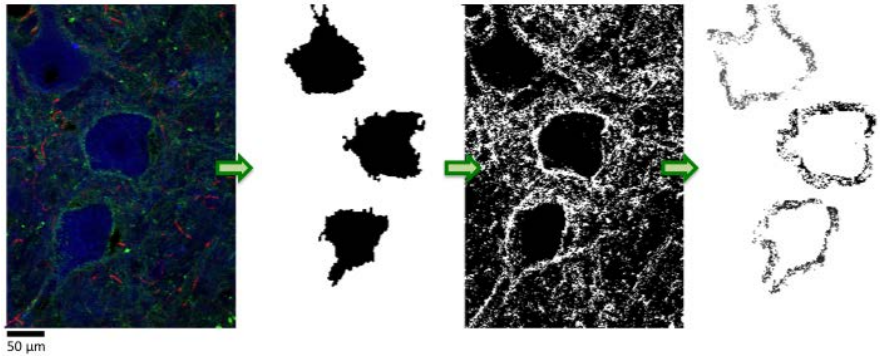


Fig 1. Analysis of the amount of immunostaining surrounding motoneurons. Automatic selection of the somata of back-labelled motoneurons, detection of the immunostaining using a fix threshold for every staining, determination of a circular perimeter area surrounding the soma of a 5µm thickness, and measurement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

Statistical analysis

For quantitative variables, normality was assessed with the 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 with the Kruskall-Wallis test. SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. A value of $P < 0.05$ was considered significant. Due to the interindividual variability previously described for some of the parameters analyzed (Alvarez et al., 2011), the values for each marker are expressed as percentage of decreased/increased density versus the contralateral side of each animal. For the relevant values, experimental power was estimated using R version 3.1.1 (free software).

RESULTS

Measurement of the size of motoneuron somas indicated that TA motoneurons had slightly lower areas (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 from 130 to 160 μm^2 in TA and from 170 to 200 μm^2 in GM motoneuron pool. VGlut1 density was also similar in all the neurons, ranging from 100 to130 μm^2 for TA and from 140 to170 μm^2 for GM motoneurons. In TA motoneurons, Gephyrin density ranged from 35 to 45 μm^2 and in GM ones from 50 to 70 μm^2. 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. The experimental power estimated for the relevant data (Syn, VGlut and PNN measurements) was about 0.8 in the different experiments.

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 in Synaptophysin density, with a maximum reduction at 2 weeks, and a progressive recovery at 4 and 8 weeks (Fig 2.2A). 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 to those for controls. VGlut1 immunoreactivity was also decreased at all post-injury times, with a negative peak at 2 weeks (Fig 2.3A). Comparatively, the decrease in VGlut1 density was greater 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 VGlut1 levels still remained significantly lower (27±2%) than in the contralateral side

(Fig 2.3A). In contrast to Synaptophysin and VGlut1 immunoreactivity, Gephyrin labeling surrounding the axotomized motoneurons was significantly increased at all times post-injury ($p< 0.05$) compared to the intact contralateral motoneurons. Again, the maximum changes were observed at 2 weeks, with a peak increase of 42±6% in TA and 45±5% in GM motoneurons. At 8 weeks, the Gephyrin density remained slightly increased by about 10% (Fig 3.2A).

Perineuronal nets and glial cell reactivity

After peripheral nerve injury, the PNN surrounding axotomized neurons showed signs of destructuring (Fig 3). When assessing the immunoreactivity of Wisteria Floribunda, which specifically labels PNN, in a 5µm-wide perimeter surrounding the motoneuron, we observed a significant decrease in 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 4.2A). Partial recovery was observed at 4 and 8 weeks, with 7±1% in TA motoneurons and 20±2% in GM motoneurons at 8 weeks.

To evaluate the role of glial cells in the stripping process, we also performed immunolabeling against Iba1 to identify microglia and against GFAP to label astrocytes. In non-injured motoneurons, the presence of glial cells close to the soma was minimal, but 1 week after axotomy we observed a marked increase in microglia immunolabeling (280±20 Iba1/µm^2, $p< 0.01$), which decreased at 2 weeks post-injury (80±10 Iba1/µm^2, $p< 0.05$) (Fig 4.1A). 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 markedly at 8 weeks post-injury (270±20 GFAP/µm^2, $p< 0.01$) (Fig 4.1B).

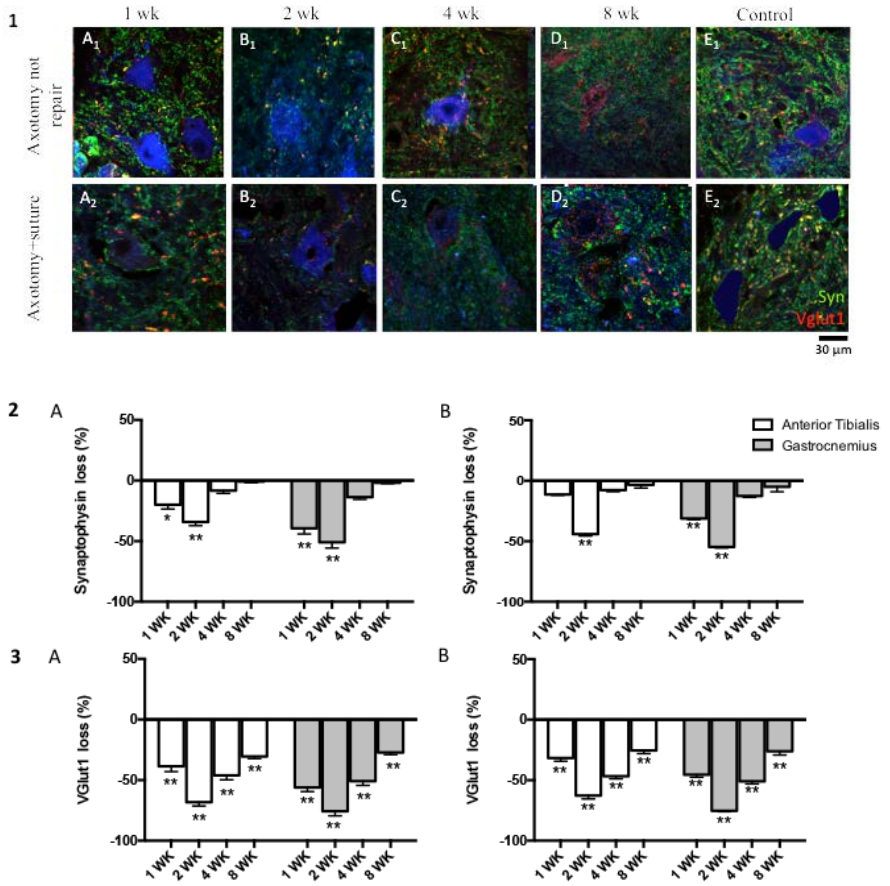


Fig 2. Evaluation of synaptic stripping after axotomy. 1. Synaptophysin (green) and VGlut1 (red) immunostaining were evaluated in confocal images of spinal cord regions containing back-labelled motoneurons (blue) from TA and GM at 1 week (A), 2 weeks (B), 4 weeks (C), and 8 weeks (D) after nerve cut (axotomy not repaired) or nerve cut repaired with direct suture (axotomy+suture). Control motoneurons (E_1, E_2) show strong density of all labelings. **2/3.** 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 back-labelled motoneurons from TA (white bars) and GM (grey bars). VGlut1 and Synaptophysin reach a low point of depletion 2 weeks after injury in both motor pools, and then recover with time. In contrast to Synaptophysin, VGlut1 never returns to baseline 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).

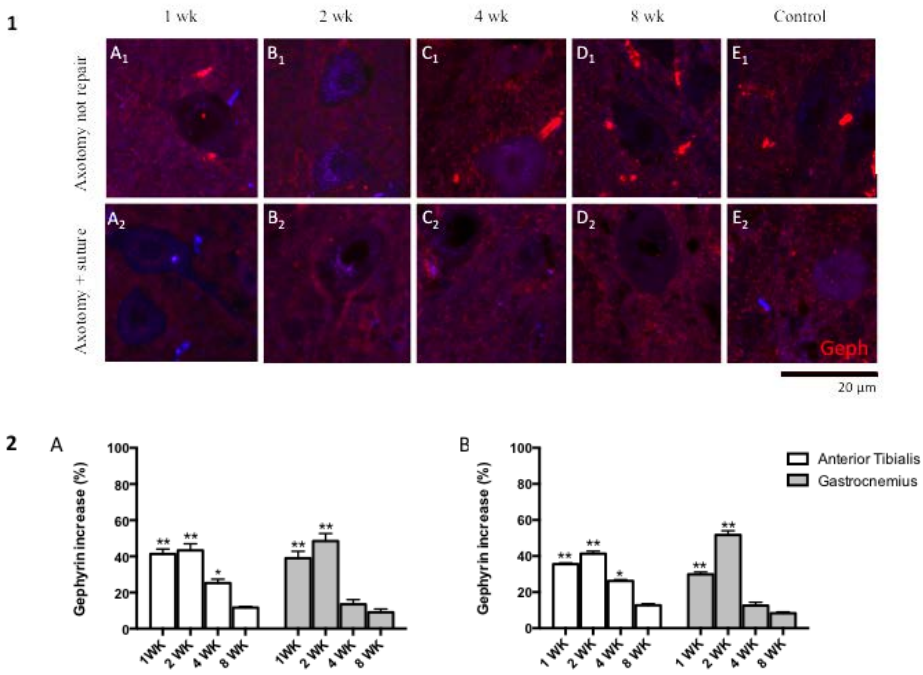


Fig 3. Gephyrin staining in axotomized motoneurons. 1. Confocal images of spinal cord regions stained against Gephyrin at 1 week (A), 2 weeks (B), 4 weeks (C), and 8 weeks (D). Back-labelled motoneurons from TA and GM in blue, Gephyrin in red. Control motoneurons (E_1, E_2) show less immunoreactivity to Gephyrin, whereas after axotomy, there is an increase with 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 back-labelled 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 in 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).

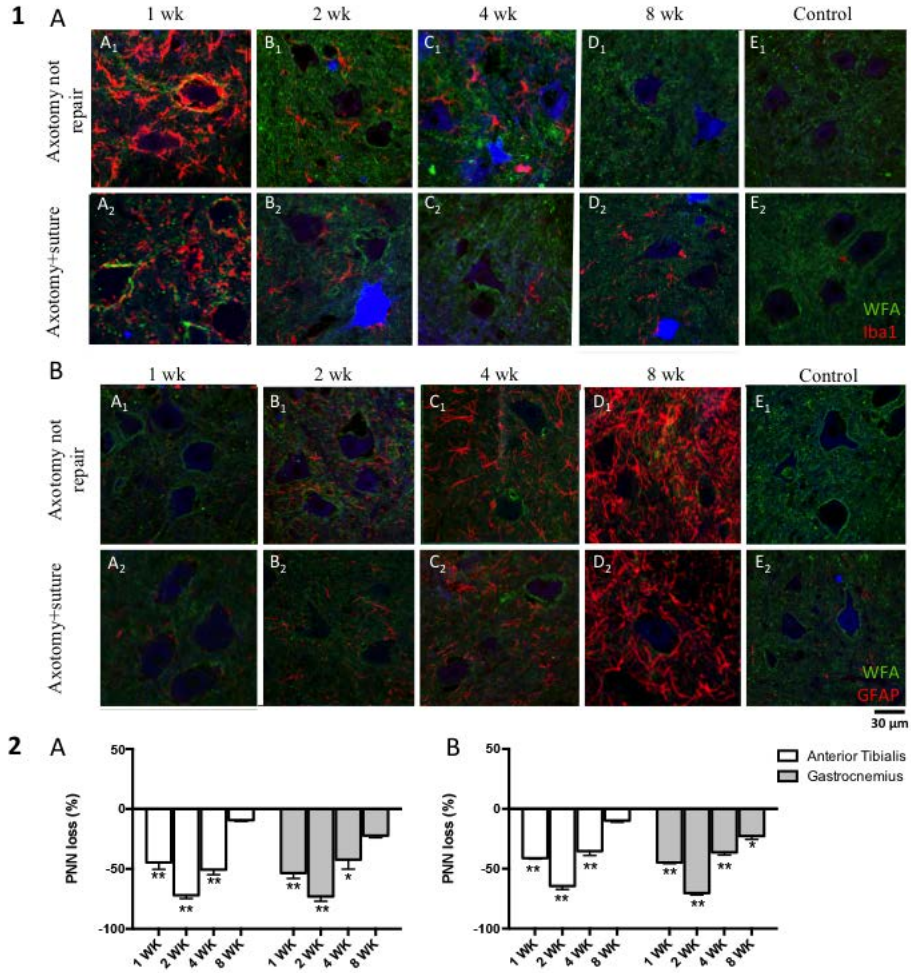


Fig 4. Glia and PNNs surrounding axotomized motoneurons. **1**. Confocal images of spinal **c**ord regions stained for microglia or astroglia and PNNs 1 week (A), 2 weeks (B), 4 weeks (C), and 8 weeks (D) after injury 1A. Back-labeled motoneurons from TA and GM in blue, PNNs (Wisteria Floribunda) in green and microglia (Iba1, panel A), and astrocytes (GFAP, panel B) in red. Control motoneurons (E_1, E_2) have no glial processes around their somas, 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 back-labeled motoneurons from TA (white bars) and GM (grey bars). Repair of the nerve does not change the evolution of PNN destructuring, with a maximum loss of staining at 2 weeks. There is a marked increase in microglial processes around axotomized motoneurons 1 week after injury, which decreases with time; whereas astroglia processes 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).

Effects of nerve repair on spinal synaptic changes of axotomized motoneurons

In order to analyze whether repair of the nerve section, that favors axonal regeneration, affects 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 axotomy only and those with axotomy and repair (Figs 2-4).

DISCUSSION

In this work 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 also observed a marked decrease in proximal glutamatergic synapses, with a maximum peak at 2 weeks post-axotomy, which was partially reversed with time. This decrease was accompanied by an increase in Gephyrin immunoreactivity and a disintegration of PNN surrounding the motoneurons.

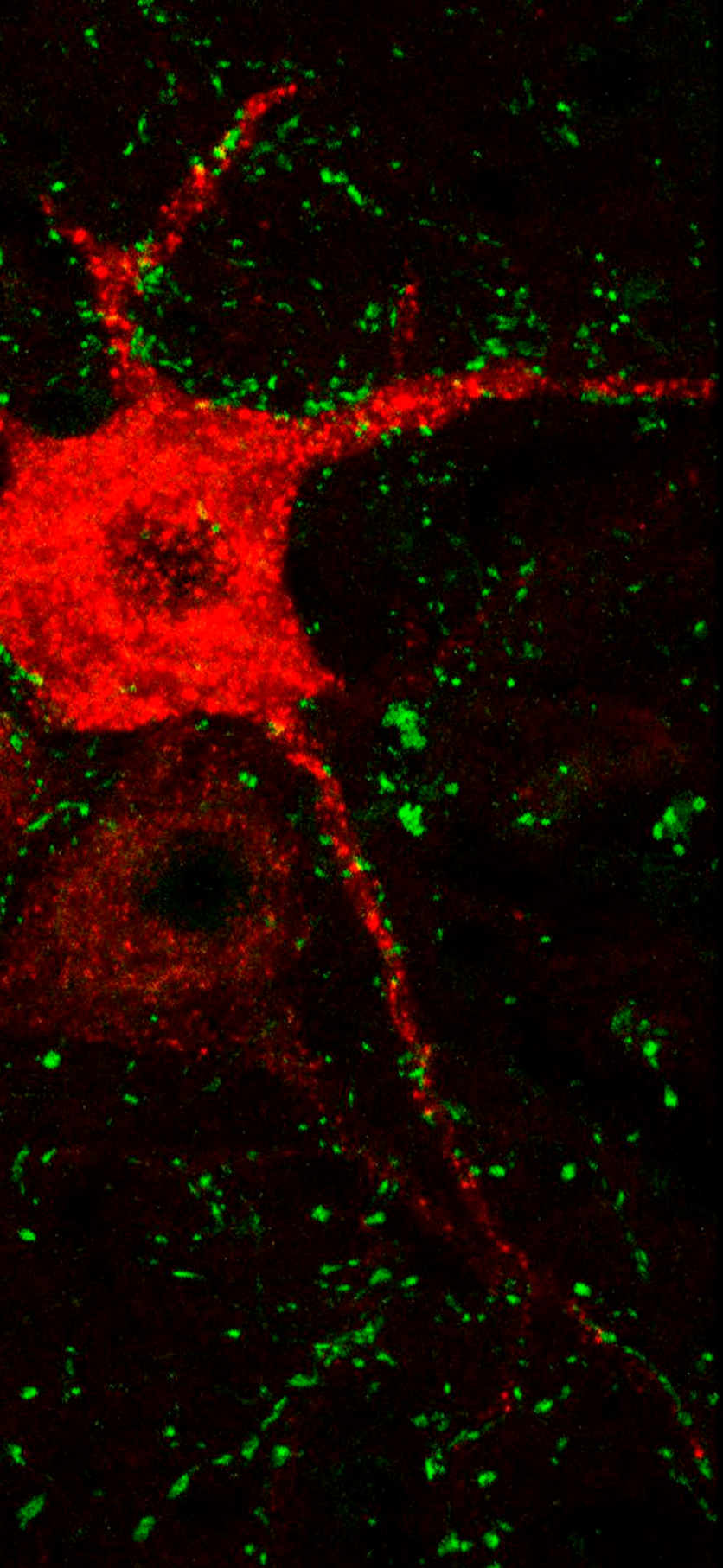
Due to the high variability described in the synaptic content of motoneurons (Alvarez et al., 2011, 2010), we specifically evaluated two motoneuron pools, the extensor GM and the flexor TA, both innervated by the sciatic nerve, and we compared the changes induced by axotomy with the contralateral intact side. In fact, we observed greater synaptic depletion in axotomized GM motoneurons. For synaptic characterization, we used an immunohistochemical approach to identify different synaptic populations. Although these are indirect markers of innervation, they normally correlate well with the presence of terminals measured by electron microscopy (Rask-Andersen et al., 2000; von Krosigk et al., 1992).

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 related to distribution of information to one where mechanisms for survival and repair

are warranted (Aldskogius 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 in microglial processes surrounding 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 lowest point at 2 weeks. This reduction may be partially attributed to the loss of glutamatergic synapses, among the synapses most affected by stripping after injury (Alvarez et al., 2011; Novikova et al., 2000). VGlut1 is a specific marker for Ia sensory afferents from the muscle spindle (Todd et al., 2003), a key element in the stretch reflex circuit. The group of Cope (Alvarez et al., 2010) proposed that VGlut1 synapses never recover after injury and that 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 recovered baseline levels after axotomy. In parallel to the VGlut1 decrease, we observed a marked increase in Gephyrin immunoreactivity around motoneurons. Gephyrin organizes the postsynaptic density of the inhibitory GABAergic and glycinergicreceptors (Fritschy et al., 2008; Tretter et al., 2008; Yu and De Blas, 2008), and it is a key inhibitory scaffolding protein. Up-regulation of Gephyrin may be a protective action to compensate for the increased excitability observed in motoneurons after axonal injury.

Accompanying the synaptic stripping induced by axotomy, we observed disorganization in the PNN surrounding injured motoneurons. A previous paper pointed out that motoneurons do not lose PNN after nerve injury (Kalb and Hockfield, 1988), although they did not perform a quantitative analysis of these nets. In fact, we were still able to detect PNN staining around axotomized motoneurons, although there was a clear reduction of immunoreactivity, with a minimum point at two weeks.

It seems logical that these nets which contribute to stabilize synapses, are affected by the process of synaptic stripping.



Chapter 2

Characteritzacion of the dendritic arbor and the spinal changes that postnatal motoneurons suffer after peripheral nerve injury

Abstract

After peripheral nerve injury (PNI), the interruption between the axons and their peripheral target organs is accompanied by a disorganization of the central circuitry, in part due to the massive stripping of central synapses that axotomized motoneurons suffer in adult rats. One of the synapses more affected is the proprioceptive Ia afferent, that directly synapses with the motoneuron, constituting the stretch reflex, a key player in the neuromuscular self-control. In the adult, this central Ia synapses never recover basal values. In this study, we want to evaluate how a PNI is affecting postnatal motoneuron, that suffer the injury at p10. At 7 and 14 dpi, there is a massive loss of synapses, mainly VGlut1, accompanied with an increase of inhibitory ones (VGat). Microglia and astroglia around motoneurons are markedly reactive already at one week post injury. PNN are still not well developed at early postnatal stages, whereas can be clearly detected at 24 postnatal day. In contrast, motoneurons that received the injury 14 days before did not have defined PNN at these stage. Two months after the injury, electrophysiological tests showed that motor reinnervation recovered basal values in proximal muscles. H amplitude got normalized with time, even when VGlut1 contacts were not completely recovered.

*Study in collaboration with **Dr. Alvarez** (Alvarez group), partially realized in **Emory University School of Medicine**, Atlanta (EUA) during a 4 month stage.*

INTRODUCTION

The outcomes after a peripheral nerve injury (PNI) can be very different depending on the age of the patient. In the adult, injured axons are able to regenerate and eventually reinnervate their target organ. In contrast, if the injury is suffered during the postnatal period, massive loss of peripheral neurons can occur. In experimental models, it is assumed that motoneurons are most susceptible to die between 1-5 days postnatal (Lowrie and Vrbová, 1992).

Even if peripheral neurons survive to axotomy and regenerate, functional recovery is usually poor. This discordance between good regeneration and bad functionality is mainly due to problems in the specificity of reinnervation of target organs, but also to disorganization of the central circuitry. Nerve injury induces a massive loss of synapses in motoneurons, the so called synaptic stripping, with alterations in the balance of excitatory and inhibitory synaptic content (Haftel et al., 2005; Novikova et al., 2000). One of the synapses that suffers a more drastic reduction is the one from Ia proprioceptive afferents, that never will recover basal values again, thus leading to a poor recovery of a functional stretch reflex (Alvarez et al., 2011).

In contrast to the adult, no studies have evaluated the changes that axotomy is inducing in postnatal motoneurons that survive the injury. Most of the studies at this stages have evaluated the susceptibility of these neurons to death (Kashihra et al., 1987; Kemp et al., 2015; Miyata et al., 2012). It is know that motoneuron dependence on target contact decreases during postnatal development (Lowrie and Vrbová, 1992). It has long been established that immature motoneurons are critically dependent upon interaction with their target muscle for survival and normal development (Hamburger, 1934; Low et al., 2003). However, how the injury will affect the motoneurons that survive to the injury during the early postnatal period and its consequences on the synaptic content of the dendritic arbor have not been addressed. Besides the synaptic stripping that motoneurons suffer after the injury, there is also an important glial reactivity around their somas.

It is known that the pattern of activation of microglia and astrocytes differs between adult and young rats (Vega-Avelaira et al., 2007), but how these differences

can influence the fate of the spinal circuitry following axotomy remains unknown. Another key element to take into account when analyzing the integrity of the spinal circuitry after injury are the PNNs, whose main function is stabilize synapses and limit plasticity in the adult nervous system. In fact, PNNs appear in the postnatal period and end the critical periods of different brain functions during which synapses are still open to experience (Foscarin et al., 2011) by blocking the formation of new synapses (Brückner et al., 1993). In the adult, PNI induces a reduction in the thickness of the PNN that surround motoneurons, thus facilitating disorganization of the spinal circuitry. It is not addressed in the literature how a PNI in the postnatal period, when PNN are still in development, would affect the maturation of the spinal circuits and the function of these in the future.

Therefore, the purpose of this study is to characterize the central changes that motoneurons suffer 7 and 14 days after sciatic nerve crush in 10 days old rats. At this age, about 60% of the injured motoneurons survive and thus, it is quite relevant to evaluate what happen on these neurons and how these central changes could affect long term functional recovery.

MATERIAL AND METHODS

Experimental animals

Adult female Sprague Dawley rats (n = 20, 10 days old, 40-55 g) were housed with free access to food and water at room temperature of 22 ± 2 °C under a 12:12-h light–dark cycle. All experimental procedures were approved by the ethics committee of Universitat Autònoma de Barcelona and followed the guidelines of the European Commission on Animal Care (EU Directive 2010/63/EU). For surgical intervention, neonatal rats were anesthetized rats were anesthetized by intraperitoneal administration of ketamine (0.3 ml/kg; Imalgen 2000) supplemented with xylazine (0.17 ml/kg; Rompun 2%). Animals were divided into different groups depending on what were aimed for and the follow-up required (Table 1).

Study	Follow-up
1.Changes surrounding motoneurons (right sciatic nerve crush)	7 dpi (n=4)
	14 dpi (n=4)
2. VGlut1 along dendrite analyses (right sciatic nerve crush)	7 dpi (n=5)
	14 dpi (n=5)
	60 dpi (n=5)
3. Electrophysiology (right sciatic nerve crush)	60 dpi (n=6)
4. Naïve group	7 dpi (n=5)
	14 dpi (n=5)
	60 dpi (n=5)

Table 1. Experimental design. Groups and follow up time. Animals were divided into 3 main experimental groups. In all of them except naïve group, sciatic nerve was crushed. **1**. Analysis of central changes after PNI. One subgroup was followed-up for 1 week and other one for 2 weeks. **2.** Analyses of VGlut1 distribution along dendrites Animals were divided based on follow-up times: 7,14 and 60 dpi. A subgroup of animals was used as a naïve group. **3.** Electrophysiology study. A group of animals were followed-up for 60 dpi to study CMAP and H/M amplitude ratio. **4.** Naïve group: non injured animals were used as control for all the analyses.

Retrograde labeling

To identify motoneuron pools from gastrocnemius medialis muscle (GM) retrograde tracing was applied to the muscle 5 days before any intervention. In all the animals followed up 1 and 2 weeks post injury, 1% CTb (Sigma–Aldrich, Taufkirchen, Germany) was applied bilaterally with a glass pipette to identify both motoneuron pools in the same animal. In the group of animals follow up for 8 weeks, 2.5% Fast Blue (Polysciences, Eppelheim, Germany) was injected. Two injections (1 µl/injection) were distributed throughout the body of the muscle with a glass pipette.

Surgical procedure

At 10 postnatal days, animals where anesthetized and the sciatic nerve was exposed at the mid-thigh and crushed during 30 secs in three different orientations by means of a fine forceps. Afterwards, skin was sutured in layers, iodine povidone was applied to the wound, and the rats were allowed to recover in a warm environment under close observation. Animals were followed for 7, 14 and 60 days. A subgroup of animals was not injured and used as a naïve group.

Electrophysiology tests

For the long term follow-up, motor reinnervation and H reflex were assessed by means of nerve conduction tests 30 and 60 days after surgery, using an electromyography apparatus (Synergy Medelec, Viasys HealthCare). Electrophysiological evaluation was performed under anesthesia (0.9 ml/kg; Imalgen 2000) supplemented with xylazine (0.5 ml/kg; Rompun 2%) anesthesia.

During the test, the rat body temperature was kept constant between 34 and 36°C by means of a thermostated flat coil. The sciatic nerve was stimulated by two needle electrodes percutaneously inserted at the sciatic notch, applying single rectangular pulses of 0.1 ms duration up to the voltage required to obtain a maximal evoked response. The compound muscle action potentials (CMAP) evoked by stimulation of motor nerve fibers were recorded from the TA, GM and plantar (PL) muscles with microneedle electrodes. All potentials were amplified and displayed at the appropriate settings for measurements of the onset latency and the amplitude from baseline to the maximal negative peak of the direct M wave and the reflex H wave. The maximal H/M amplitude ratio was calculated for each muscle tested.

Histological processing and immunohistochemistry

At the end of short-term follow-up, deeply anesthetized animals were transcardially perfused with 4% Paraformaldehyde in PBS. The L3–L6 spinal cord segment was removed, cryoprotected in 30% sucrose and stored at 4 °C until use.

Changes surrounding motoneurons analyses

Samples were embedded in Tissue-Tek, serially cut (20 µm thickness) in the transverse plane with a cryostat, and collected onto gelatin-coated glass slides. All sections were first blocked with 10% normal bovine serum for 1 h, followed by overnight incubation at 4 °C with combinations of primary antibodies: rabbit anti Synaptophysin (1:200, Covance), guinea pig anti VGlut1 (1:300, Millipore), guinea pig anti VGat (1:200, Synaptic Systems), Lectin from Wisteria Floribunda (1:100, Sigma),

mouse anti GFAP (1:1000, Millipore) and rabbit anti Iba1 (1:500, Wako). After washes, immunoreactivity sites were revealed by using species-specific secondary antibodies conjugated to 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 40×/1,3 Oil DIC M27).

Image analysis, processing and regression analysis from motoneurons labeling quantification 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 a perimeter of 5μm thickness surrounding the soma as we described previously. For each animal, 10 to 15 motoneurons of each pool and each side were analyzed.

VGlut1 contacts along dendrite analyses

Parasagittal longitudinal sections were obtained serially at 50 μm thickness on a vibratome (VT-1000) and processed to reveal VGlut1 immunofluorescence. The sections were collected for free floating and after blocking with 10% normal donkey serum for 1 h they were incubated overnight in a solution containing VGlut1 anti rabbit antibody (diluted 1:1000; Synaptic Systems) diluted in 0.01 M phosphate buffer with 0.3% Triton X-100 (PBS-TX0.3). The following day the sections were washed in PBS and incubated for 2 h in donkey anti-rabbit IgG antibodies conjugated to fluorescein isothiocyanate (FITC; Jackson Immunoresearch) diluted 1:100 in PBS-TX0.3 and then washed in PBS, mounted serially on glass slides, and coverslipped with Vectashield (Vector Laboratories).

Confocal imaging and neuron reconstruction

Labeled motoneurons were localized and images captured with a scanning confocal microscope Olympus FV1000 using a 60x objective (NA, 1.35, oil-immersion) with no digital zoom applied, to observe VGlut1 contacts along the dendrites. Since the field-of-view includes only a small region of the whole dendritic arbor in the section, we imaged the whole section using overlapping image tiles, each containing a z-stack of all optical planes (z-step=1 um). Up to 42 tiles were necessary in some sections to image all dendritic segments. For each animal, 5-6 motoneurons of each side were analyzed.

Neuron reconstructions for VGlut1 contacts along dendrite analyses

Sections were mounted in Neurolucida (v10.0, Microbrightfield) from the z-stack image tiles and all labeled dendrites traced. The position of each synapse was labeled using a "marker" that was then "attached" to that dendritic position. The cell body was reconstructed through a series of contours traced in each optical plane. Once all labeled dendrite and axon segments were traced in single sections and their synapses marked, we reconstructed the whole neuron by manually splicing dendrite and axon pieces through serial sections.

The traced neurons and their synaptic markers were analyzed using Neuroexplorer (v10.0, Microbrightfield). All basic morphological features were measured based on previous papers from the group of Alvarez (Alvarez et al., 2011, 2010; Rotterman et al., 2014). Briefly, cell body surface, volume, aspect ratio, and diameters were measured. For dendritic arbor: number of primary dendrites, overall surface, total length, number of branches and dendrite segments, number of dendrites of different order, maximum path distance from the cell body, and maximum branch order. The distribution of synapses was analyzed by using path distance measurements and also Sholl analysis (Fig 1.3) to further test VGlut1 distributions along dendrites.

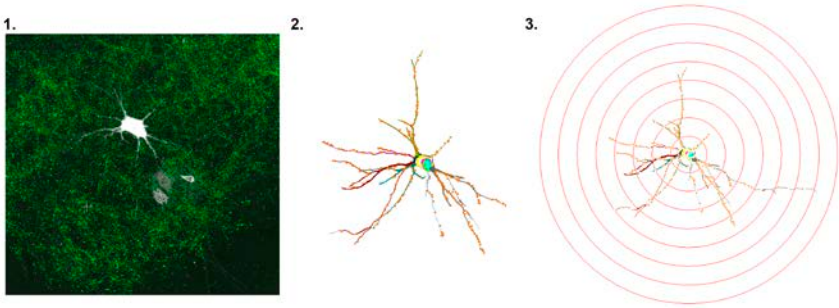


Fig 1. Neuron reconstructions and Sholl analysis procedure. **1.** Confocal image of VGlut1 contacts (Green) and GM motoneurons labeling by CTb (White). **2.** Dendritic and VGlut1 contacts delineation using Neurolucida software. **3.** Sholl Analysis: quantitative analysis in which concentric spheres are placed in increasing radii around the motoneuron. The amount of dendritic length and number of synapses in each bin were then computed.

Statistical analysis

For quantitative variables, normality was assessed with the 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 with the Kruskall-Wallis test. SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. A value of $P < 0.05$ was considered significant. Due to the interindividual variability previously described for some of the parameters analyzed (Alvarez et al., 2011), the values for each marker are expressed as percentage of decreased/increased density versus the contralateral side of each animal.

RESULTS

Spinal synaptic changes induced by axotomy of motoneurons

In the 5µm perimeter surrounding the soma of postnatal gastrocnemius (GM) motoneurons, the average density of synaptophysin ranged from 172 to 180 contacts/µm^2. VGlut1 density was also similar in all the neurons, ranging from 150 to 174 contacts/µm^2. No significant differences were observed in Syn and VGlut1

immunolabeling between control animals and contralateral sides and consequently, values obtained at the contralateral side were used as control.

Synaptic stripping was studied 7 and 14 days after crushing the sciatic nerve, by comparing the immunoreactivity against Synaptophysin (Syn) and VGlut1 between the injured and the contralateral sides. Motoneurons showed an important decrease in Syn density (around 50%) already 7 dpi post injury, that was similarly low at 14 dpi (Fig 2.1). Moreover, VGlut1 immunoreactivity was also decreased at all post-injury times, with no significant differences between 7 and 14 dpi (Fig 2.1); it is important to note that the decrease in VGlut1 density was greater than for Syn. Regarding inhibitory synapses, immunolabeled using VGat, we observed a 30-40% increase of immunostaining after crush injury at both 7 and 14 dpi (Fig 2.1).

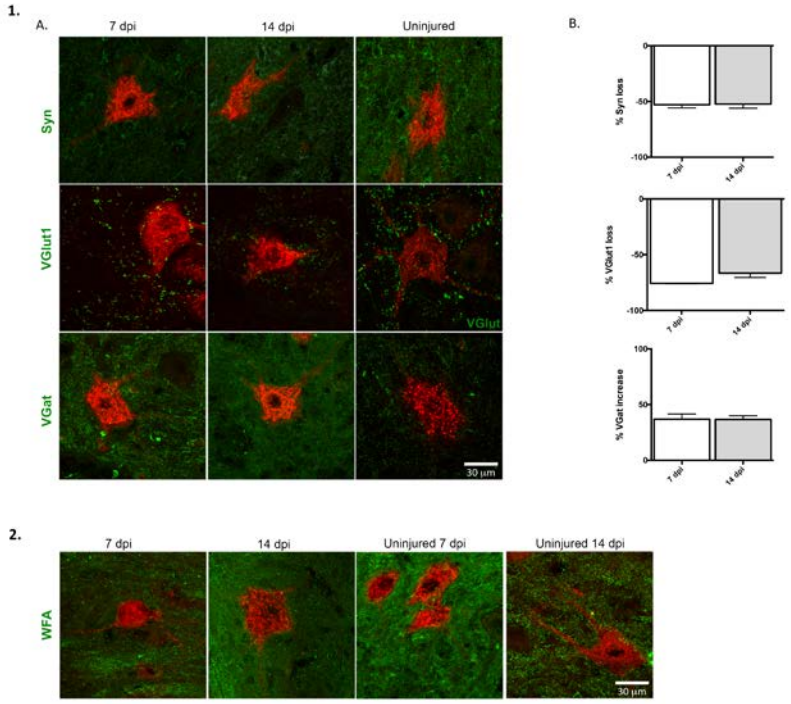


Fig 2. Evaluation of synaptic stripping after axotomy. 1A. Synaptophysin, VGlut1 and VGat immunostaining were evaluated in confocal images of spinal cord regions containing back-labelled motoneurons (red) from GM at 1 and 2 weeks post injury. **1B.** Quantitative analysis of motoneuron synaptic stripping after axotomy. Evaluation of Syn, VGlut1 and VGat staining at 1 week and 2 weeks after axotomy. **2.** WFA immunostaining to evaluate PNN changes after injury. (Data are expressed as mean ± SEM, *p<0.05, **p<0.01).

Regarding PNN around motoneurons, immunostaining for WFA was not clearly defined in the contralateral side neither at 10 nor 17 postnatal days. Consequently, an accurate quantification of these PNN and comparison with the contralateral injured side was not possible. However, at 24 postnatal days a clear structure reactive against WFA could be observed around motoneurons. In contrast, in the contralateral injured side, no PNN could be observed, thus indicating that axotomy was affecting the normal development of spinal PNN (Fig 2.2).

Glia reactivity

To evaluate glia reactivity around injured motoneurons, we identified microglia using Iba1 and astroglia using GFAP immunolabeling. In this case, significant differences were observed between the contralateral side of injured animals and naïve animals. While non-injured animals had no Iba1 activation surrounding motoneurons, the contralateral side of axotomized animals showed an increase of microglia (140±10 Iba1/µm^2, $p< 0.01$), which was also present, although slightly decreased, 2 weeks post-injury (102±15 Iba1/µm^2) (Fig 3). In parallel, a marked increase of Iba1 immunollabeling was observed in injured motoneurons (272±25 Iba1/µm^2) at one week, increase that was significant compared to contralateral side ($p<0.05$). 14 days after injury, microglia reactivity at the homolateral side was lower (172±5 Iba1/µm^2), but still significantly higher compared to the contralateral side.

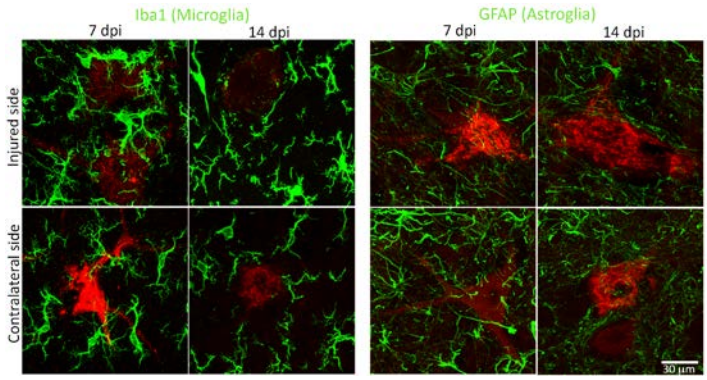


Fig 3. Microglia and astroglia surrounding axotomized motoneurons. **1.** Confocal images of spinal cord regions containing back-labelled motoneurons (red) stained for microglia (A) and astroglia (B) at 7 and 14 days post injury in both injured and contralateral side.

On the other hand, a high astrocytic reactivity was observed at all times post injured and in both injured and contralateral side, without significant differences between groups (212±35 GFAP/μm^2) (Fig 3).

VGlut1 contacts along dendrite analyses

Firstly, the distribution of VGlut1 contacts in the dendritic arbor of intact motoneuron was analyzed in 10 and 24 days postnatal animals. When we compared the different parameters analyzed in naive animals with the ones obtained from the contralateral side of injured animals, no differences were found, (data no shown), thus validating the use of the contralateral side as a "control". Therefore, the changes on excitatory synapses were analyzed 7, 14 and 60 days after crush of the sciatic nerve, by comparing the immunoreactivity against VGlut1 between the injured and the contralateral side.

The density of VGlut1 synapses were significantly decreased in injured MNs compared to contralateral side ($p>0.01$) at all times post injury. Control MNs received an average of 90±33 VGlut1 contacts over the entire dendritic arbor and an average of 25±13 on the cell body. In contrast, injured MNs showed an average of about 44±33 VGlut1 contacts over the entire dendritic arbor and of about 10±6 on the cell body both 7 and 14 days post axotomy. However, 2 months after injury, there was an increase of VGlut1 contacts in axotomized motoneurons (65±25 and 7±6 VGlut1 contacts over the entire dendritic arbor and on the cell body, respectively) but these values were still lower than the ones from the contralateral side. In fact, intact contralateral motoneurons also had an slightly increased amount of VGlut1 contacts (98±38 VGlut1 contacts over the entire dendritic arbor and an average of 31±10 on the cell body) compared with intact motoneurons at 17 and 24 days postinjury. It is important to note that the distribution of VGlut1 synapses over the dendritic arbors were not uniformly distributed on the dendrites of MNs, with the highest numbers located proximal to the cell body. Consequently, the changes were analyzed in the proximal 50, 100 and 150 µm dendrites, using both linear and Sholl analyzes.

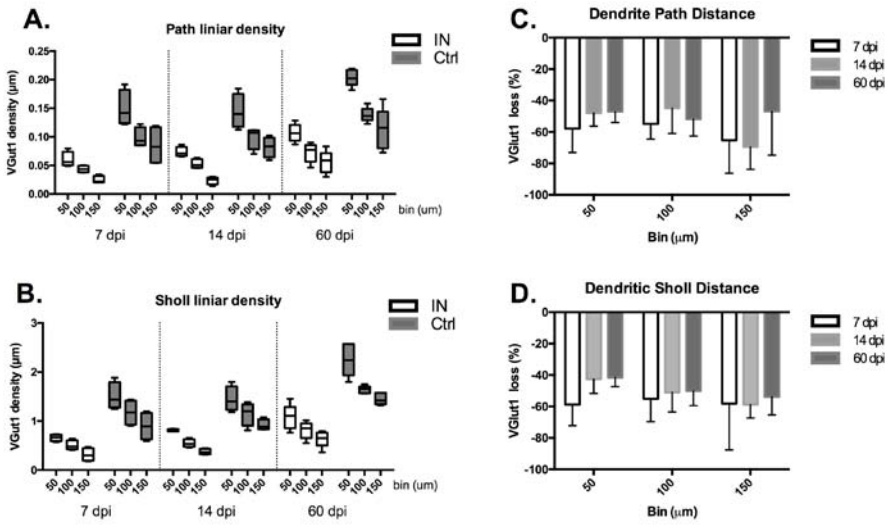


Fig 4. Distribution of VGlu1 contacts in different bins 7, 14 and 60 days post injury based on linear density. **A.** Path linear VGlut1 density**.** Injured groups of animals are showed in whit bars, controls ones in grey. **B.** Sholl linear VGlut1 density. Injured groups of animals are showed in whit bars, controls ones in grey **C.** Percentage of path linear VGlut1 contacts loss after 7 dpi (white bars), 14 dpi (grey bars) and 60 dpi (dark grey bars). **D.** Percentage of Sholl linear VGlut1 contacts loss after 7 dpi (white bars), 14 dpi (grey bars) and 60 dpi (dark grey bars). Data are expressed as mean ± SEM.

The number of VGlut1 contacts was divided by the available length of dendrites (linear density) or dendritic surface area (surface density). In length of dendrites path analysis, control motoneurons showed a VGlut1 average of 0,26±0,02 in 50µm and 0,10±0,03 in 100 and 150 µm distance from dendrite origins. In contrast, in first 50 µm proximo-distal bins dendrite origins linear density estimates 0,15±0,05 VGlut1 contacts in 7 dpi injured MNs, whereas the range was from 0,02 to 0,05 in both 100 and 150µm of distance. After 14 dpi, linear density was 0,060±0,02 VGlut1 contacts in the first 50 µm and 0,04±0,02 VGlut1 contacts in 100 and 150 µm of distance. 60 dpi, linear density was 0,10±0,02 VGlut1 contacts in the first 50 µm, 0,07±0,03 VGlut1 contacts in 100 µm of distance and 0,06±0,02 in the last and 150 µm (Fig 4A,6). This decrease of VGlut1 contacts in injured motoneurons was around 40-60% compared to contralateral side and was similar at all times post injury (Fig 4C).

When Sholl linear distance was studied, a very similar pattern from path distance was reported (Fig 4B,4D).

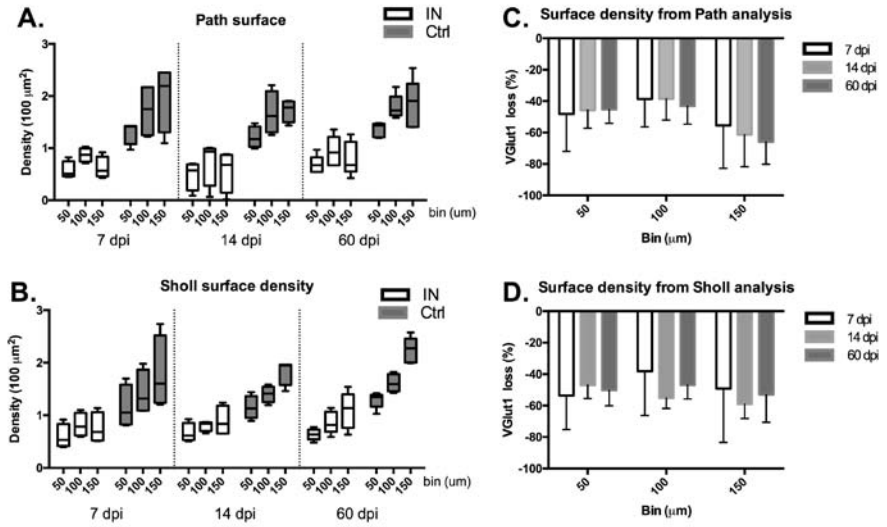


Fig 5. Distribution of VGlu1 contacts in different bins based on surface density at 7, 14 and 60 days post injury. A. Path surface VGlut1 density. Injured groups of animals are showed in white bars, controls ones in grey- **B.** Sholl surface VGlut1 density. Injured groups of animals are showed in white bars, controls ones in grey. **C.** Percentage of VGlut1 contacts loss in surface density from Path analysis after 7 dpi (white bars), 14 dpi (grey bars) and 60 dpi (dark grey bars). **D.** Percentage of VGlut1 contacts loss based on surface Sholl analyses after 7 dpi (white bars), 14 dpi (grey bars) and 60 dpi (dark grey bars). Data are expressed as mean ± SEM.

In control MNs, surface densities at 50 µm distance from dendrite origins were, on average, 1±0,5 VGlut1/µm^2 whereas at 100 µm and 150µm were of about 1,9±0,4 VGlut1/µm^2(Fig 6). After injury, although VGlut1/µm^2 contacts loss was slightly higher in the first 50 µm, no significant differences were obtained between different distances; in all of them we reported a loss of around 50% (Fig 4C). Similar conclusions were obtained using Sholl analyses in bins of 50 µm incremental distances from the cell body center (Fig 4D). Close parallelism between path distance and Sholl analyses are expected because MN dendrites fan out linearly in radial directions from the cell body center (Fig 5). Sholl bins in the first 0-50 µm from the cell body averaged 1,16 VGlut1/µm^2 of available surface in control motoneurons, and 1,42 and 1,78 VGlut1/µm^2 in 100 and 150 µm respectively, whereas in injured motoneurons, we

again observed a loss of around 40-50% in all distances (average of 0,59 VGlut1/µm^2 in first 50 µm from the cell body, and 0,81 and 1,03 VGlut1/µm^2 in 100 and 150 µm) (Fig 6).

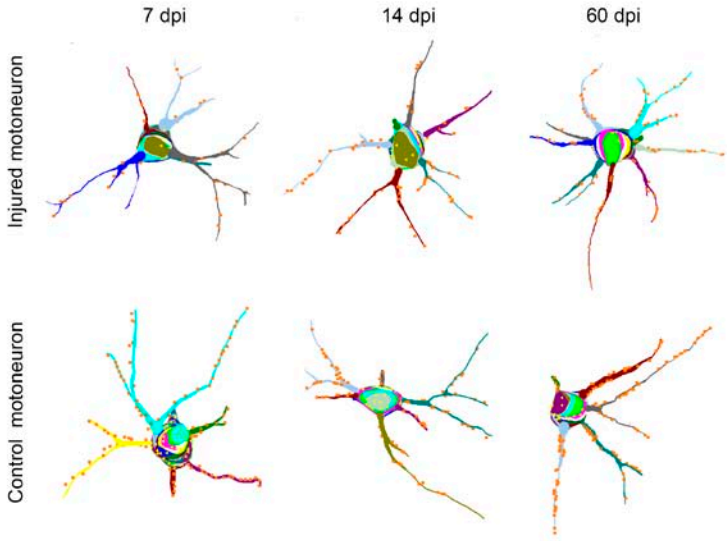


Fig 6. Neurolucida reconstruction of both injured and control motoneurons at different times postinjury. Different dendrites are represented with different colors. Orange plots indicate a VGlut1 contact along the dendrite, whereas yellow ones indicate VGlut1 on the soma.

Muscle reinnervation

Electrophysiological studies were performed to evaluate motor reinnervation 2 months after the injury. M values of the muscles at the contralateral side was similar in all animals, with no differences between groups. In the injured side, at 30 dpi, M values for GM and TA were about 32±8 and 22±2 mV respectively, compared to 50±5 mV in GM and 32±1 mV in TA recorded in the contralateral side. In contrast, at 60 dpi, amplitudes of CMAPS were about 58±6 and 33±2 mV for GM and TA respectively, similar to contralateral values, fact that suggest whole recovery of amplitudes and thus successful reinnervation of these muscles at the end of follow up. (Fig 7). In contrast, when recording the plantar muscle, a more distal target, no potential was recorded at the end of follow up at the injury side (data no shown).

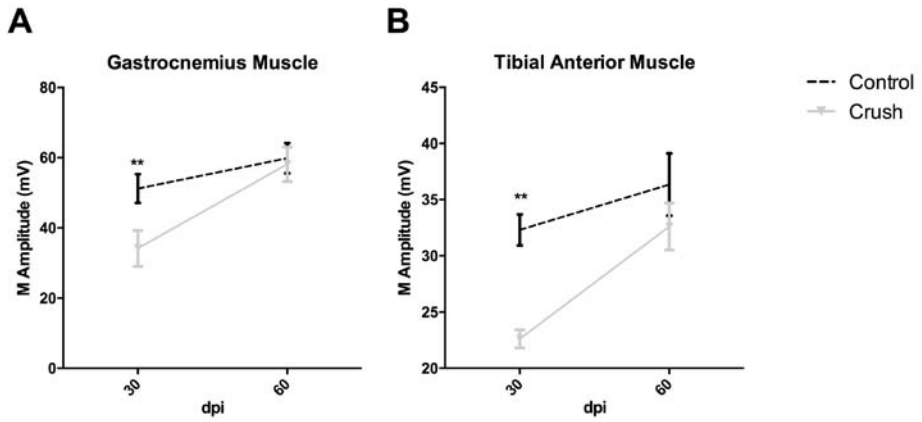


Fig 7. Electrophysiological tests performed. Results of compound muscle action potentials (CMAP) recorded in GM (A) and TA (B) muscles at 30 and 60 dpi at control and injured side. Data are expressed in absolute values as mean ± SEM, *p<0.05, **p<0.01.

At 30 dpi. when motor reinnervation is still in process, we observed an increase in the *H/M* ratio in the injured side, indicative of hyperreflexia (p<0.01). At 60dpi the H/M ratio decreased, as M increases, in both TA and GM and no differences were found in H/M ratios between injured and contralateral side (Fig 8).

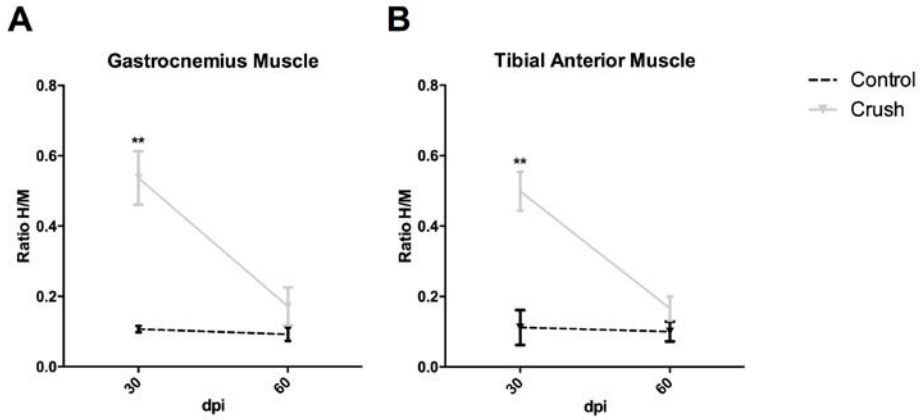


Fig 8. Plots of the H/M ratio (ratio between the amplitude of the H reflex wave and the amplitude of the direct M wave after electrical stimulation of the nerve). Results recorded in GA(A) and TA(B) muscles at 30 and 60 dpi at control and injured side. Data are expressed in absolute values as mean ± SEM, *p<0.05, **p<0.01.

DISCUSSION

In this study we evaluated the central changes that postnatal motoneurons suffer 1 and 2 weeks after the crush of the sciatic nerve and also we studied in detail synaptic VGlut1 distribution along their dendritic arbor 1, 2 and 8 weeks after injury. Moreover, we also evaluated the degree of muscle reinnervation by means of electrophysiological studies 30 and 60 days after injury.

In non-injured motoneurons we could observe an increase of VGlut1 contacts in 2 months old animals compared to postnatal ones. It has already been described that excitatory influences to the motoneurons which innervate hind limb muscles increase with development (Westerga and Gramsbergen, 1990) and this excitatory input is probably glutamate-mediated (O'Brien and Fischbach, 1986).

When focusing on the injured site, we observed a significant decrease of glutamatergic synapses (VGlut1) in motoneurons 1 week after crush injury, which was maintained 14 dpi. This decrease was accompanied with an increase of inhibitory (VGat) synapses, although VGlut1 decrease was more marked that VGat increase. When analyzing in more detail the distribution of VGlut1 contacts and their fate after injury, we found that at proximal dendrites, axotomy induces around a 50% loss of these synapses. This is a marked loss, similar to the one reported in adult motoneurons 14 days after injury. Moreover, this 50% reduction in VGlut was maintained 2 month after the injury, whereas in the adult the loss is just of 10% at this time-point (Alvarez et al., 2011; Rotterman et al., 2014). Interestingly, the studies of Alvarez et al also reported that VGlut1 contacts never recovered basal values, even after successful reinnervation of peripheral targets. This permanent loss of IA afferent/VGLUT1 synapses of injured motoneurons can explain the poor recovery of a functional stretch reflex (Alvarez et al., 2010; Rotterman et al., 2014).

The most important loss of VGlut1 synapses when the PNI is suffered at postnatal stages compared to adults can indicate that these terminals are more susceptible during development, when the circuitries are still wiring. However, it is also possible that the injury affects survival of sensory neurons. In fact, about 50% of

sensory neurons die within 2 to 3 weeks in the neonate (Whiteside et al., 1998). However, it has not been studied which subpopulations of sensory neurons are more susceptible to death when injured in postnatal stages. Thus, further studies characterizing if postnatal proprioceptive neurons die when injured are needed in order to investigate a relationship between sensory neurons loss and central VGlut1 decrease at these stages.

However, we applied the injury at 10 days old animals, where neuron death is already limited (Kemp et al., 2015). In fact, when analyzing the amount of muscle reinnervation achieved by these animals 2 months after the injury, we could observe a fully recovery in proximal muscles, although not in distal ones. After a crush injury, axons from the proximal stump are adequately guided by the intact endoneurial tubes, thus facilitating reinnervation of the correct peripheral targets (Valero-Cabré et al., 2004). Therefore, good regeneration and reinnervation is expected after a crush injury, at least in the adult. In 10 days old animals, more than 60% motoneurons survived after injury (unpublished data from our group). Although this loss if significant higher than in adults (Greensmith et al., 1993; S. Kemp et al., 2015; Lowrie and Vrbová, 1992), does not seems to limit the recovery of proximal muscles. Longer follow up would be necessary in order to evaluate if reinnervation of distal plantar muscles is affected when PNI occur at the postnatal stages. Some studies in the literature suggest that regeneration could be hampered when young animals suffer a nerve injury compared to adult ones (Nave and Salzer, 2006; Syed and Kim, 2011).

In our model we also found a good recovery of both the H/M ratio and the amplitude of the H wave 60 dpi. The ratio H/M estimates the amount of excitability of the spinal reflex. We observed an increase ratio at 30 dpi, that was normalized at 60 dpi, thus indicating that, as in the adult, PNI induces a facilitation of the reflex at earlier stages of reinnervation, but excitability is reduced progressively as muscle reinnervation advances (Burke et al., 1999; Valero-Cabré and Navarro, 2001). On the other hand, recovery of the H wave in animals with muscle reinnervation after nerve injury indicates that central sensory–motor connections were reestablished. We also

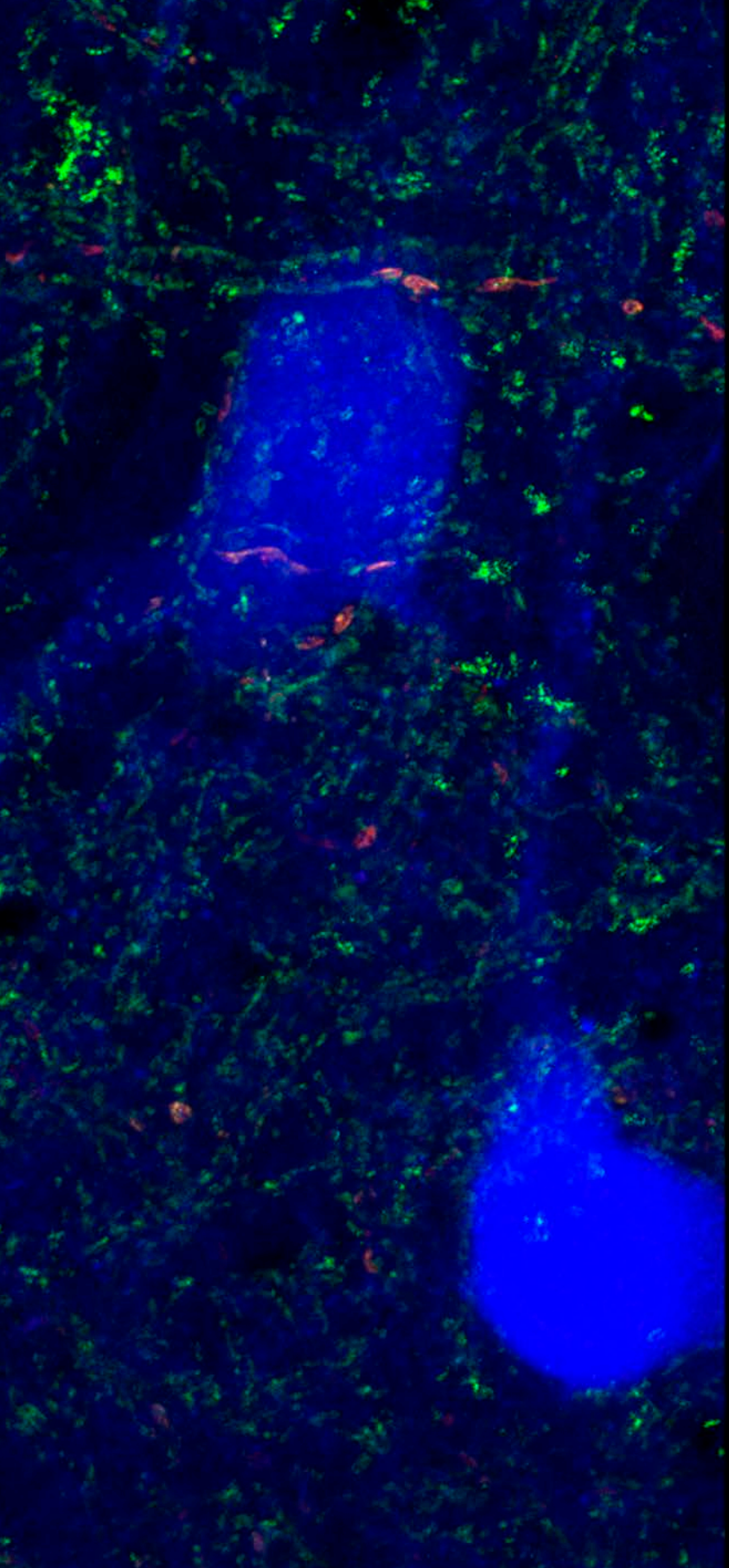
recorded H waves in our injured animals, despite we did not observe a complete recovery of VGlut1 contacts along dendrites. However, we have to take into account that after reinnervation, reinnervated muscles may fail to elicit stretch reflexes by activation of muscle spindles (Cope and Clark, 1993) even when an electrophysiological H reflex can be elicited. Therefore, it is possible that other types of afferents directly synapse with motoneurons, inducing an electrophysiological reflex that does not correlate with a functional stretch reflex.

The possibility that other afferents could centrally synapse with motoneurons can be linked with the reduction in the amount of PNN around axotomized motoneurons induced by the PNI.

PNNs, which can serve as a biomarker of the closing of the critical period during development, does not appears until 24 days after injury in the spinal cord. Extrapolating from brain areas, lack of PNNs is characteristic of an immature state of development, where there is greater plasticity, and a greater amenability to intervention (DeFelipe, 1997; Kwok et al., 2011). Thus, injuries at these periods, when PNN are still not well established could favor plasticity. In fact, after crush injury, we do not observe these membrane proteoglycans even 2 weeks post injury, when the animals are 24 days old; although this maturational delay in PNN expression may be interpreted as an open-window for plasticity in spinal cord after axotomy in neonates, it can also favor maladaptive plasticity and facilitates erroneous wiring of spinal circuits. Therefore, a more functional evaluation of the stretch reflex and the locomotor capacity of this animals is needed in order to clarify the consequences of the severe loss of VGlut1 synaptic contents in motoneurons that have been axotomized at postnatal stages.

Interestingly, when animals suffer a PNI at postnatal stages, they do not develop neuropathic pain (Mckelvey et al., 2015), being this phenomenon a paradigm of maladaptive plasticity after nerve injuries. However, this lack of neuropathic pain has been linked to a different profile in the inflammatory response observed in the spinal cord of postnatal animals compared to adult ones (Fitzgerald and McKelvey, 2016). In fact, when animals reached adulthood the inflammatory reaction shifts to

pro-inflammatory profile, and can unmask a 'latent' neuropathic pain response to the earlier nerve postnatal injury (Fitzgerald and McKelvey 2016). Therefore, this study suggest that the plastic changes induced by the PNI in the postnatal spinal cord are similar to the ones observed in the adult, but these are temporally attenuated due to a different inflammatory response. When analyzing microglia reactivity, we also found a distinct response compared to the one observed in the adult one. We found a marked increase in microglial processes surrounding axotomized motoneurons already at one week. This increase was accompanied by an increase in astrocytes immunoreactivity. In contrast microglia immunoreactivity peaks at 2 weeks in the adult, whereas astrocytes are fully activated at 4 weeks (Arbat-Plana et al., 2015; Chapter one). Thus, as in other studies, we found a more robust astrocytic activation in young animals than in adults (Vega-Avelaira et al., 2007). However, the role of this different glia reactivity is not clear. Previous studies have linked synaptic stripping with a high activation of microglia, which withdraw their processes, proliferate, and migrate towards the axotomized motoneuron cell body (Greaber et al., 1993). Therefore, the greater microglia reactivity in postnatal stages could facilitate the more severe synaptic stripping observed in young motoneurons. But microglia reactivity is also fundamental to phagocyte dead neurons and debris (Aldskogius, 2011; Polazzi and Monti, 2010). Regarding astrocytes, even when these have long been considered main player in the inhibition of CNS repair via formation of glial scar, it is now accepted that they also play an important role in repair (Barnett and Linington, 2013; Skripuletz et al., 2013). Thus, further studies to evaluate the specific role of glia activation on synaptic stripping of postnatal motoneurons after nerve injury are also needed.



Chapter 3

Therapies to modulate the stretch reflex arc after peripheral nerve injury

ABSTRACT

After peripheral nerve injury there are important changes at the spinal level and even if the peripheral axons are able to regenerate and successfully reinnervate their target organs, these changes at the central level can compromise functional recovery. Maintenance of neurotrophic support and appropriate training and/or provision of inputs in the neural circuits after lesions can be a key-point in reducing these plastic changes that neurons suffer due to the loss of synaptic and neurotrophic inputs. The aim of this study was to analyze how the addition of trophic factors into the injured stump and provision of activity-dependent strategies could modulate these changes after sciatic nerve injury in rats.

Direct application of neurotrophins at the proximal stump and electrical stimulation were not able to reverse the synaptic loss and the reduction of PNN that motoneurons suffer after axotomy. In contrast different protocols of exercise, as forced treadmill running, voluntary free running in a wheel and passive bicycling, reduced the observed disorganization of perineuronal nets and the loss of glutamatergic synapses of motoneurons two weeks after injury. Interestingly, physical exercise was not able to reverse PNN loss after elimination of sensory inputs from the homolateral hindlimb, suggesting that the effects of exercise on motoneuron PNN depend on increased sensory activity. As a conclusion, 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.

INTRODUCTION

Rehabilitation is one of the cornerstones of the treatment of injuries of the nervous system. Exercise and other activity-dependent therapies have been extensively used to improve functional recovery after spinal cord injuries (Hutchinson et al., 2004b; 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). The effect of exercise on PNI has been primarily evaluated using forced exercise, such as treadmill running. For example, different studies have demonstrated that forced treadmill running following peripheral nerve injury was effective in promote axon regeneration (Cobianchi et al., 2013; Gordon and English, 2015; Sabatier et al., 2008). Moreover, it is known that passive and active exercise also produced slight improvement in regeneration after axotomy (Udina et al., 2011b). Passive exercise of the denervated muscle before their reinnervation preserves the structure of the end-plates and enhance reinnervation (Patcher, 1989). Another activity dependent strategy extensively used in the literature is electrical stimulation (ES) of the nerve. Several authors evidenced that ES of injured peripheral nerve accelerated axon outgrowth from both motor and sensory neurons following injuries (Al-majed et al., 2000; Brushart et al., 2002; Geremia et al., 2007).

It is assumed that repeated activity will 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 promotes neurite outgrowth and functional plasticity (Ghiani et al., 2007; Rampon et al., 2000; Sale et al., 2007; Vaynman and Gomez-Pinilla, 2005). Activity-dependent plasticity has been linked with changes in neurotrophin expression, neuronal growth genes and regulatory substances (Cobianchi et al., 2013; R. Molteni et al., 2004). However, other studies note the importance of a specific rehabilitation therapy to improve functional recovery after neural damage (García-Alías and Fawcett, 2012; Wang et al., 2011).

However, just how these therapies can influence the plasticity of the central circuits where spinal motoneurons are involved is not clear. It is known 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 modulating 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 (Kwok et al., 2011), suggesting that PNN behavior 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 massive stripping of their central synapses, related to the loss of trophic support from the muscle. Brain-derived neurotrophic factor (BDNF) and neutrotrophin-3 (NT-3) are two key neurotrophins that regulate the synaptic plasticity, formation and density of synaptic innervation of motoneurons and, when exogenously delivered, are able to prevent/reverse the synaptic stripping suffered by axotomized motoneurons (Davis-López de Carrizosa et al., 2009; Novikova et al., 2000).

When neurons regenerate and reinnervate target organs, they partially recover their synaptic arbor, but in contrast to other excitatory and inhibitory inputs, muscle spindle Ia excitatory synapses, among the most affected by synaptic stripping, never recover baseline levels, even when the muscle spindle and the muscle are correctly reinnervated (Bullinger et al., 2011; Haftel et al., 2005). This reduced connectivity may explain the lack of recovery of a functional stretch reflex (Bullinger 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 not only specific reinnervation of peripheral target organs but also adequate reconnection of the central circuitry between sensory afferents and motoneurons (Bullinger et al., 2011). 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, which can be detrimental to 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 neurotrophic support and activity in the neural circuits after lesions can be a key-point in reducing the plastic changes that neurons suffer due to the loss of synaptic and neurotrophic inputs. Thus, the aim of this study was to analyze how the addition of neurotrophins BDNF and/or NT-3 into the injured stump and provision of activity-dependent strategies, like ES of the nerve or different types of physical exercise, can modulate the changes that axotomized motoneurons suffer after peripheral nerve injury.

MATERIAL AND METHODS

Experimental animals

Adult female Sprague Dawley rats (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 cycle. All experimental procedures were approved by the ethics committee of our institution and followed the European Commission on Animal Care 2010/63/EU. 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%). Axotomized animals were divided in different groups depending of the treatment that they received. A subgroup of animals was performed to study the influence of homolateral hindlimb sensory inputs on PNN after high intensity treadmill running protocol (Fig 1). A group of animals were not treated, just submitted to nerve injury.

Retrograde labeling

To identify motoneuron pools from tibialis anterior (TA) and gastrocnemius medialis (GM) muscles, retrograde tracing was applied to the muscle 1 week before any intervention. Bilaterally, two retrotracers, True Blue Chloride (TB, Setareh Biotech) and Fluorogold (FG, Fluorochrome), were applied to identify both motoneuron pools in the same animal. 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 with a glass pipette using a Picospritzer.

Surgical procedure

Under anesthesia, the sciatic nerve was exposed at the mid-thigh and cut by using microscissors. The proximal and distal stumps were rejoined with two epineural sutures. Afterwards, muscles and skin were sutured in layers, iodine povidone was

applied to the wound, and the rats were allowed to recover in a warm environment under close observation.

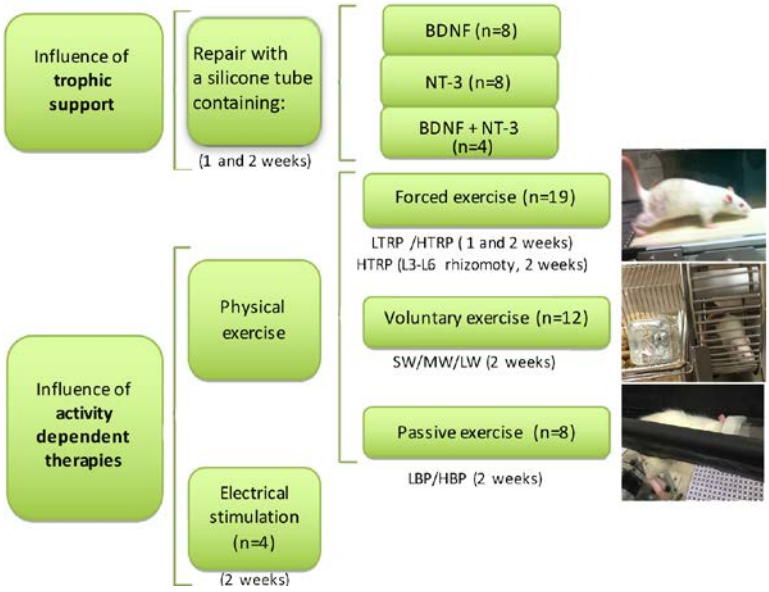


Fig 1. Treatments applied in different groups of animals. 1. Influence of trophic support on the synaptic changes. BDNF, NT-3 or a mixture of the two neurotrophic factors was applied at the injury site using a silicone tube **2**. Influence of activity dependent therapies on the synaptic changes. Three days after nerve repair animals were submitted physical exercise or to electrical stimulation. The groups of animals submitted to physical exercise were subdivided in forced, voluntary and passive exercise. Different intensities of each type of exercise were studies. In the group of animals submitted to forced exercise (treadmill running), LTRP or HTRP were performed, for 1 or 2 weeks. A subgroup of animals where submitted to HTRP after a L3-L6 rhizotomy to study the role of homolateral sensory inputs. Voluntary exercise (wheel) animals were submitted to SW, LW or LW, for 2 weeks. Finally, the group of animals submitted to passive exercise (bicycle) were subdivided to LBP or HBP, and trained for 2 weeks.

Neurotrophic factor application

In another group of animals (n=4 for each time and condition) we applied BDNF, NT-3, 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. The collagen matrix was enriched with 14 µl of phosphate-

buffered saline (PBS, 0.1M) containing 2 ng/µl of BDNF, NT-3 or a mixture of both (Ionova). 14 µl of PBS was added to the tubes of the control side. The homogenous solution was carefully pipetted onto the mid-zone of 8 mm-long silicone tubes (2.0 mm i.d.), and the tubes were incubated overnight at 37ºC. The sciatic nerve was exposed mid-thigh and sectioned as described above. The right sciatic nerve was repaired with tubes containing a collagen matrix with neurotrophins, whereas the left side was repaired with tubes containing control collagen matrices. 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.

Electrical stimulation

Electrical stimulation was applied transcutaneous for 1h at the proximal stump of the sutured sciatic nerve (20Hz, 0.1ms pulse duration, supramaximal intensity) for 2 weeks.

Treadmill running

Treadmill running exercise consisted of two different protocols, each carried out during 1 and 2 weeks, involving four 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 one 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 maximum speed of 30 cm/s for 60 min was reached 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 cm/s in two sessions of 30 minutes each with 10 minutes resting period, daily during all the follow-up (Fig 2).

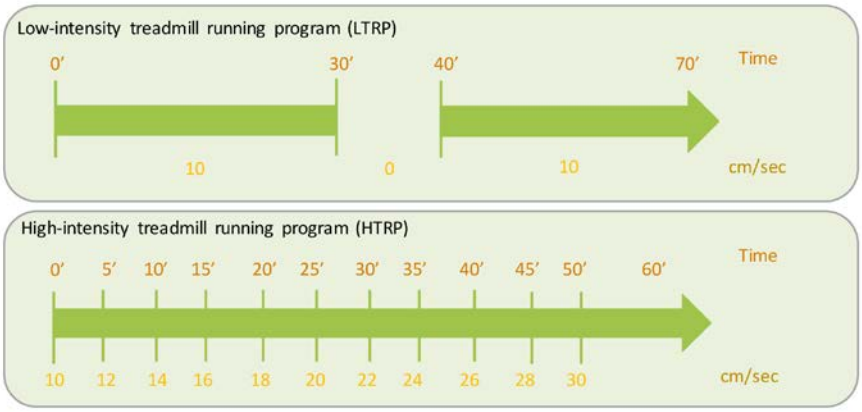


Fig 2. Treadmill running protocols. LTRP consists in a constant velocity of 10 cm/sec. In contrast, in HTRP velocity was progressively increased until 30 cm/sec.

Bicycle protocol

Bicycle Apparatus for rats consists of a motor driving two pedals that can rotate at variable rates, installed on a support frame. Animals were suspended in a full-body sling with wide openings for the hindlimbs to pass through. The feet were secured with tape to the pedals of the cycling machine, which moved the limbs through a complete circular motion to cause flexion and extension movement of the hip, knee, and ankle joints of both hindlimbs (Udina et al., 2011b). Care was taken to not stretch the limb beyond a normal extended state. Animals were maintained under isoflurane anesthesia during the sessions.

Three days after the injury, animals where submitted to two different programs of passive exercise in the bicycle. In the low-intensity bicycle program (LBP) group, each daily session consisted of two periods of 30 minutes of passive exercise at a cycling rate of 45 rpm separated by 10 minutes of rest. The other group was submitted to a high-intensity bicycle program (HBP), where bicycle velocity was progressively increased for 60 min; cycling started at a locomotion speed of 45 rpm

that was increased 7 rpm every 10 min, until a maximum speed of 75 rpm that was reached during the final training session (Fig 3).

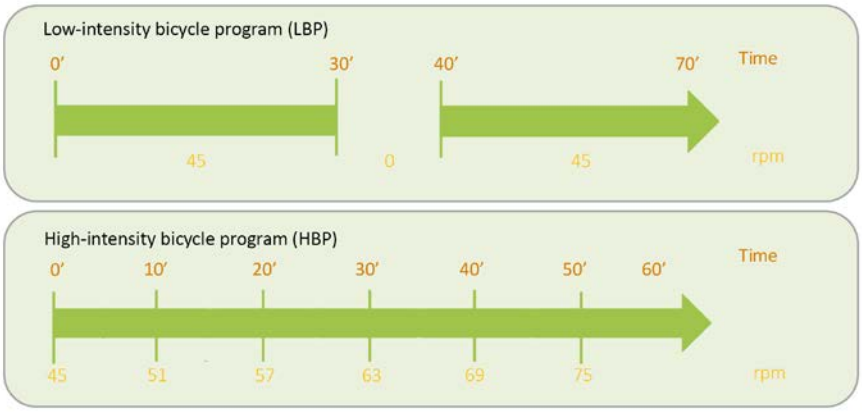


Fig 3. Bicycle protocols. Cycling velocity in the LBP program was constant at 45 rpm. In contrast, in HBP program, velocity was progressively increased from 45 to 60 rpm.

Wheel protocol

Rats in the voluntary exercise group were housed in pairs in cages with stainless-steel running wheels (Panlab,LE904) and were allowed free access to the wheel 24 h per day. Running distance was monitored daily. All wheels were film daily and analyzed using Smart Video Tracking (Panlab) in order to ensure that all the animals in the cage were running similar distances. However, there is a great individual variation in the time and distance run in the wheels and therefore we divided the animals into: short (SW), medium (MW) or long (LW) distance runners. Animals in SW run 4-5 km/day, in MW run 5-6 km/day and in LW could run up to 10-12 km/day (Fig 4).

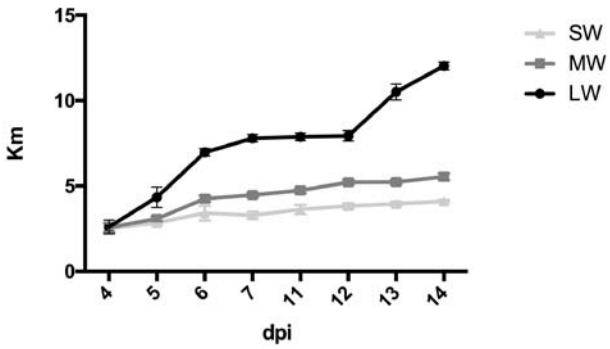


Fig 4. Distance run into the Free access wheel. Three different groups were defined based on running distances. Animals running 4-5 km/day were assigned to SW group, the ones running 5-6 km/day to LW group and animals in group LW could run up to 10-12 km/day. (Data are expressed as mean ± SEM).

Suppression of homolateral sensory inputs

In order to evaluate the role of segmentary sensory inputs from the homolateral hindlimb on motoneurons and the PNN after exercise, in a group of animals (n=3) we performed unilateral preganglionic rhizotomy of L3 to L6 dorsal roots and 3 days after the surgery, was submitted to HTRP for 2 weeks, following the same protocol described above. A subgroup of animals (n=3) was not trained and followed for 2 weeks. Since motoneurons were not injured in this model, the changes observed may be exclusively attributed to the loss of the segmentary sensory inputs from the homolateral hindlimb. For the surgery, 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. Post-operative analgesia was provided with buprenorphine (0.05mg/kg).

Immunohistochemical analysis of spinal changes

At the end of follow-up, deeply anesthetized animals were transcardially perfused with 4% paraformaldehyde in PBS. The L3–L6 spinal cord segment was removed, post-fixed for 24h, cryoprotected in 30% sucrose, and stored at 4ºC until use. 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 (Table 1). After washes, immunoreactive sites were revealed by using species-specific secondary antibodies conjugated to 488 Alexa Fluor (1:200, Invitrogen), 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***).***

Labeling	Antigen	Immunogen	Host type	Working dilution	Manufacture
Synaptic stripping	Synaptophysin	C-terminus of human Synaptophysin	Rabbit polyclonal	1:500	Invitrogen
Excitatory synapses	VGlut1	Synthetic peptide from rat VGlut1	Guinea pig polyclonal	1:300	Millipore
Perineuronal nets	Perineuronal nets	Wisteria floribunda lectin	----	1:200	Sigma
Inhibitory synapses	Gephyrin	Rat Geph (aa 569-726)	Mouse	1:200	BD
	VGat	Strep-Tag fusion protein of rat VGat (aa 2 -115)	Guinea Pig	1:200	Synaptic systems
Astroglia	GFAP	Purified GFAP from porcine spinal cord	Mouse	1:1000	Millipore
Microglia	Iba1	C-terminus of Iba-1 synthetic peptide	Rabbit polyclonal	1:500	Wako

Table 1 Primary antibodies used in this study.

Image analysis and processing, and regression analysis were performed by means of in-house software implemented in Matlab R2014a (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 a perimeter of 5µm thickness surrounding the soma. For each animal, 10 to 15 motoneurons of each pool and each side were analyzed.

Statistical analysis

For quantitative variables, normality was assessed with the 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 with the Kruskall-Wallis test. A nested design ANOVA test was used in order to determine if the variability was due to the difference between the motoneurons or between animals in the experimental groups SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. A value of $P < 0.05$ was considered significant. For the relevant values, experimental power was estimated using R version 3.1.1 (free software).

RESULTS

As we previously described in chapter 1, motoneurons suffered a massive synaptic stripping of their central arbor after PNI, with a marked decrease of glutamatergic synapses and an increase of inhibitory ones. There was also a disorganization of their PNN around these motoneurons, evidenced by reduction of its immunoreactivity. The experimental power estimated for the relevant data (Syn, VGlut1, Gephirin, VGat and PNN measurements) was about 0.8 in the different experiments.

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

To evaluate whether addition of trophic factor support could ameliorate the changes in synaptic inputs found after axotomy, neurotrophins BDNF and NT-3 were applied to the proximal stump by means of a silicone tube containing a collagen matrix enriched with these factors. The contralateral sciatic nerve was also cut and repaired with the tube containing the collagen matrix without trophic factors. Due to the interindividual variability the values for each marker are expressed as percentage of decreased/increased density versus the contralateral side of each animal.

At 1 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 for Syn labeling in GM motoneurons 1 week postinjury (P=0.025, Fig 5A). In contrast, NT-3 application did not affect the changes induced by axotomy on Synaptophysin, VGlut1, and Gephyrin immunolabeling surrounding the motoneurons. Neither the application of BDNF nor NT-3 affected the reduction in PNN immunoreactivity observed after axotomy (Fig 5. A,B).

Application of a mixture of BDNF and NT-3 had no noticeable effect on Synaptophysin, VGlut1, Gephyrin and PNN labeling after axotomy (data not shown).

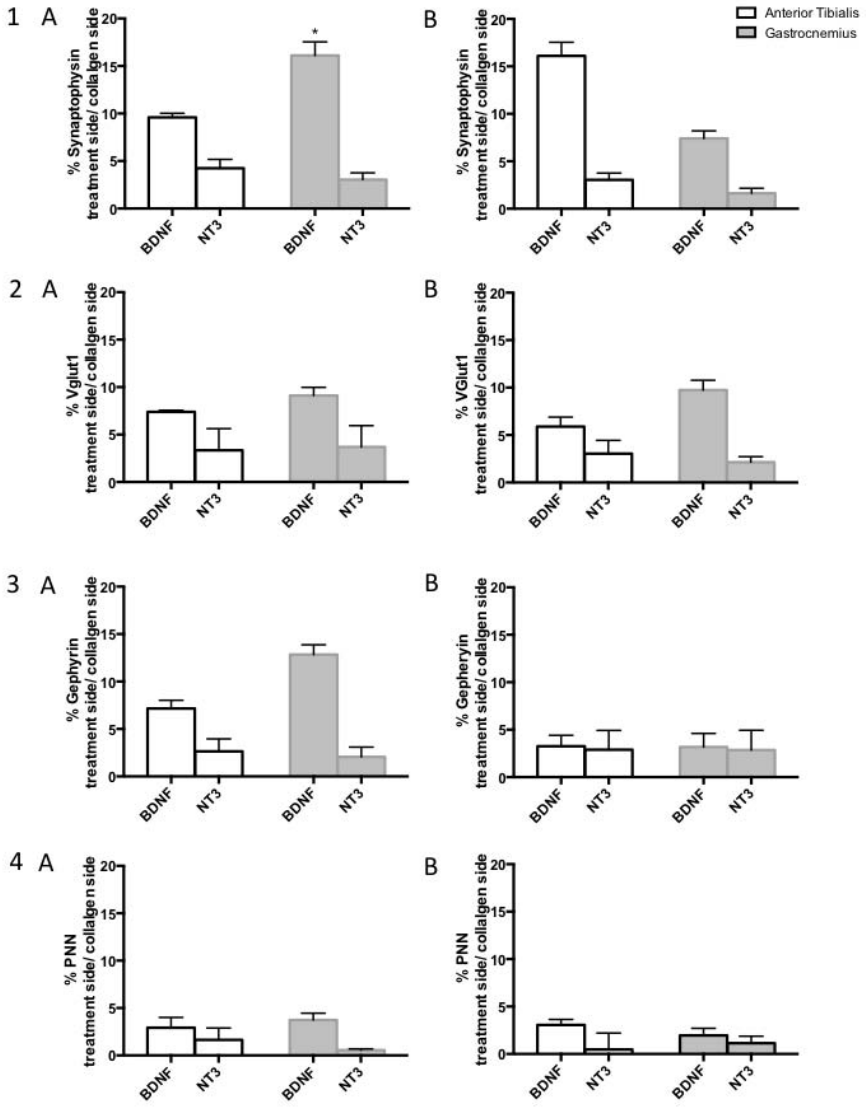


Fig 5. Quantitative analysis of synaptic stripping after axotomy and application of neurotrophic factors. Evaluation of Synaptophysin (1), VGlut1 (2), Gephyrin (3) and PNN (4) staining 1 week (A) and 2 weeks (B) after repairing the sciatic nerve with a tube containing a collagen matrix with BDNF or NT-3, expressed as percentage versus contralateral side, which was repaired with a tube with collagen matrix. BDNF application shows a trend toward reduction of synaptic stripping, although it is not significant. NT-3 application has no effect. (Data are expressed as mean ± SEM, *p<0.05, **p<0.01).

Influence of electrical stimulation on the synaptic changes induced by axotomy of motoneurons

Similar to neurotrophic factors application, electrical stimulation of the injured nerve for 1h was not able to revert the changes observed in the spinal cord after axotomy. The synaptic loss and the reduction in the number of glutamatergic synapses that MNs suffer after axotomy were similar in control animals and animals that received electrical stimulation. PNN loss was also similar in these two groups, suggesting that the protocol of electrical stimulation used is not able to prevent the effects that axotomized motoneurons suffer at the central level (Fig 6).

Regarding glia activation, no significant differences were observed between stimulated and untreated animals (data non shown).

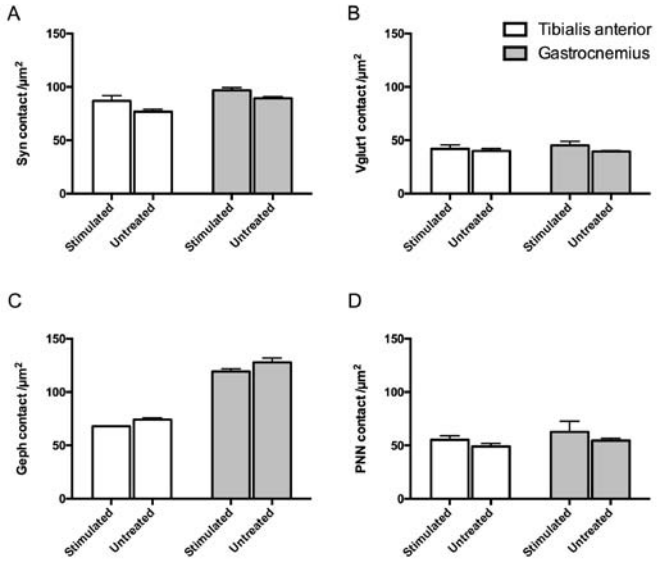


Fig 6. Quantitative analysis of synaptic stripping after axotomy in animals submitted to electrical stimulation. Evaluation of Synaptophysin (A), VGlut1 (B) and VGat (C) and PNN (D) staining at TA (white bars) and GM (grey bars) motoneurons. Data are expressed as mean ± SEM, *p<0.05, **p<0.01. No significant differences were observed between stimulated and untreated animals.

Effects of different protocols of exercise on the synaptic changes induced by axotomy in motoneurons

Forced exercise: treadmill running

In order to learn whether forced exercise could modulate the plastic changes affecting motoneurons after axotomy, two different treadmill protocols were performed. No significant differences were found in the contralateral sides between groups.

HTRP was able to partially prevent the decrease in Synaptophysin immunoreactivity surrounding axotomized motoneurons 2 weeks post-injury (96±5 Syn/μm^2 and 102±4 Syn/μm^2 in TA and GM versus 130±3 Syn/μm^2 in both muscles, respectively, in non-trained animals) ($p<0.001$) (Fig 7.1). The VGlut1 decrease observed after axotomy was also partially reduced with HTRP already at 1week post-lesion in both motoneuron pools, and excitatory synapses labeling was 65±8 VGlut1/μm^2 at 2 weeks, in contrast to the 40±2 VGlut1/μm^2 observed in non-trained animals (Fig 7.2). VGat increase was partially reduced by HTRP, at 1 and 2 weeks for GM motoneurons (Fig 7.3). In contrast, the low intensity treadmill exercise (LTRP) was not able to reverse the synaptic stripping changes observed after axotomy (Fig 7).

When analyzing PNN immunoreactivity surrounding the motoneurons, we found that both protocols of exercise reduced the decrease that these nets suffer 2 weeks after axotomy. Moreover, the high intensity protocol (HTRP) prevented this decrease already at 1 week (Fig 8). Unexpectedly, we observed that PNN immunoreactivity of intact motoneurons in the contralateral side was greater in animals submitted to the high-intensity protocol compared to non-exercised rats (Fig 8).

At 1 week post-injury, TA motoneurons in LTRP animals had a density of 94±5 PNN/μm^2, whereas in GM motoneurons the PNN labeling was 105±3 PNN/μm^2. At 2 weeks, levels were 74±10 PNN/μm^2 for both pools of motoneurons. In the HTRP group, 1 week after axotomy, TA motoneurons had 107±1 PNN/μm^2, whereas this fall

reached 124±4 PNN/µm^2 in GM motoneurons. At 2 weeks, the density was 76±3 PNN/µm^2 in TA motoneurons and 83±4 PNN/µm^2 in GM ones. In fact, GM motoneurons of animals receiving HTRP for 2 weeks showed a significant increase in PNN staining compared to motoneurons from untrained animals (49±4 PNN/µm^2 and 54±2 PNN/µm^2 in TA and GM respectively, p<0.001) (Fig 8.2).

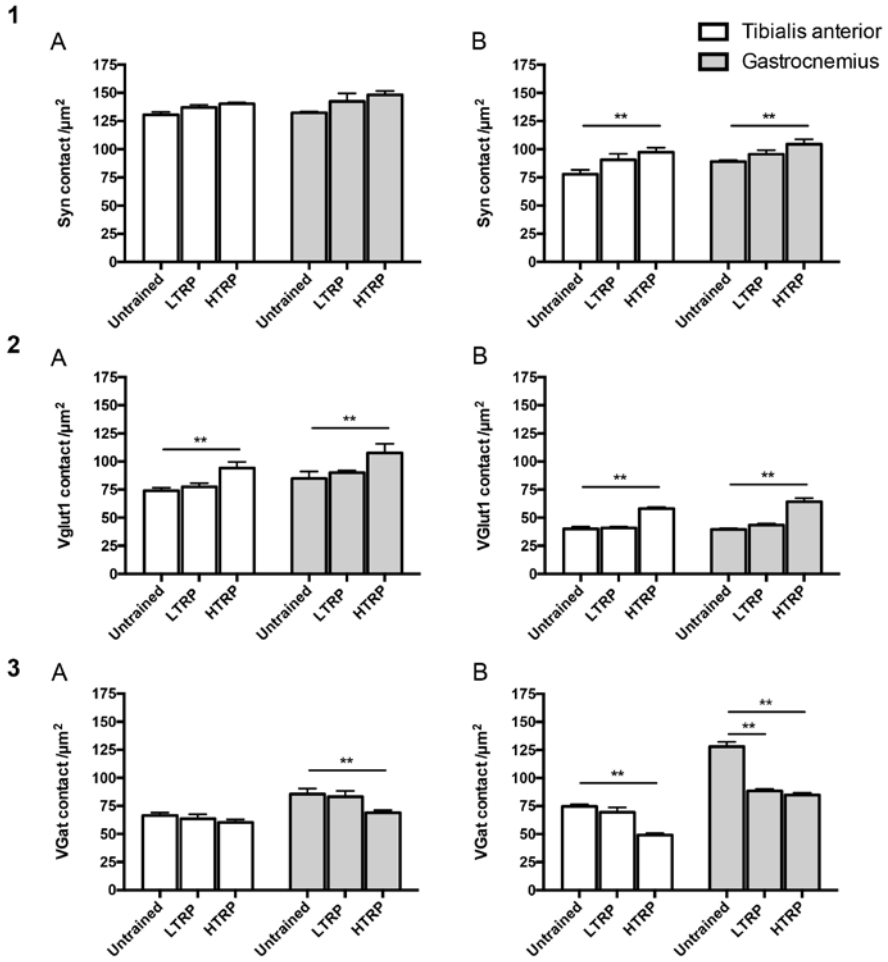


Fig 7. Quantitative analysis of synaptic stripping after axotomy in animals submitted to different protocols of treadmill. Synaptophysin (1), VGlut1 (2) and VGat (3) staining at 1 week (A) and 2 weeks (B) were evaluated in animals submitted to a low intensity (LTPR) and a high intensity treadmill running program (HTRP) and in untrained animals. 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 and Synaptophysin loss in motoneurons 2 weeks after injury. The low intensity protocol was not able to influence synaptic stripping.

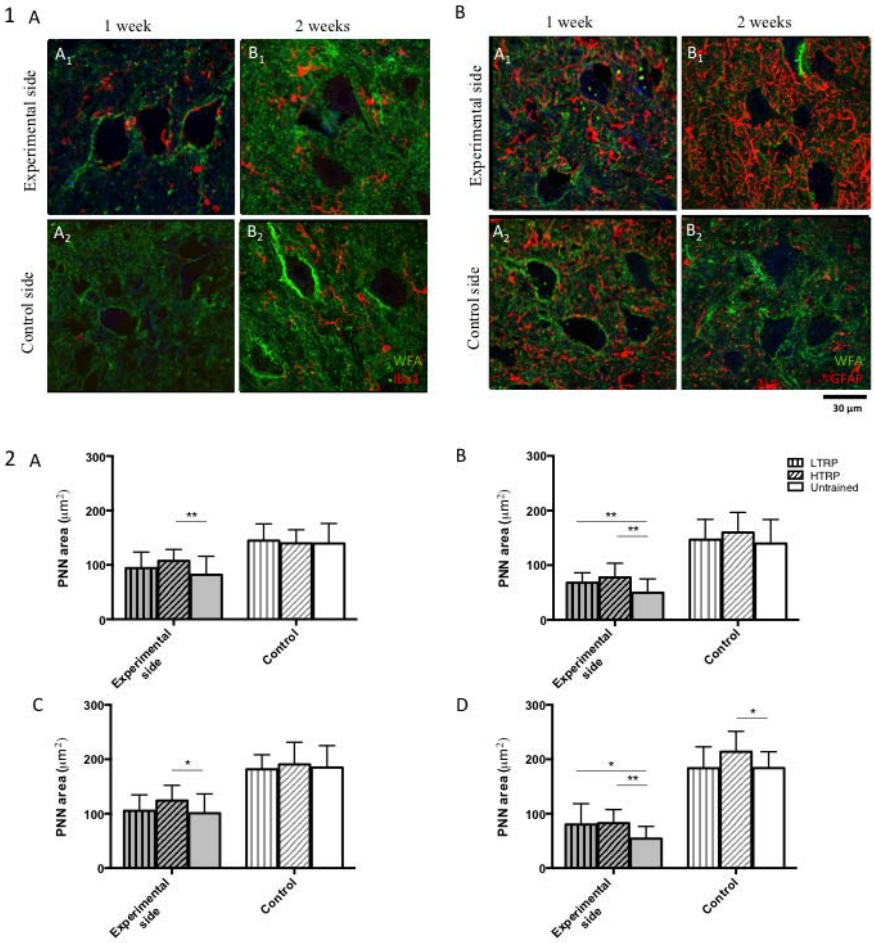


Fig 8. Glia and PNNs surrounding axotomized motoneurons in animals submitted to different treadmill protocols 1. Confocal images of spinal cord regions stained for microglia (A), astrocytes (B) and PNNs (A, B) 1 week and 2 weeks after injury in HTRP animals. Back-labeled motoneurons from TA and GM muscle in blue, microglia (Iba1) and astrocytes (GFAP) in red, and PNNs (Wisteria Floribunda) in green**. 2.** Evaluation of PNN staining in animals submitted to low intensity treadmill (LTPR, vertical dashed bar), high intensity treadmill (HTRP, dashed bar), and untrained (solid bar) in TA (white bar) and GM (grey bar) motoneurons axotomized for 1 week (2A, 2B) or 2 weeks (2C, 2D) with cut and suture of the sciatic nerve. Data are expressed in absolute values, since the contralateral non-injured side was also submitted to the treatment. Exercise reduced the loss of motoneuron PNN observed after injury, with the effect being more marked with the more intense protocol. At 2 weeks, animals submitted to HTRP also showed increased PNN immunostaining surrounding MN of the contralateral non-injured side. (Data are expressed as mean ± SEM, *p<0.05, **p<0.01).

HTRP also modulated glial activation. Animals submitted to HTRP showed reduced microglia reactivity compared to non-trained animals. There was lower immunoreactivity to Iba1 surrounding axotomized motoneurons in exercised rats (60±10 Iba1/μm^2, $p<0.05$, Fig 8.1A) than in non-trained animals. In contrast, 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, $p<0.01$, Fig 8.1B). For the low intensity protocol (LTRP) no changes in astroglia or microglia immunoreactivity were observed between exercised and non-trained animals.

Passive exercise: motorized cycling

We also study the effects of passive exercise, by means of a motorized cycling apparatus adapted to rats, in the plastic changes affecting motoneurons after axotomy. Two different bicycle protocols were applied. No significant differences were found between contralateral sides in none of them.

LBP was not able to prevent synaptic stripping and PNN loss after axotomy. Simultaneously, there were not significant effects between LPB and untrained animals in both excitatory and inhibitory synapses. On the other hand, synaptic stripping was partially prevented with HBP when we analyze Synaptophysin labeling surrounding axotomized motoneurons (90±10 Syn/μm^2 versus 130±3 Syn/μm^2 in both TA and GM muscles, respectively, in non-trained animals) ($p<0.001$) (Fig 9.A). Moreover, the group of animals submitted to HBP, was able to prevent the decrease of VGlut1 observed after axotomy (50±5 VGlut1/μm^2, in contrast to the 40±2 VGlut1/μm^2 observed in non-trained animals) (Fig 9.B). Regarding inhibitory synapses, the increase of VGat observed after injury was partially reduced ($p<0.001$) (Fig 10.C). Lastly, HBP was able to reduce the decrease of PNN observed after axotomy (78±4 PNN/μm^2 and 85±3 PNN/μm^2 versus 49±4 Syn/μm^2 and 54±2 PNN/μm^2 in TA and GM, respectively, in non-trained animals) (Fig 9.D).

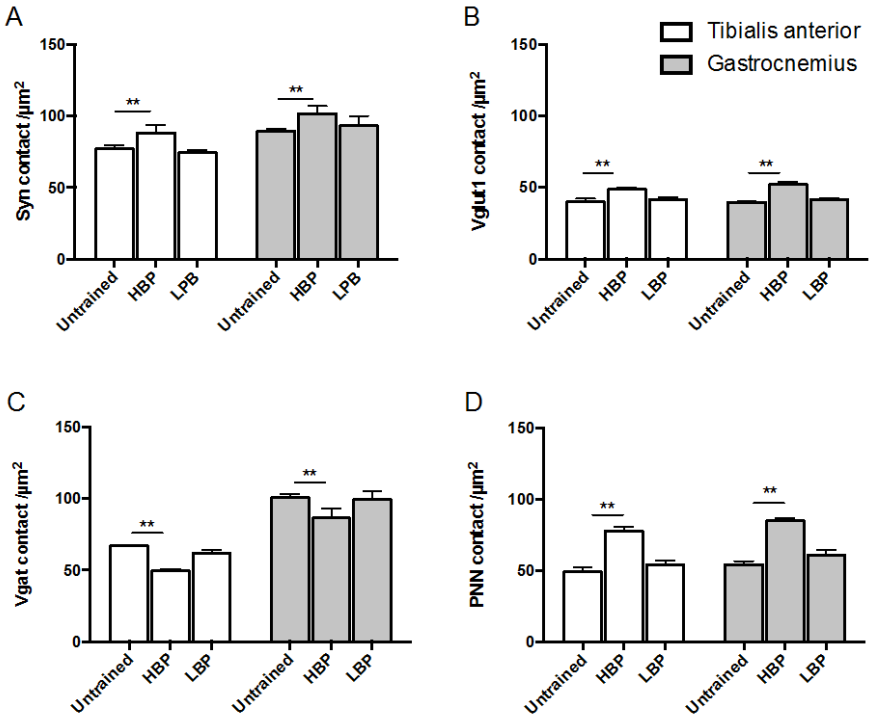


Fig 9. Quantitative analysis of synaptic stripping and PNN after axotomy in animals submitted to passive exercise. Two different groups were performed based on the intensity of the exercise: low (LBP), and high (HBP) bicycling program, and compared to untrained animals. Evaluation of Synaptophysin (A), VGlut1 (B), VGat (C) and PNN (D) in TA (white bars) and GM (grey bars). Data are expressed as mean ± SEM, *p<0.05, **p<0.01. The HBP group of animals was able to reduce synaptic stripping, partially protecting VGlut1 reduction in both motoneuron pools, and Synaptophysin loss after axotomy. Moreover, animals submitted to HBP shows an increase of PNN surrounding MNs. The low distance groups were not able to influence synaptic stripping and PNN.

In relation to glia activation, there was a significant increase in GFAP immunoreactivity surrounding axotomized motoneurons in both groups of passive exercise compared to untrained ones. No significant differences were found in astroglia between HBP and LBP.

Moreover, there was not significant differences in microglia reactivity between HBP trained and non-trained animals. In contrast, animals submitted to LPB

showed a more marked increase of microglia reactivity (334±8 Iba1/μm^2), $p>0.01$ compared to axotomized ones (Fig 11).

Voluntary exercise: free running in a wheel

To know how voluntary exercise could modulate the plastic changes affecting motoneurons after axotomy, animals were allowed to run freely in a wheel during 2 weeks. It is well known that velocity of the animals submitted to wheel exercise in progressively increased every day. On the other hand, since the amount of exercise was free, animals were subdivided in three different groups depending on the cumulative distance run at the end of follow up: short (SW), medium (MW) or long (LW)). No significant differences were found at the contralateral sides among groups in none of the parameters analyzed.

When animals run short or medium distances, synaptic stripping and PNN loss of axotomized motoneurons was similar to control animals that did not run in a wheel. Similarly, no significant differences were found between untrained animals and SW and MW animals when we analyze excitatory and inhibitory synapses. In contrast, LW was able to partially prevent the decrease in Synaptophysin immulabeling surrounding axotomized motoneurons compared to untrained or low intensities ones (92±3 Syn/μm^2 in LW group of animals versus 80±9 Syn/μm^2 in non-trained animals in both TA and GM muscles) ($p<0.001$) (Fig 10.A). When excitatory synapses were analyzed, we observe that the VGlut1 decrease observed after axotomy was also partially reduced in the group of animals running longer distances (50±4 VGlut1/μm^2 in LW group in contrast to the 40±2 VGlut1/μm^2 observed in non-trained animals) (Fig 10.B). The increased amount of inhibitory synapses, labeled by VGat observed after axotomy, was partially reduced in LW group (Fig 10.C). Similarly to what we observed in the other parameters, only when animals were running longer distances, PNN loss was partially prevented 2 weeks after axotomy (79±4 PNN/μm^2 and 88±4 PNN/μm^2 in

TA and GM versus 49±4 Syn/µm^2 and 54±2 PNN/µm^2 in TA and GM, respectively, in non-trained animals) (Fig 10.D).

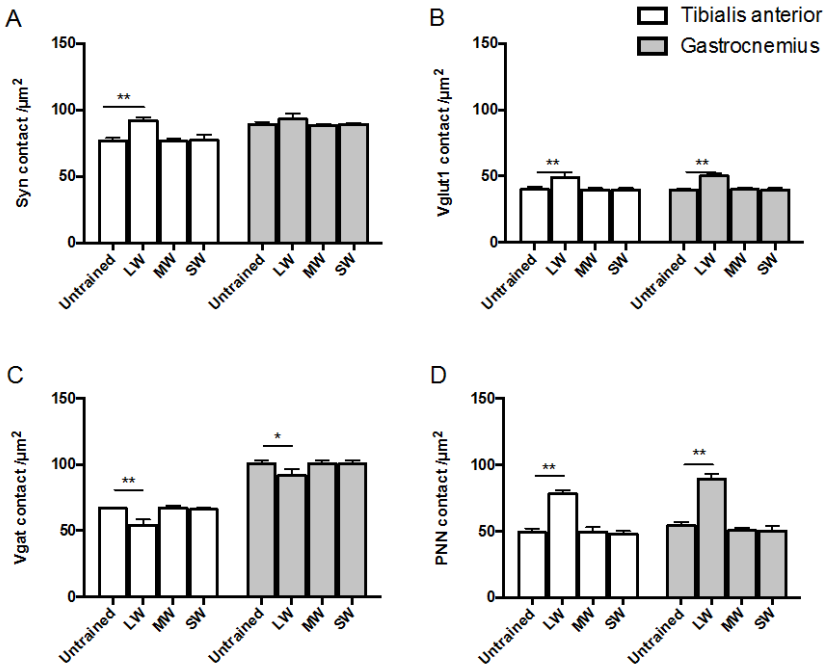


Fig 10. Quantitative analysis of synaptic stripping and PNN after axotomy in animals submitted to voluntary exercise. Three different groups were performed based on the distances they reached the last day of their follow up/cumulative distance run at the end of follow up: short (SW), medium (MW) or long (LW), and compared to untrained animals. Evaluation of Synaptophysin (A), VGlut1 (B), VGat (C) and PNN (D) in TA (white bars) and GM (grey bars). Data are expressed as mean ± SEM, *p<0.05, **p<0.01. The LW group of animals was able to reduce synaptic stripping, partially protecting VGlut1 reduction in both motoneuron pools, and Synaptophysin loss after axotomy. The low distance groups were not able to influence synaptic stripping and PNN.

When analyzing microglia reactivity, we could observe that there were not significant differences in microglia immunoreactivity surrounding axotomized motoneurons in large and medium distances of wheel exercise. Interestingly, the group of animals that runs less distances (SW) showed a more marked increase of microglia reactivity compared to non-trained animals. (331±12 Iba1/µm^2, p<0.001) (Fig 11.). In contrast, there was an increase in astrocyte reactivity compared with non-trained animals in all the animals of the free wheel groups (Fig 11).

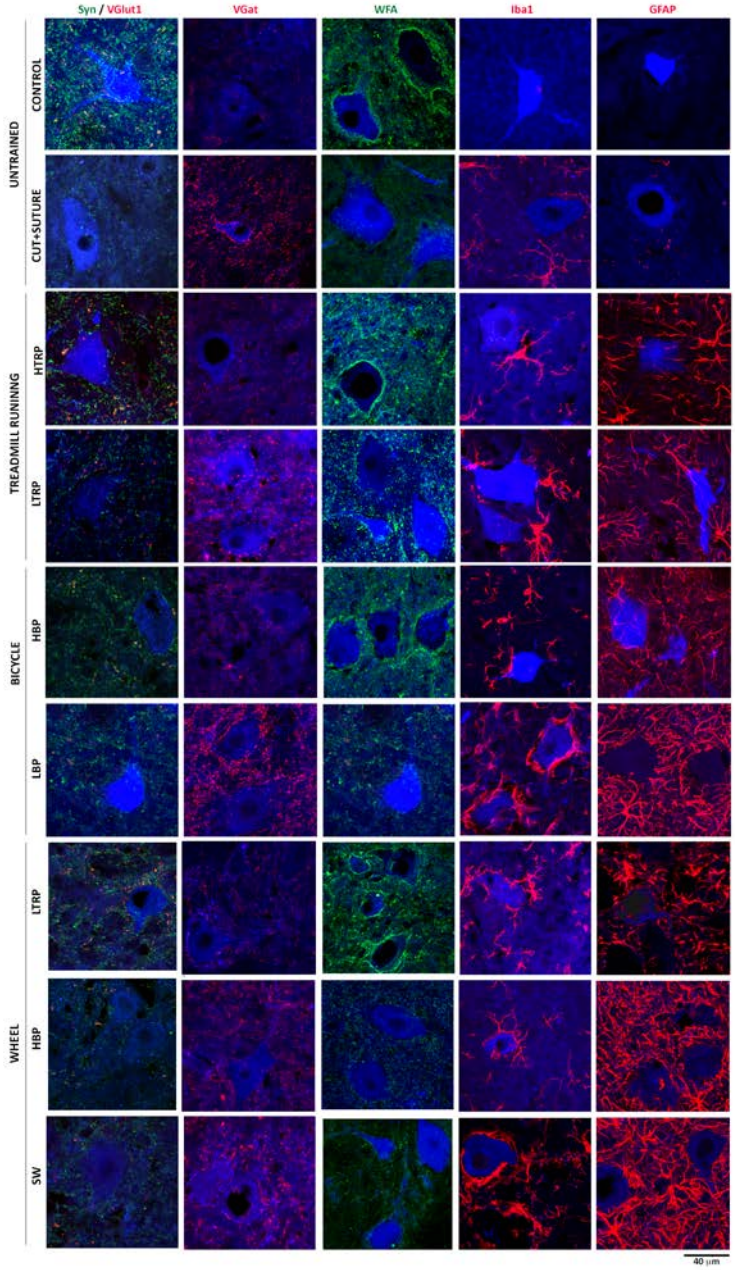


Fig 11. Evaluation of excitatory and inhibitory synapses, PNN, astroglia and microglia reactivity surrounding axotomized motoneurons of animals that freely run in a wheel short, medium or long distances. All immunostainings were evaluated in confocal images of spinal cord regions containing back-labeled motoneurons (blue) of TA and GM muscles after nerve cut repaired with direct suture in the different groups of animals.

Influence of homolateral hindlimb sensory inputs on PNN after HTRP

In order to assess the influence of sensory inputs on motoneuron changes after axotomy, we sectioned dorsal roots L3 to L6 at the preganglionic level. To confirm 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 muscles was similar to control values, whereas the H wave was abolished (data not shown).

As expected, disruption of peripheral sensory inputs 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 to non-trained ones.

Two weeks after rhyzotomy, even if motoneurons were not axotomized, we found a 22±2% reduction in the density of PNN around motoneurons of TA and GM muscles pools. This reduction was also observed in animals receiving HTRP for 2 weeks; therefore, exercise was not able to reverse the effects of sensory deprivation on PNN (Fig 12).

Dorsal root lesion did not induce 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 in 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 on the contralateral side a significant increase in astroglia reactivity around motoneurons was observed (100±10 GFAP/μm^2 *P>0.05*) (Fig 12).

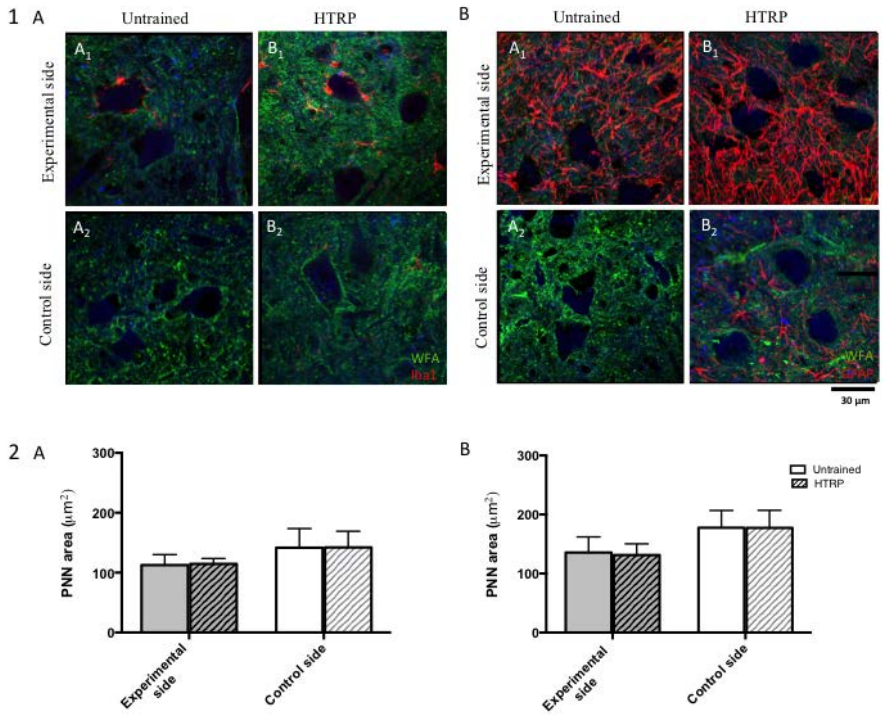


Fig 12. **Glia and PNNs surrounding intact motoneurons that lost the segmentary sensory inputs of the homolateral hindlimb**. **1.**Confocal images of spinal cord regions stained for PNNs (Wisteria Floribunda, green) and microglia (Iba1, red, A) or astroglia (GFAP, red, B) 2 weeks post-injury in untrained animals (A) and animals submitted to HTRP. **2.** Quantitative analysis of motoneuron PNN of animals submitted to dorsal rhyzotomy (L3 to L6) to disrupt the homolateral sensory inputs of the limb. TA (2A) and GM (2B) motoneurons of untrained animals (solid bars) and 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 reverse the loss. In the control non-injured side of trained animals there was reduction in microglia reactivity, a mild increase in astroglia, and PNN were similar to controls. (Data are expressed as mean ± SEM, **p*<0.05, ***p*<0.01).

DISCUSSION

In this paper we have evaluated the effects of different therapies in the central changes that motoneurons suffer after a peripheral nerve injury. Application of neurotrophins or electrical stimulation at the proximal stump were not able to reverse these effects. In contrast, different protocols of exercise, either forced running

in a treadmill, passive cycling in a motorized bicycle or free running in a wheel reduced the destructuring of PNN and the loss of glutamatergic synapses 2 weeks after injury.

Synaptic stripping has been related to loss of trophic support of the neurons after axotomy (Davis-López de Carrizosa et al., 2009; Novikova et al., 2000). Therefore, direct application of trophic factors in the injured nerve may be an alternative therapy to prevent synaptic loss of axotomized motoneurons. In fact, in oculomotor neurons, addition of BDNF and NT-3 was able to reverse synaptic stripping. These neurotrophins differentially affected excitatory and inhibitory synapses (Davis-López de Carrizosa et al., 2009). Therefore, we wanted to test whether addition of these neurotrophins to the proximal stump of the nerve could reverse this stripping, by mimicking the trophic support normally received from the muscle. However, we found only marginal preservation of VGlut1 synapses after BDNF administration. Direct application of these factors into the spinal cord could be a more effective method to reverse synaptic stripping (Davis-López de Carrizosa et al., 2009; Novikova et al., 2000), but application at the injury site is a better clinical strategy, since it can be performed in conjunction with surgical repair and it also enhances axonal regeneration. Continuous delivery of these factors, either by slow-release microparticles or by viral vector-mediated transfer of neurotrophins (Blits et al., 2003; Ruitenberg et al., 2002) may be a more effective strategy to guarantee trophic support for longer periods of time. Furthermore, other trophic factors may be important in spinal motoneurons to maintain the synaptic contacts. However, finding the correct combination of factors, with the appropriate dose and timing-pattern, may be too complex to perform. In fact, when the mixture of both BDNF and NT-3 were applied, no effect was observed. TrkB mediates the effects of BDNF but also NT-4 and NT-3 (Klein et al., 1991; Yamamoto et al., 1996). Thus, perhaps NT-3 is interfering with BDNF action. Since activity-dependent therapies have the capacity to up-regulate trophic factors (Gomez-Pinilla et al., 2012; Gómez-Pinilla et al., 2001; Gordon and English, 2015; Krakowiak et al., 2015; Vaynman et al., 2003) these strategies may be a better clinical strategy to modulate the trophic support of axotomized motoneurons. Interestingly, different

activity-dependent therapies can modulate the plastic changes observed after peripheral nerve injury, as reported for the modulation of the H reflex (Asensio-Pinilla et al., 2009; Udina et al., 2011b; Vivó et al., 2008) and of pain responses (Cobianchi et al., 2013, 2010), thus, being good candidates as strategies to prevent/reduce synaptic stripping in motoneurons after PNI. In fact, the different types of exercise applied were able to partially prevent synaptic stripping and PNN loss of axotomized motoneurons. However, although ES also upregulates BDNF in motoneurons (Gordon, 2010) its application was not able to mimic the effects of exercise. Probably, one hour of electrical stimulation just after the injury was not inducing enough amount of activity in axotomized motoneurons to reverse the effects of the injury. In fact, the protocols of exercise that were more intense induced more significant changes than the protocols of lesser intensity. Similarly, only if rats freely run up to 10-12 km/day (long distances wheel runners), the effects of axotomy on motoneurons were partially reversed. However, we use this protocol of ES since is the one more extensively used in the literature, and different studies demonstrate that promotes nerve regeneration and that upregulate BDNF, among other factors (Brushart et al., 2002; Gordon and English, 2015; Gordon, 2010; Vivó et al., 2008). In contrast, daily application of 1h of ES had detrimental effects on nerve regeneration (Asensio-Pinilla et al., 2009).

It is noticeable that the three types of exercise used, when applied at sufficient intensity, were able to partially reverse synaptic stripping and modulate the spinal changes observed after PNI. Moreover, we observe that the most significant changes were obtained after 2 weeks of forced exercise, in form of TR. Therefore, we only study the effects of passive and active exercise for 2 weeks. In fact, not only intensity but duration of the protocol can be important.

The effects of exercise on motor function after peripheral nerve injury are a bit controversial, with some 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., 2011b) and others indicating it is detrimental (Gutmann and Jokoubek, 1963; Herbison et al., 1974, 1080; Meeteren et al., 1997; Soucy , M Seburn

and Gardiner, 1996). In general, it is accepted that a mild physical activity applied at the onset of the denervation phase would accelerate motor recovery, whereas long term hyperactivity or forced exercise would be detrimental (revised in Udina et al., 2011).

In our study, the key element to induce positive effects was the intensity and not the type of exercise used, thus indicating that the amount of activity received by motoneurons is fundamental to prevent synaptic stripping and PNN in motoneurons. Therefore, interruption of the sensory inputs of the hind limb by multiple rhyzotomy, PNN loss was not reversed when animals were forced to run on the treadmill, suggesting that the effects of exercise on motoneuron PNN depend on increased input activity mediated by sensory afferents (Raffaella Molteni et al., 2004). We performed a wide dorsal rhyzotomy to suppress most of the sensory inputs of the hind limb, and not only the sciatic afferents. Even during the denervation period, exercise can stimulate afferents from proximal non-denervated muscles (Koerber et al., 2006).

Even if it seems logical that activity is fundamental to maintain the integrity of the system, it was quite surprising that activity, in the form of different types of exercise, was able to partially prevent the loss of PNN observed after PNI. Whereas PNN stabilizes synapses and limits plasticity of the nervous system, some of the positive effects of exercise on the nervous system has been linked to increased plasticity. Enzymatic degradation of PNN reopens the critical period for experience-dependent plasticity in the visual cortex (Pizzorusso et al., 2006, 2002) and enhances 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 after peripheral nerve injury and when activity dependent therapies are applied. In a spinal cord injury model, it has been described that rehabilitation increased motoneuron PNN (Smith et al., 2015; Wang et al., 2011). In our study we characterized the fate of motoneuron PNN after sciatic nerve injury, and how exercise might affect these structures that are critical for the stability of the neural circuits.

It is interesting to note that in other neurons of the central nervous system, such 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) and the cerebellum (Carulli et al., 2013), PNN formation and maintenance are influenced by the activity of sensory afferents, with increased activity reducing PNN. After a peripheral nerve injury, there is loss of normal sensory inputs from Ia afferents that are also injured and disconnected from their target organ, the muscle spindle. Accompanying this sensory loss, motoneurons are also injured and switch to a pro-regenerative state that includes changes in their dendritic arbor. Therefore, to further understand the mechanism that may explain the reduction of PNN after injury, we also evaluated their fate when motoneurons were not axotomized but had lost sensory inputs from the limb. Interestingly, after transection of the dorsal roots innervating the lower limbs (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.

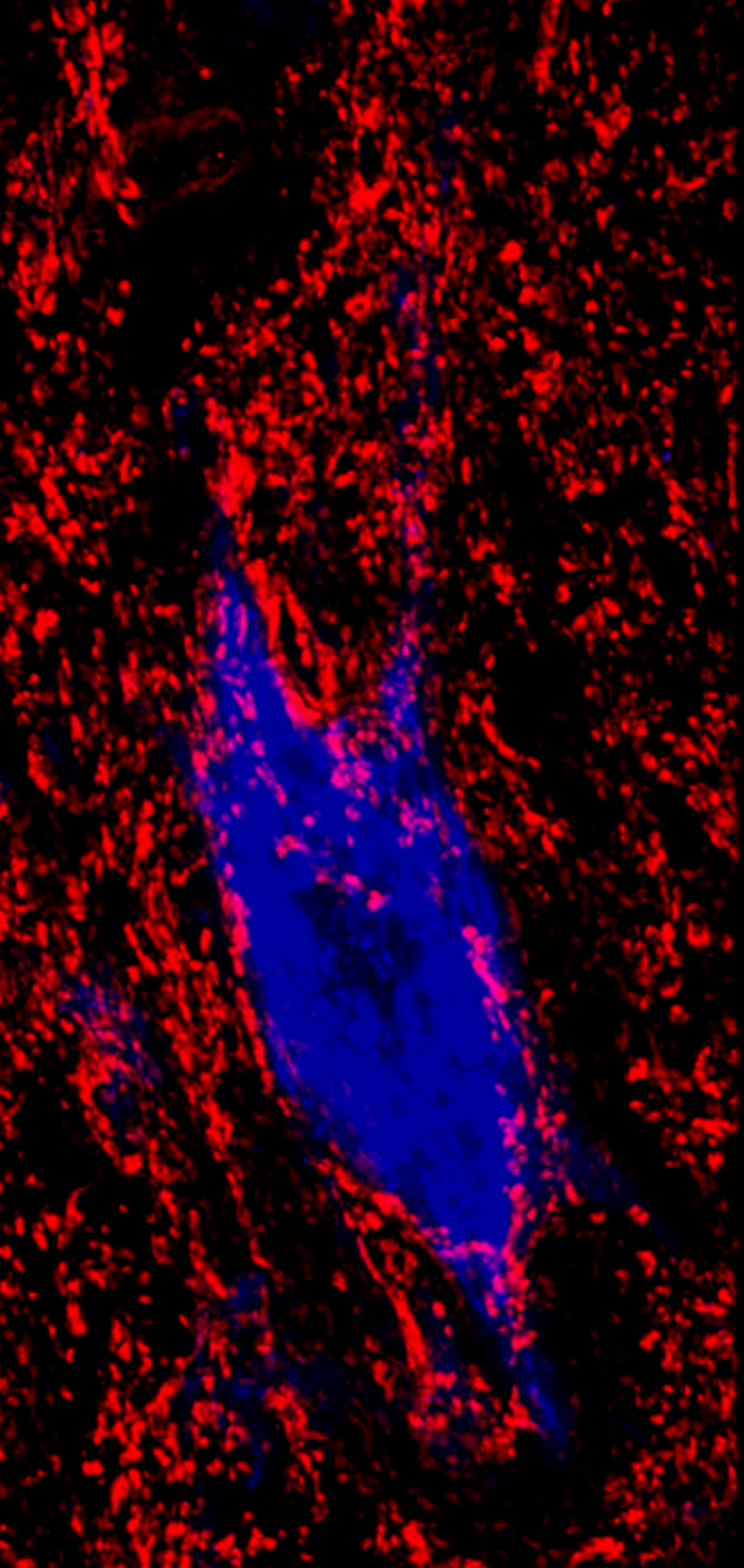
Increased activity in the form of treadmill exercise also modulated glial activation surrounding axotomized motoneurons. The same protocol that we used was able to reduce microglia activation after chronic constriction injury in mice (Cobianchi et al., 2010). In agreement with this, we observed a significant reduction in microglia processes around motoneurons already at one week. The intensity of exercise was related to the amount of microglia reactivity attenuation, since low intensity treadmill had no effect on microglia activity. Although it has been proposed that reduction in microglia reactivity can prevent neuronal loss (Milligan and Watkins, 2009), others suggest that activated microglia play a predominantly protective role (Cullheim and Thams, 2007). In our study, reduction in microglia activation at early time points was associated with less severe synaptic stripping observed in the animals that were running at high intensity. However, when voluntary and passive exercise were applied we observed a high activation of microglia in these animals that

runs/cycles shorter distances that the ones that run/cycles longer ones. In contrast to treadmill running, which is a forced exercise, it is possible that wheel and bicycle exercise generate less stress than treadmill running. In fact, it is well known that the noradrenergic system is activated by acute stress. Since noradrenaline has an anti-inflammatory effect in the periphery (Sanders and Straub, 2002) it is possible that higher intensity protocols, by generating more stress at the system, attenuated microglia reactivity through activation of descendant noradrenergic inputs. In contrast, in situations with lower stress component, like mild run in a wheel or low velocity in a bike, where the locus coeruleus is not so active, microglia reactivity would not be attenuated. Interestingly, these groups of animals showed less or no preservation of synapses and PNN, thus suggesting that the reduction in glia activation could facilitate preservation of synapses and PNN after injury. Thus, besides the direct effects of increased activity, stress-related changes could also influence the fate of synapses and PNN (Van Meeteren et al., 1997).

Since inflammatory mediators released by activated microglia may activate astrocytes (Ji et al., 2007), we expected that reduced reactivity of microglia induced by exercise would be accompanied by a reduction in astrocyte reactivity. Nevertheless, we observed a marked increase in astrocytes 2 weeks post-injury in the trained animals, a fact that suggests that astrocyte activation is independent of microglia activation. This increased astrocyte reactivity may be related to the preservation of PNN in axotomized motoneurons of trained animals. A previous study showed that the formation of synapses promoted by astrocytes paralleled the emergence of PNNs in embryonic hippocampus (Pyka et al., 2011). However, low intensity exercise protocols also show a high GFAP activation, suggesting that there are other factors involved in preservation of PNN, such as microglia.

In conclusion, our results provide a proof of concept that enhancing functional afferent inputs to spinal neurons by means of different types of exercise, when sufficiently intensives, may help to prevent the central changes that these neurons suffer after axotomy. Further studies analyzing the functional recovery of the

central circuits after high intensity exercise and its relation to motoneuron PNNs changes would help elucidate the complex effects of activity-dependent therapies on the nervous system.



Chapter 4

Endogenous modulation of TrkB signaling by treadmill exercise after peripheral nerve injury

Abstract

Peripheral nerve injury (PNI) induces target denervation but also massive stripping of the central synapses of axotomized motoneurons that disrupts spinal circuits. Even when axonal regeneration is successful, the non-specific target reinnervation and the limited rebuilding of spinal circuits impair functional recovery. Therefore, strategies aimed to preserve spinal circuits after nerve lesions may improve the functional outcome. Activity dependent therapies, such as treadmill running (TR), reduce synaptic stripping and disorganization of perineuronal nets on axotomized motoneurons. The mechanism underlying these effects remains unknown, although the benefits of exercise are often attributed to an increase of neurotrophin BDNF. In this work, TrkB agonist and antagonist were administered to rats subjected to sciatic nerve injury in order to shed light on the role of BDNF. The maintenance of synapses on axotomized motoneurons induced by TR was partially dependent on TrkB activation, since a high dose of the TrkB agonist favored maintenance of inhibitory synapses whereas markedly reduced excitatory glutamatergic synapses. Therefore, the complex pattern of activation induced by specific regimes of physical exercise seem a better strategy to modulate the maladaptive plastic changes induced by PNI than systemic modulation of the BDNF pathway.

INTRODUCTION

Peripheral nerve injury (PNI) results in loss of motor, sensory and autonomic functions in the denervated territory. Despite peripheral axons have the capacity to regenerate, functional recovery is usually limited, due to misdirection of regenerated axons leading to non- specific reinnervation of the target organs, and also to maladaptive changes of the neural central circuits (Navarro et al., 2007). The interruption between the axons and their target organs is accompanied by disorganization of the central circuitry, in part due to the massive stripping of central synapses suffered by axotomized neurons (Alvarez et al., 2011).

Exercise and other activity dependent therapies have been extensively used to improve nerve regeneration after PNI in animal models (Gordon and English, 2015; Park and Höke, 2014; Teodori et al., 2011; Udina et al., 2011a). Particularly, rats submitted to treadmill running exercise (TR) after sciatic nerve injury showed improved axonal regeneration (English et al., 2011), increased muscle reinnervation and higher numbers of regenerated myelinated axons (Asensio-Pinilla et al., 2009).

It is assumed that repeated activity may reinforce the circuitry of the nervous system and facilitate functional recovery, by promoting structural plasticity, maintaining a trophic support for the paralyzed muscles and enhancing axonal growth (Ghiani et al., 2007; Rampon et al., 2000; Sale et al., 2007; Vaynman and Gomez-Pinilla, 2005). Maladaptive plastic changes associated with PNI, such as hyperreflexia (Asensio-Pinilla et al., 2009; Vivó et al., 2008) and neuropathic pain (Cobianchi et al., 2010; Fu et al., 2004; Nam et al., 2001) can be modulated by activity dependent therapies, probably by reducing the excitability of sensory neurons (Modol et al., 2014) and the synaptic stripping and perineuronal nets (PNN) loss that motoneurons suffer after axotomy (Arbat-Plana et al., 2015).

Accumulating evidences suggest that brain derived neurotrophic factor (BDNF) along with its specific receptor tropomyosin-related kinase (TrkB) may be a key pathway underlying the beneficial effects of TR after injuries to the nervous system (Ding et al., 2011; Hutchinson et al., 2004a; Raffaella Molteni et al., 2004). Increased

BDNF induced by exercise has been proposed to underlie the significant enhancement of axonal elongation into peripheral nerve grafts (Sabatier et al., 2008) and the reduction of synaptic stripping suffered by axotomized motoneurons (Krakowiak et al., 2015). By using a neuron-specific TrkB knockout mice, Krakowiak et al. (2015) demonstrated that the source of BDNF mediating beneficial effects of exercise is neuronal. Other studies showed that exercise reduces microglial expression of BDNF, a mechanism that would mediate the reduction of neuropathic pain after PNI in exercised animals (Cobianchi et al., 2013, 2010; López-Álvarez et al., 2015). Particularly, BDNF released by activated microglia induces down-regulation of the neuron specific K^+-Cl^-cotransporter 2 (KCC2) (Coull et al., 2003; Ferrini and Koninck, 2013), which is responsible for setting the reversal potential for GABA and glycine receptors in the control of sensory-motor inputs at the spinal cord (Boulenguez et al., 2010; Payne et al., 2003; Zeilhofer et al., 2012).

Although the effects of exercise and other activity dependent therapies are quite promising in animal models, and rehabilitation is one of the cornerstones for the treatment of neural injuries, it is important to note that the intensity of the activity and the time of application after the injury are key factors that influence the beneficial effects. Despite some controversy in the literature, it seems that moderate exercise training during the denervation phase would benefit regeneration, whereas long term hyperactivity or forced exercise could be detrimental (Tam and Gordon, 2003; Udina et al., 2011b). On the other hand, when focusing on the ability of exercise to modulate the plastic changes after PNI, different studies point that a high intensity running protocol within a short time after PNI is more beneficial than low intensity protocols (Arbat-Plana et al., 2015; Cobianchi et al., 2013). Delayed application of exercise after PNI is also less effective (Brandt et al., 2015). In the clinical practice, early application of activity dependent therapies of moderate or high intensity can be difficult to achieve in traumatic patients. Therefore, a pharmacological approach that would mimic the effects of exercise on plastic changes observed after PNI and that could also enhance axonal regeneration would allow an early treatment of these injuries in the clinic.

7-8-dihydroxyflavone (7,8-DHF) is a small-molecule compound that crosses the blood brain barrier and binds with high affinity and specificity to the BDNF receptor TrkB (Jang et al., 2010). Recent studies demonstrate that 7,8-DHF confers neuroprotective, cognitive-enhancing and antidepressant effects in animal models (Andero et al., 2011; Blugeot et al., 2011; Devi and Ohno, 2012; Jang et al., 2010). In contrast, ANA-12 is a non-peptide antagonist of TrkB receptor that elicits strong and specific effects in vivo (Cazorla et al., 2011). This molecule binds to the extracellular domain of TrkB, prevents BDNF-induced TrkB activation, and abolishes the biological effects of BDNF on TrkB-expressing cells but not those of NGF or NT-3 on TrkA and TrkC expressing cells. Moreover, systemic administration of this compound inhibits TrkB function in the brain (Ambrogini et al., 2013; Cazorla et al., 2011).

In the present study we describe the effects of the activation or blockage of TrkB by administering either 7,8-DHF or ANA-12 to rats after PNI. We evaluated the effects of these drugs on the synaptic stripping and PNN disorganization that motoneurons suffer after axotomy and in the development of neuropathic pain, with the aim to elucidate if pharmacological modulation of BDNF pathway underlies the effects of exercise on these spinal changes. We also further evaluated the role of the TrkB pathway in the improvements observed when TR is applied after PNI.

MATERIAL AND METHODS

Experimental animals

Adult female Sprague Dawley rats (n = 45, 8 weeks old, 200–280 g) were housed with free access to food and water at room temperature of 22 ± 2 °C under a 12:12-h light–dark cycle. All experimental procedures were approved by the ethics committee of our institution and followed the guidelines of the European Commission on Animal Care (EU Directive 2010/63/EU). 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 of tibialis anterior (TA) and gastrocnemius medialis (GM) muscles, retrograde tracing was applied one week before intervention. Bilaterally, two retrotracers, True Blue Chloride (TB, Setareh Biotech) and Fluorogold (FG, Fluorochrome), were used to identify both motoneuron pools. Firstly, the muscle was exposed by making a small cut to the skin and then two injections (2.5 µl/injection) were distributed throughout the body of the muscle with a glass pipette using a Picospritzer.

Surgical procedure

Animals where anesthetized and the sciatic nerve was exposed at the mid-thigh and cut by using microscissors. The proximal and distal stumps were rejoined with two epineural 10-0 sutures. Afterwards, muscle and skin were sutured in layers, iodine povidone was applied to the wound, and the rats were allowed to recover in a warm environment under close observation. Animals were followed for 2 weeks.

Pharmacological treatments

One group of animals was used as control group and did not receive any treatment. However, to evaluate potential toxicity of the vehicle solution, DMSO at 17% was administered twice a day in a subgroup of animals. A third control group was submitted to exercise from 3 days after the surgery without receiving any pharmacological treatment. Regarding pharmacological modulation of TrkB, two subgroups of animals were treated with the high-affinity TrkB receptor agonist 7,8-DHF (TCI-Europe), injected intraperitoneally twice a day at a high dose (10 mg/kg b.w). in 17% DMSO) (Jang et al., 2010) or once daily at a low dose (5 mg/kg b.w). in 17% DMSO), respectively (Agrawal et al., 2015; English et al., 2013). In another group, the TrkB receptor antagonist ANA-12 (Sigma-Aldrich) was administered intraperitoneally twice a day (0.5 mg/Kg b.w. in 1% DMSO) (Cazorla et al., 2011). A subgroup of animals receiving ANA-12 was also submitted to a high-intensity TR protocol from 3 days after

the surgery (Table 1). All pharmacological treatments started 3 days post injury and were carried out during 2 weeks.

All animals submitted to high intensity training exercise (HTRP) were placed on the treadmill for 60 min twice a week prior to surgery in order to acclimatize them to a motor-driven rodent treadmill (Treadmill LE 8706, LETICA, 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 started 3 days after surgery and was carried out during 2 weeks. TR consisted of one session of treadmill running 5 days/week 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 maximum speed of 30 cm/s for 60 min was reached (Cobianchi et al., 2013).

Experimental groups	**Treatment**	**Exercise protocol**	
		Immunohistochemistry, mechanical nociceptive threshold	**Immunoblotting**
1**. TrkB activation** (right sciatic nerve injury)	7,8-DHF treatment (5 mg/kg once a day)	No exercise (n=5)	No exercise (n=6)
	7,8-DHF treatment (10 mg/kg twice a day)	No exercise (n=5)	-
2. **TrkB inactivation** (right sciatic nerve injury)	ANA-12 treatment (0.5 mg/kg twice a day)	Treadmill running (n=5)	-
		No exercise (n=5)	
3.**Control** (right sciatic nerve injury)	DMSO 17% (twice a day)	No exercise (n=4)	-
	No drug treatment	Treadmill running (n=5)	Treadmill running (n=6)
		No exercise (n=5)	No exercise (n=6)
4. **Naïve**	No drug/ No injury	No exercise (n=5)	No exercise (n=6)

Table 1. Experimental design. Groups and treatments applied. Animals were divided into 4 main experimental groups. In all of them except naïve group, sciatic nerve was cut and repaired by direct suture **1.** TrkB agonist administration. Two subgroups with different doses of 7,8-DHF treatments were evaluated. **2.** TrkB antagonist administration. The group of animals treated with ANA-12 was divided in two subgroups: one submitted to treadmill running and another one was untrained. **3**. Control group: Vehicle (DMSO 17% twice a day) was administered in a group of animals. Another group was untreated, with half of the animals submitted to TR and the other half left untrained. **4.** Naïve group: no injured animals without treatment and exercise.

Mechanical nociceptive threshold measurement

Seven days before surgery, all the animals were habituated to the experimental device, by leaving them to explore the apparatus for 20 min and then starting the test for recording baseline nociceptive thresholds. The nociceptive behavior tests were performed on both hind paws before and at different days after injury. In postoperative tests, the experimenter was blind to rat assignment to the different groups.

Sensitivity to mechanical stimulus was measured by means of an electronic Von Frey algesimeter (Bioseb, Chaville, France). Rats were placed on a wire net platform in plastic chambers 10 min before the experiment for habituation. A non-noxious pointed probe was gently applied to medial plantar paw area, and then slowly increasing the pressure. The threshold was expressed as the force (expressed in grams) at which rats withdrew the paw in response to the stimulus. A cutoff force was set at 40 grams, when the stimulus lifted the paw without response. Threshold was calculated as the mean of three measurements per test site, with 3 min interval between each measurement. Algesimetry tests were performed during the morning, while TR sessions were carried out during the afternoon.

Immunohistochemical analysis of spinal changes

At the end of follow-up, deeply anesthetized animals were transcardiacally perfused with 4% paraformaldehyde in PBS. The L3–L6 spinal cord segment was removed, post-fixed for 4 h, cryoprotected in 30% sucrose, and stored at 4°C until use. Samples were embedded in Tissue-Tek, serially cut (20 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 1 h, followed by overnight incubation at 4°C with combinations of primary antibodies: rabbit anti Synaptophysin (1:200, Covance), guinea pig anti VGlut1 (1:300, Millipore), guinea pig anti VGat (1:200, Synaptic Systems), Lectin from Wisteria Floribunda (1:100, Sigma), mouse anti GFAP (1:1000, Millipore) and rabbit anti Iba1 (1:500, Wako). After washes, immunoreactive sites were revealed by using species-specific secondary antibodies conjugated to 488

Alexa Fluor (1:200, Invitrogen), 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 cover-slipped with Fluoromount-G (SouthernBiotech). The samples processed for KCC, were also counterstained with DAPI and mounted with Mowiol (Sigma). Labeled motoneurons were localized and images captured with a scanning confocal microscope (LSM 700 Axio Observer, Carl Zeiss 40×/1,3 Oil DIC M27).

Image analysis, processing and regression analysis of labeling quantification was performed by means of in-house software implemented in MatlabR2014b (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 a perimeter of 5 μm surrounding the soma (Arbat-Plana et al., 2015). For each animal, 10 to 15 motoneurons of each pool and each side were analyzed.

Three sections of three samples per group were randomly used for quantification of KCC2 immunoreactivity in L4-L5 spinal cord. Images of sections were acquired with the confocal microscope at 20X magnification, and analyzed using ImageJ software (NIH, USA). A threshold for subtracting background was applied by defining a fixed gray scale cutoff point set from the same image of control rats. Then, the integrated density (InDen) of KCC2 labeling was measured.

Immunoblot

Rats were anesthetized and decapitated at 7 days post axotomy for sample preparation (n=4 for each treatment). The L4–L5 spinal cord segments were removed and divided into quarters to isolate the ventral part of each side. The ipsilateral and contralateral ventral parts of L4–L5 cord segments were separately homogenized in RIPA modified buffer [50mM Tris-HCl pH 7.4, 1% Triton X-100, 0.5% DOCNa, 0.1% SDS, 150mM NaCl, 2mM EDTA] with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and phosphatase inhibitor cocktail (Roche). After clearance, protein concentration was measured by the BCA method assay (BCA Protein Assay kit, Pierce,

Rockford, IL, USA). An equal amount of protein (40 µg/lane) was resolved in 7.5% SDS-PAGE and electrotransfered to PVDF membranes (Millipore) in transfer buffer [25mM trizma-base, 192mM glycine, 20% (v/v) methanol, pH 8.4]. Membranes were blocked with 6% non-fat dry milk in TBS plus 0.1% Tween 20 buffer for 1 h at room temperature and incubated overnight with the corresponding primary antibody diluted in blocking buffer: goat anti-TrkB and goat anti-TrkC (1:1000) (R&D Systems, Minneapolis, MN, USA), rabbit anti-phospho-AKT (S473)(D9E) and rabbit anti-pan-AKT (C67E7) (1:1000) (Cell Signaling Technology; Beverly, MA, USA), and mouse anti-β-Actin (Sigma-Aldrich). After several washes, membranes were incubated for 1 h with an appropriate secondary antibody conjugated with horseradish peroxidase (1:3000) anti-rabbit-HRP, anti-goat-HRP or anti-mouse-HRP (Bio-Rad Laboratories, Berkeley, CA, USA). Proteins were visualized by enhanced chemiluminiscence method (ECL Clarity kit, Bio-Rad Laboratories, Berkeley, CA, USA), and the images captured and analyzed with Image Lab software (Bio-Rad Laboratories).

Statistical analysis

For quantitative variables, normality was assessed by Shapiro-Wilk test (Royston, 1993). For variables with a normal distribution 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. A nested design ANOVA test was used in order to determine if the variability was due to the difference between the motoneurons or between animals in the experimental groups. Statistical analysis of mechanical threshold was made by two-way ANOVA with group and time after injury as factors, followed by Bonferroni post hoc comparisons. Statistical significance for KCC2 immunofluorescence was calculated by two-way ANOVA (for multiple groups comparison) followed by Tukey post-hoc test when necessary. SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. A value of $p< 0.05$ was considered significant. Data are presented as mean±SEM.

RESULTS

As we previously described (Arbat-Plana et al., 2015), axotomized motoneurons presented a massive synaptic stripping of their central arbor, with a marked decrease of proximal glutamatergic synapses and an increase of inhibitory ones after nerve injury. There was also disorganization of their PNN, evidenced by reduction of its immunoreactivity.

Since each drug required a different concentration of DMSO, we tested the higher dose of DMSO in control rats, to discard a toxic/beneficial effect of the vehicle. No significant differences were found between the non-treated group and the group injected with DMSO at 17% in any of the analyzed stainings (Supplementary Fig 1). Although statistical comparisons were done using both DMSO and non-treated control groups, since no differences were found, to simplify the graphs and the results only the control non-treated group is described.

Effects of 7,8-DHF administration on central changes after axotomy

To evaluate whether pharmacological activation of TrkB could mimic the effects of TR on synaptic stripping after PNI, two different dose of 7,8-DHF were evaluated.

Synaptophysin (Syn) immunolabeling, a general marker of synaptic coverage, showed that the number of synaptic contacts was reduced after sciatic nerve injury. Both groups of animals treated with the TrkB agonist had significantly higher contacts on TA motoneurons (about 87±4 Syn/μm^2) compared to control injured animals (79±4 Syn/μm^2; $p<0.001$), whereas this preservation was not significant in GM motoneurons of the animals treated with 7,8-DHF. However, preservation of Syn labeled contacts by 7,8-DHF was slightly lower than observed after TR (97±5 Syn/μm^2 in TA motoneurons, and 104±5 Syn/μm^2 in GM motoneurons) (Figs 1A, 2.1). Interestingly, the high dose of 7,8-DHF decreased Syn contacts on motoneurons of the contralateral side whereas the low dose did not affect contralateral non-injured motoneurons.

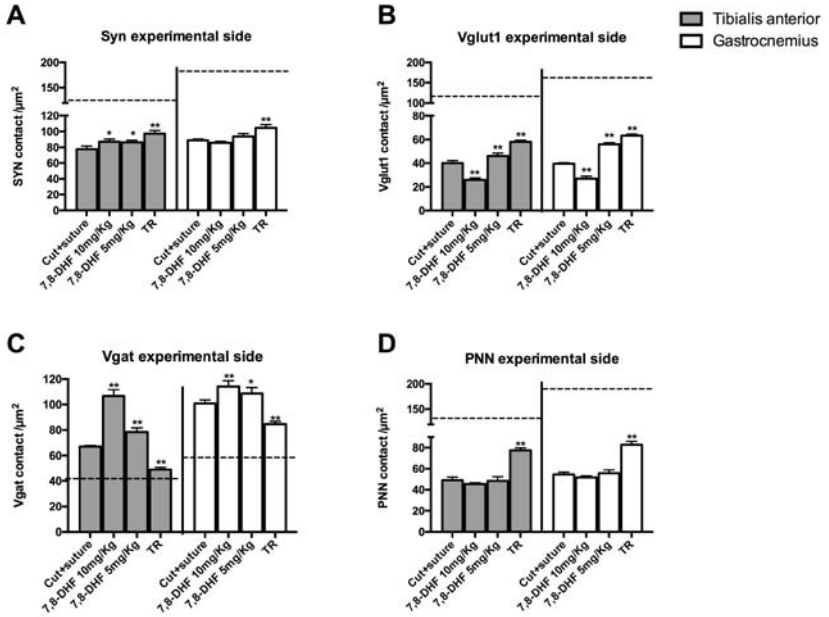


Fig 1. Quantitative analysis of synaptic contacts and PNN immunoreactivity on motoneurons after axotomy in animals treated with different doses of the TrkB agonist 7,8-DHF. Evaluation of Synaptophysin (A), VGlut1 (B), VGat (C) and PNN (D) in back-labeled motoneurons of tibialis anterior (TA, gray bars) and gastrocnemius medialis (GM, white bars) muscles from injured animals untreated, treated with a low and a high dose of 7,8-DHF, and animals submitted to TR. Horizontal dotted lines indicate the average of motoneuron labeling in the non-injured side of both TA and GM motoneurons. Data are expressed in absolute values as mean ± SEM, *p<0.05, **p<0.01.

When focusing on the excitatory VGlut1 containing synaptic contacts, we found that axotomized TA and GM motoneurons had a density of about 40±2 per µm^2, less than half of the intact motoneurons (Fig 1B,2). Rats treated with 10 mg/kg 7,8-DHF showed a significant decrease of VGlut1 synapses on axotomized motoneurons of both TA and GM pools compared to the non-treated group (26±3/µm^2 in both TA and GM motoneurons; p<0.001). In contrast, the low dose of 7,8-DHF increased VGlut1 density (45±3/µm^{2}in TA motoneurons; 56±2/µm^2 in GA motoneurons; p<0.005), although less than in the group submitted to TR (57±2/µm^2 in TA motoneurons; 62±2/µm^2 in GA motoneurons; p<0.001 vs control group). Unexpectedly, we observed that VGlut1 immunoreactivity in contralateral intact motoneurons was lower in rats receiving 10mg/kg 7,8-DHF (24±3/µm^2) than in the

control animals (61±2/µm^2; p<0.001) and animals receiving the low dose of 7,8-DHF (58±4/µm^2; p<0.001) (Figs 1B, 2).

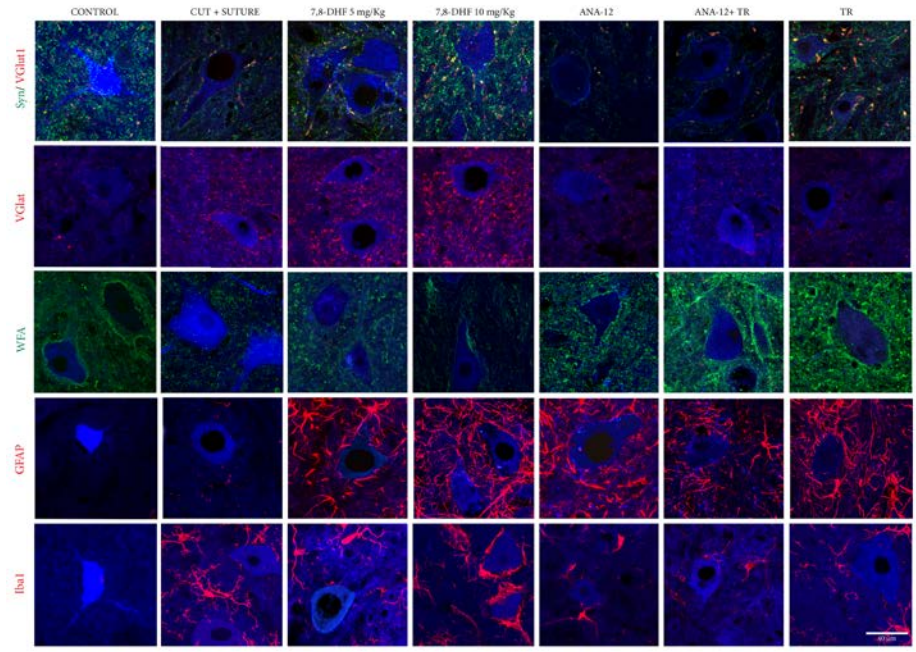


Fig 2. Evaluation of excitatory and inhibitory synapses, PNN, astroglia and microglia reactivity surrounding axotomized motoneurons of animals submitted to different pharmacological treatments. All immunostainings were evaluated in confocal images of spinal cord regions containing back-labeled motoneurons (blue) of TA and GM muscles after nerve cut repaired with direct suture in the different groups of animals.

In contrast to Syn and VGlut1 immunoreactivity, VGat labeling surrounding axotomized motoneurons increased after injury (67±1 VGat contacts/µm^2 and 100±3/µm^2 in TA and GM motoneurons, respectively) compared to intact motoneurons (42± 2 VGat/µm^2 and 61± 2 VGat/µm^2 in TA and GM motoneurons, respectively) (Figs 1C, 2). Treatment with 7,8-DHF produced a significantly higher increase of VGat contacts, more pronounced in the rats receiving the high dose (113 ± 10 VGat/µm^2 in TA and GM motoneurons) than in those with the low dose (78± 5 VGat/µm^2 and 105± 3 VGat/µm^2 in TA and GA motoneurons, respectively) compared to untreated animals (p<0.001). In contrast, in the group of animals submitted to TR, VGat density surrounding axotomized motoneurons was closer to

contralateral values and significantly lower than those found in the 7,8-DHF treated groups (49± 2 VGat/μm^2 and 85± 2 VGat/$\mu m^{2\ in}$ TA and GA motoneurons, respectively; $p<0.001$) (Figs 1C, 2).

Astroglial reactivity, analyzed by GFAP immunolabeling around motoneurons, was significantly increased (265 ± 18 GFAP/μm^2) in both groups of animals treated with TrkB agonist compared to the non-treated group (5±2 GFAP/μm^2, $p<0.0001$). In the group of animals submitted to TR, astroglia reactivity was also increased (262± 4 GFAP/μm^2) similarly to 7,8-DH treated animals (Fig 2).

Iba1 immunoreactivity around axotomized motoneurons was used to evaluate microglial reactivity. 15 days after nerve injury, there was a marked increase of microglia (80±20 Iba1/μm^2). The high dose of 7,8-DHF significantly increased microglial reactivity (150±18 Iba1/μm^2), whereas the low dose drastically reduced it (8±2 Iba1/μm^2), similarly to that observed in the exercised group (6±2 Iba1/μm^2) (Fig 2).

The PNN, analyzed by assessing the immunoreactivity of Wisteria floribunda, showed a reduction in axotomized motoneurons, that was partially prevented if animals were submitted to exercise, but was not affected by administration of neither low nor high doses of 7,8-DHF (Figs 1D, 2).

Effects of ANA-12 administration on central changes after axotomy

To further evaluate if inactivation of TrkB could be involved in the spinal changes mediated by TR following PNI, ANA-12 was administrated to two subgroups of rats, one of them submitted to TR.

The reduction of Syn immunoreactivity observed in motoneurons after axotomy was significantly more reduced with ANA-12 administration (66±5 Syn/μm^2 and 80±3 Syn/μm^2 in TA and GM motoneurons, respectively; $P < 0.001$). In contrast, TR was able to partially prevent the decrease in Syn immunoreactivity of axotomized motoneurons (97±5 Syn/μm^2 and 104±5 Syn/μm^2 in TA and GM motoneurons, respectively). Administration of ANA-12 to exercised animals blocked this positive effect of TR on maintenance of synaptic contacts (46±3 Syn/μm^2 and 90±6 Syn/μm^2 in

TA and GM motoneurons, respectively), and similar values of immunoreactivity between ANA-12 untrained and ANA-12 trained animals were observed (Figs 2, 3A).

ANA-12 administration also induced a more marked decrease of VGlut1 synapses on injured motoneurons (28±4 VGlut1/μm^2 in both TA and GM versus 40±2 VGlut1/μm^2 in non-treated animals). When animals treated with ANA-12 were submitted to TR, the decrease in VGlut1 was less marked (45±6 VGlut1/μm^2), but still significantly lower than with TR alone (57±2 μm^2 in TA motoneurons and 62±2 μm^2in GA motoneurons; $p<0.005$) (Fig 2, 3B).

The noted increase of VGat inhibitory synapses was less marked in ANA-12 treated animals (56±3 VGat/μm^2 and 96±3 VGat/μm^2 in TA and GM respectively; $P < 0.001$). On the other hand, animals submitted to TR, both treated and untreated with ANA-12, showed a lower increase in inhibitory synapses after axotomy (46±3 VGat/μm^2 and 90±6 VGat/μm^2 in TA and GM versus 49±2 VGat/μm^2 and 85±2 VGat/μm^2, respectively, in treated and non- treated animals) (Figs 2, 3C).

As the TrkB analogue, administration of ANA-12 also modulated glial cell responses, as shown by increased astrocyte reactivity (265± 10 GFAP/μm^2) and reduced microglia reactivity (5 ± 2 Iba1/μm^2 vs 80 ± 20Iba/μm^2, $P < 0.01$) around axotomized motoneurons compared to control untreated rats. Administration of ANA-12 did not affect the modulation of glial reactivity induced by exercise (Fig 2).

In the group of animals treated with ANA-12, we detected a slight increase of PNN immunoreactivity (60±3 PNN/μm^2 in TA and 78±6 PNN/μm^2 in GM) when compared to untreated animals (48±2 PNN/μm^2 in TA and 55±3 PNN/μm^2 in GM), although these differences reached statistical significance only for the GM motoneurons.

Axotomized motoneurons of animals receiving ANA-12 and submitted to TR showed a similar significant increase in PNN staining (61±4 PNN/μm^2 and 75±12 PNN/μm^2 in TA and GM respectively) compared to motoneurons of untrained animals. However, the PNN density in ANA-12 trained animals was similar to non-treated animals submitted to TR (76±2 PNN/μm^2 and 84±4 PNN/μm^2 in TA and GM respectively) (Fig 2, 3D).

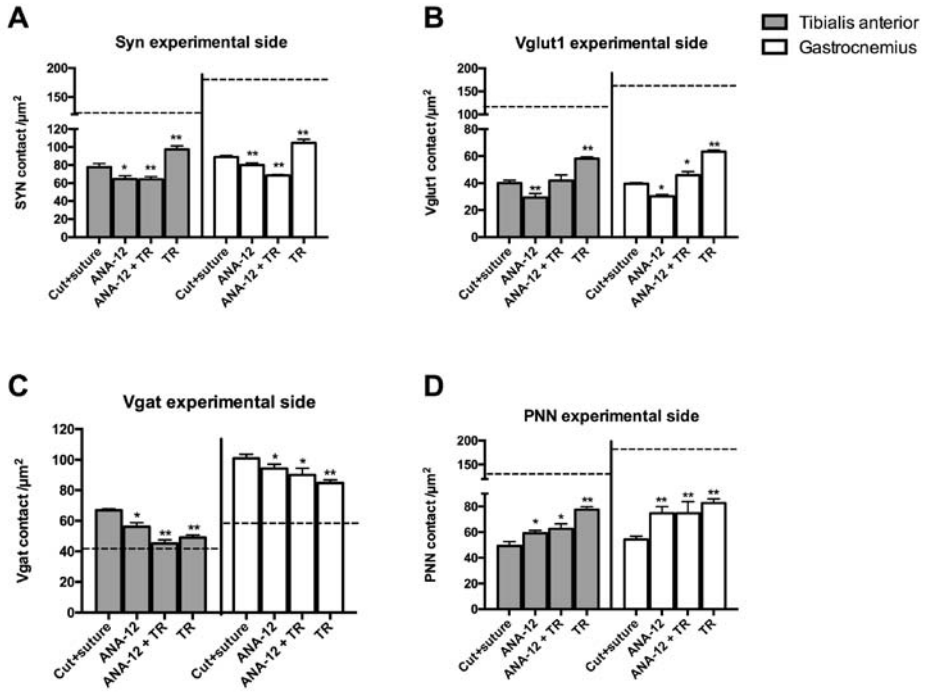


Fig 3. Quantitative analysis of synaptic contacts and PNN immunoreactivity on motoneurons after axotomy in animals treated with TrkB antagonist (ANA-12) and submitted to treadmill running. Evaluation of Syn (A), VGlut1 (B), VGat (C) and PNN (D) in back-labeled motoneurons from TA (gray bars) and GM (white bars) muscles of injured animals non treated, treated with ANA-12 and animals submitted to TR, either treated or not treated with ANA-12. Horizontal dotted lines indicate the average of motoneuron labeling in the non-injured side of both TA and GA motoneurons. Data are expressed in absolute values as mean ± SEM, *p<0.05, **p<0.01.

Effect of treadmill and drug administration on mechanical nociception

Sciatic nerve injury induced a fast decrease of withdrawal threshold in the injured paw, rapidly lowering to 58% and 38% of contralateral at 3 and 14 days postinjury respectively as showed by DMSO control group (Fig 4), reflecting a state of mechanical allodynia. Similarly to what was previously demonstrated (Cobianchi et al., 2013; López-Álvarez et al., 2015), the TR protocol used produced a significant increase in the withdrawal threshold of the injured paw compared with the control group, both at 7 (70% of contralateral; $p<0.001$) and at 14 days (71%; $p<0.001$ in DMSO vs. TR groups). Oppositely, administration of the low dose of 7,8-DHF resulted in further threshold decrease at 7 dpi compared to exercised animals, whereas the high dose of

7,8-DHF induced no changes compared with DMSO group. ANA-12 administration did not change the withdrawal threshold compared to the DMSO group; however, when combined with TR exercise, it does not change the hypoalgesic effect (67% of contralateral threshold at 7 days, $p<0.01$ in DMSO vs. ANA-12+TR groups; 82% of contralateral threshold at 14 days, $p<0.001$ in DMSO vs. ANA-12+TR groups). Finally compared with the groups of drug treatments, the injured paw mechanical threshold of TR group was significantly higher both at 7 and 14 days (respectively: $p<0.05$ and $p<0.001$ in TR vs. ANA-12 groups; $p<0.001$ and $p<0.01$ in TR vs. 7,8-DHF-5mg/Kg groups; $p<0.01$ and $p<0.01$ in TR vs. 7,8-DHF-10mg/Kg groups).

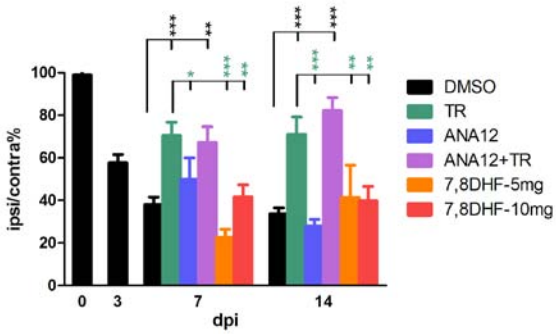


Fig 4. Mechanical algesimetry test results on animals submitted to different treatments. Values are expressed as the % ratio between the threshold force ipsilateral versus contralateral to the injured paw at different days post injury (dpi). Significant statistical comparisons are represented for DMSO and TR groups versus drugs treatment groups after injury. Data are expressed as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.

Changes in KCC2 expression in the spinal cord

A significant decrease of KCC2 immunoreactivity was observed after PNI at both the ventral and the dorsal horn of the injury side compared to rats (Fig 5A, naïve vs. DMSO groups). Neither 7,8-DHF nor ANA-12 treatments significantly changed this effect (images not shown). In contrast, TR exercise such prevented the decrease of KCC2 immunoreactivity, this effect being more evident in group combined with ANA-12 treatment. Statistical comparisons of KCC2 immunoreactivity highlighted a significant difference of naïve with all others (Fig 5B, $p<0.01$) except groups performing TR in the injured side dorsal and ventral horns and also in the contralateral

dorsal horn of the spinal cord. On the other hand, after injury KCC2 immunoreactivity was significantly higher only in the injured side dorsal horn of ANA-12+TR group ($p<0.001$, ANA-12+TR group vs. DMSO, ANA-12 and both 7,8-DHF groups).

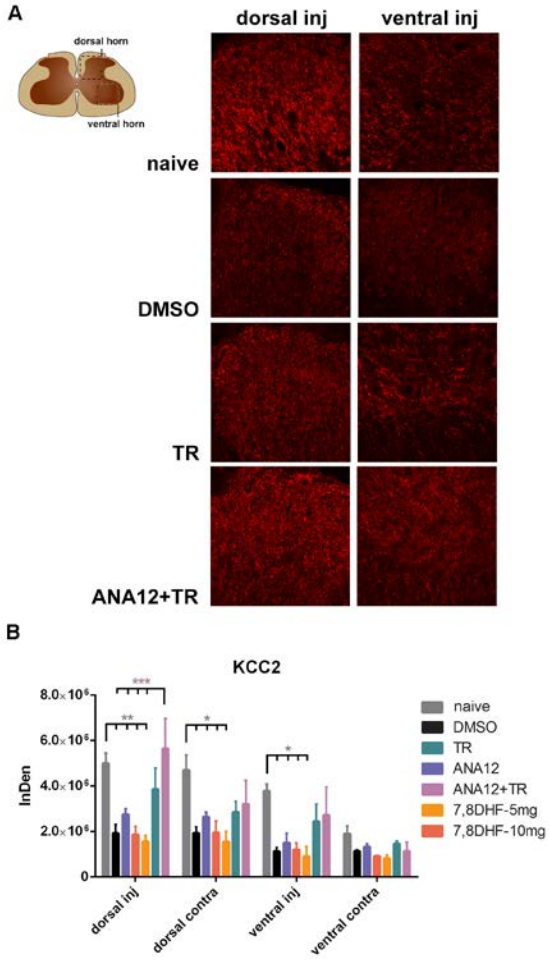


Fig 5. Changes in KCC2 expression at dorsal and ventral horns of the spinal cord. A. Immunostaining against KCC2 in indicated areas of dorsal and ventral horns of spinal cords (naïve group). PNI induced an important reduction in the immunostaining against KCC2 in injured side of both spinal horns (DMSO group). This reduction was prevented in animals performing exercise (TR and ANA-12+TR groups). On the contrary, drug treatments alone did not change KCC2 immunoreactivity after injury (images not shown). **B**. Quantification of KCC2 immunoreactivity in L4-L5 spinal cords of different experimental groups. Statistically significant differences emerged between naïve and other groups (gray asterisks) and between ANA-12+TR and other groups (violet asterisks). Data are expressed as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.

Effect of 7,8-DHF and TR on the protein levels of TrkB, TrkC and AKT

Protein levels of TrkB at the spinal cord were not affected by the PNI. TrkB expression tends to be enhanced in the contralateral side of animals submitted to exercise, although the difference is not significant. In contrast, this tendency is not observed after 7,8-DHF administration compared to non-treated animals (Fig 6 A, B). In addition, there was a slight, though not significant increase of TrkC protein levels after axotomy, which was not observed after 7,8-DHF and TR treatments (Fig 6A, B). Finally, levels of AKT, a downstream signaling pathway of tyrosine-kinase receptors were similar in 7,8-DHF and TR animals, and higher than in non-treated animals, although differences were not significant (Fig 6A, C).

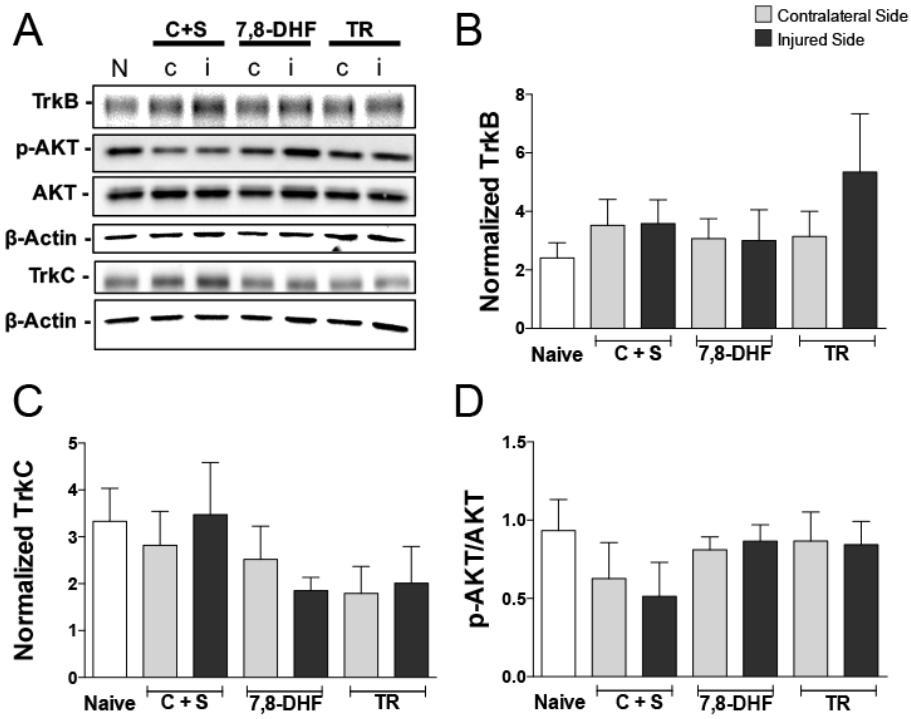


Fig 6. Western blot analysis of the tyrosine-kinase receptors. (A) Immunoblot at dpi of the contralateral (c) and injured (i) sides of the rat spinal cord ventral horn of naïve (N), axotomized (C+S), treated with 7,8-DHF (7,8-DHF) and exercised (TR) groups. (B-D). All protein levels were normalized with β-actin and analyzed by Image Lab software (Bio-Rad Laboratories, Inc, Berkeley, CA, USA). Data are expressed as mean ± SEM, *p<0.05, **p<0.01

DISCUSSION

The results of this study show that a high intensity protocol of treadmill running applied during 2 weeks is able to attenuate synaptic stripping and PNN loss around the injured motoneurons after sciatic nerve lesion in rats, corroborating to the results obtained in present studies (Arbat-Plana et al., 2015; Cobianchi et al., 2013). Since physical exercise modulates the expression of BDNF and NT-3 in the spinal cord, with specific patterns of activation for each neurotrophin (Ding et al., 2011; Krakowiak et al., 2015; Skup et al., 2000) in this study we have evaluated if systemic administration of a TrkB analog, 7,8-DHF, can mimic the ability of treadmill exercise to reduce synaptic stripping of motoneurons and neuropathic pain after PNI. To further clarify the role of TrkB activation, we also evaluated if an antagonist of TrkB could block the positive effects induced by treadmill exercise. In addition, we evaluated the TrkB receptor levels and one of their downstream targets, AKT, after treatment with 7,8-DHF and TR following PNI. We also studied TrkC expression, which is also implicated in the effects of exercise (Gómez-Pinilla et al., 2001; Ying et al., 2003).

In a previous study, administration of the TrkB agonist 7,8-DHF (10 mg/kg once a day) mimicked the effects that physical exercise and environmental exploration induce on synaptogenesis in rat dentate gyrus (Ambrogini et al., 2013). Another study, using the same agonist but at lower dose (5 mg/kg once a day) showed that TrkB activation promotes axonal regeneration in transected nerves (English et al., 2013). Therefore, in this work we evaluated the effects of administration of this TrkB agonist at both doses on the plastic changes induced by PNI in the spinal cord. After PNI, there is an important loss of excitatory synapses and an increase of inhibitory synapses on motoneurons, that normalize with recovery (Arbat-Plana et al., 2015). Interestingly, the low dose of 7,8-DHF favored the maintenance of synapses in axotomized motoneurons. In contrast, the dose of 10 mg/Kg of 7,8-DHF caused a further loss of excitatory synapses and a significant increase of inhibitory synapses in both injured and contralateral sides.

Although a high activation of TrkB does not mimic the effects of TR on synaptic maintenance in axotomized motoneurons, TrkB inactivation impedes the

positive effects exerted by exercise after PNI, like maintenance of VGlut1 terminals on axotomized motoneuron. Thus, these results suggest that the positive effects of TR depend on specific levels of BDNF and an increased activation of TrkB does not potentiate these benefits. In fact, synaptic maintenance induced by TrkB activation is dose-dependent, and over-activation can promote deleterious effects on synaptic content, affecting both injured and intact motoneurons. On the other hand, systemic TrkB activation just potentiates the BDNF pathway, whereas exercise increases both BDNF and NT-3 neurotrophins, along with their receptors, TrkB and TrkC respectively (Gómez-Pinilla et al., 2001; Krakowiak et al., 2015; Skup et al., 2000). In abducens oculomotor neurons, prevention of synaptic stripping is more effective when both neurotrophins are applied together than separately (Davis-López de Carrizosa et al., 2009). That study also showed that NT-3 and BDNF had complementary, but not compensatory effects over distinct afferents. Similar to oculomotor neurons, spinal motoneurons probably need a balanced source of both neurotrophins to maintain their normal arbor and excess of one neurotrophin over the other might disrupt their synaptic inputs. In fact, our data suggest that BDNF is preferentially favoring maintenance of inhibitory VGat synapses in spinal motoneurons.

To further elucidate the potential role of BDNF and NT-3 in the effects of exercise, and the reasons why a TrkB agonist is not mimicking these effects, we evaluated the expression of their receptors, TrkB and TrkC respectively, in animals submitted to exercise or treated with 7,8-DHF. Interestingly, levels of TrkB receptor tends to be increased in the spinal cord of exercised animals, whereas TrkB agonist was not affecting their levels. In contrast, levels of TrkC receptor were similar in both groups. To corroborate that the TrkB pathway was being activated, we also evaluated the downstream signaling AKT, that was similarly expressed in both groups, with a trend to be higher than in non-treated injured animals. We did not found significant diferences in TrkB and AKT proteins after 7,-8-DHF afministration compared to only injured animals, likely due to a overactivation of TrKB, as reported in studies showing that prolonged BDNF treatment reduces the trophic response of TrkB (Ascaño et al., 2009; Carter et al., 1995; Chen et al., 1995). However, the expression of these three

proteins was analyzed in the whole spinal cord, and specific changes in some of the cell populations could be masked. Although the major source of BDNF appears to be neuronal (Rauskolb et al., 2010) , it can also be produced by astrocytes and microglia (Dougherty et al., 2000; Parpura et al., 2010). In fact, English et al. (2013) showed that the effects of exercise are mainly mediated by activation of neuronal TrkB. In contrast, treadmill exercise seem to reduce activation of microglial TrkB (Cobianchi et al., 2010). Thus, whereas systemic administration of a TrkB analog would activate this receptor in all cell types, exercise could be acting more specifically in some cell populations, increasing activation of TrkB in neurons but reducing it in microglia.

It is known that microglia express activated TrkB, and released BDNF increases their proliferation (Spencer-Segal et al., 2011). The secretion of BDNF by microglia has been linked to the inversion of inhibitory GABAergic currents in neurons, triggered by KCC2 down-regulation (Coull et al., 2003; Ferrini and Koninck, 2013). GABAergic interneurons are particularly sensitive to altered BDNF signaling. Increased BDNF-mediated TrkB activation suppresses Cl^-dependent fast GABAergic inhibition by down-regulating KCC2 (Rivera et al., 2001), inverting the anion flux upon GABA receptor activation from inhibitory to excitatory (Beggs et al., 2012; Price et al., 2005). After PNI there is an important down-regulation of both ipsilateral and contralateral KCC2 protein and its phosphorylated active form in dorsal horn neurons, that is associated to the development of neuropathic pain (Modol et al., 2014). We recently found that TR significantly reduced microglia reactivity and BDNF expression in microglia after sciatic nerve injury, rescuing also KCC2 levels (López-Álvarez et al., 2015). Thus, exercise can counteract some of the mechanisms related by central disinhibition. Here we observed that treatment with the TrkB analog caused a trend to further reduce KCC2 levels after PNI, whereas administration of the antagonist ANA-12 in exercised animals increased these levels, suggesting that pharmacological TrkB modulation may partially affect KCC2. In contrast, administration of ANA-12 by itself is not able to mimic the analgesia induced by exercise, indicating that TR may be acting through different mechanism than TrkB modulation on excitability of sensory neurons. In fact, the reduction in microglia reactivity induced by TR could be un-related to the

effects of exercise on neuropathic pain, since this reactivity is unspecifically affected by the different treatments, whereas the modulation of neuropathic pain is clearly dependent on TrkB activation. Low doses of the TrkB analog increased the hyperalgesia induced by PNI, that was also accompanied by increased microglia reactivity in the dorsal horn. In contrast, pharmacological blockade of TrkB activity did not reproduce but might facilitate the hypoalgesia induced by TR. In fact, TR significantly reduced hyperalgesia after PNI, and this effect was potentiated by administration of the TrkB antagonist.

When focusing on PNN, we found that ANA-12 administration, either in exercised or non-exercised animals, significantly increased PNN density around axotomized motoneurons compared to control untrained animals. Therefore, maintenance of PNN can be mediated by blockade of TrkB activation, probably on glial cells. In fact, during development, blockade of TrkB-mediated signaling by chondroitin sulfate proteoglycans, the main component of PNN, leads to closure of the critical period (Kanato et al., 2009; Kurihara and Yamashita, 2012) and thus, the restriction of neural plasticity. Thus, although some of the effects of exercise on central circuitry and plasticity after PNI are mediated through TrkB activation, pharmacologic activation of TrkB receptor is not able to mimic all the positive effects of exercise and may even have deleterious effects on synaptic maintenance on motoneurons and development of neuropathic pain. Therefore, the complex pattern of activation induced by specific regimes of physical exercise seem a better strategy to modulate the maladaptive plastic changes induced by PNI than systemic modulation of the BDNF pathway.

SUPPLEMENTARY MATERIAL

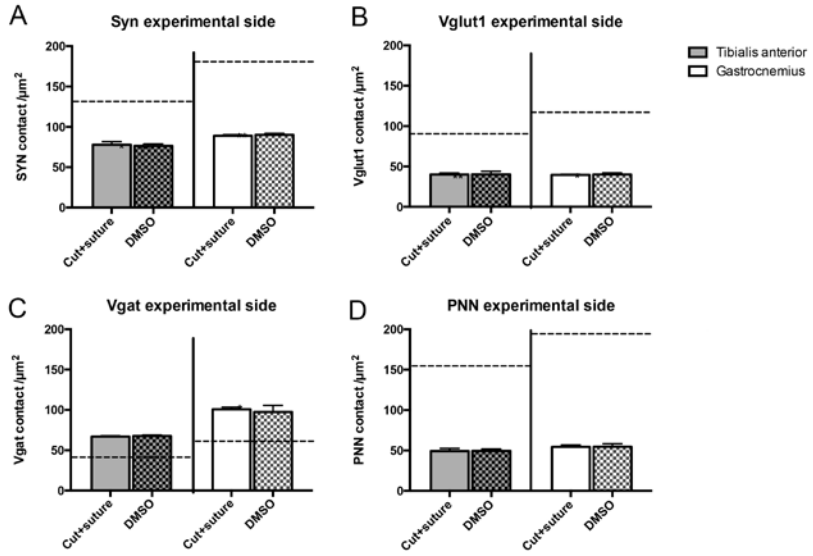
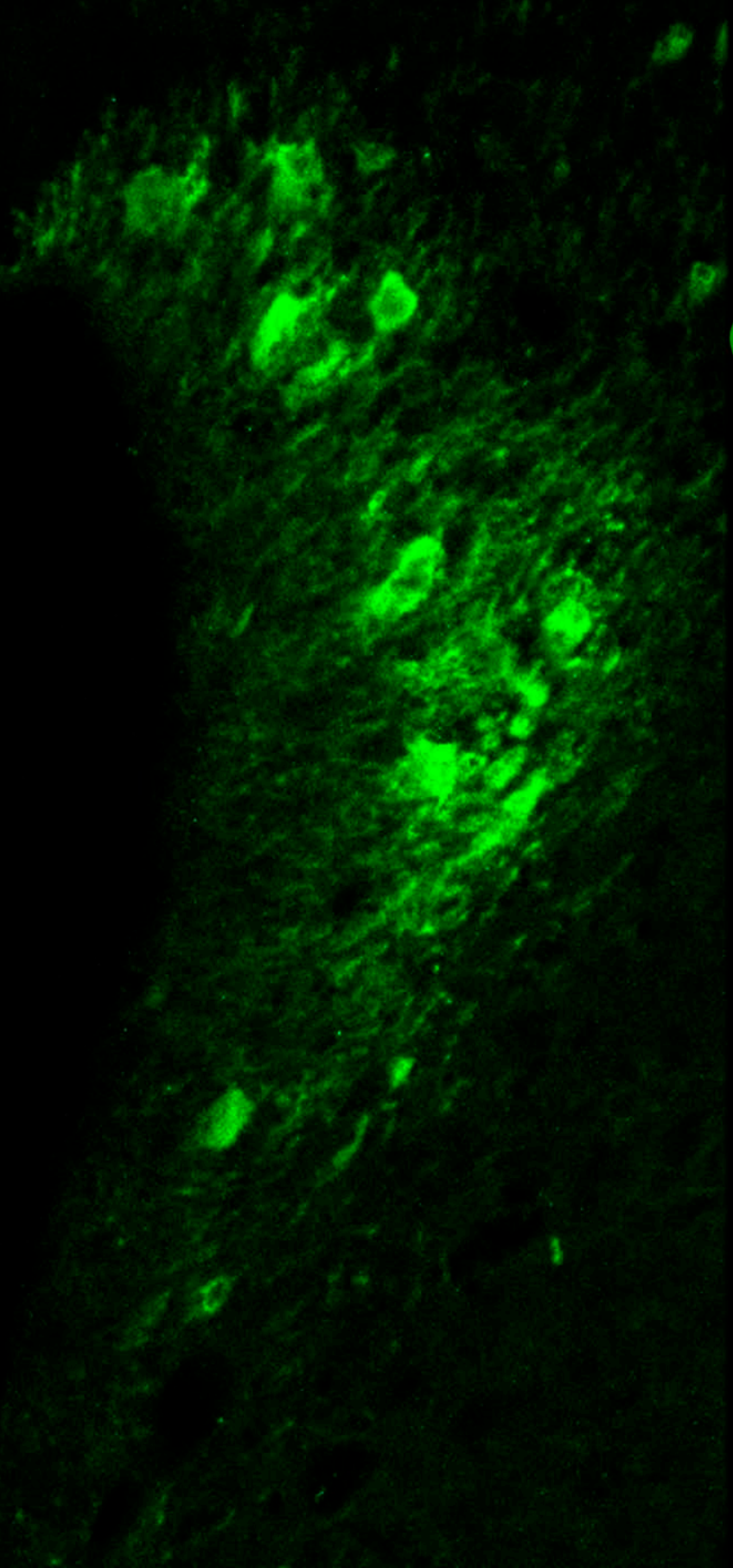


Fig 1. Quantitative analysis of the effects of DMSO administration on synaptic stripping of motoneurons after sciatic nerve injury. Evaluation of Synaptophysin (A), VGlut1 (B), VGat (C) and PNN (D) in back-labeled motoneurons from TA (gray bars) and GM (white bars) from injured animals untreated or treated with DMSO 17%. Data are expressed in absolute values as mean ± SEM. No significant differences were found between groups.



Chapter 5

Effects of physical exercise and noradrenergic descending inputs on changes in spinal motoneurons after peripheral nerve injury

ABSTRACT

Physical rehabilitation is one of the cornerstones for the treatment of injuries of the nervous system. After peripheral nerve injuries, activity dependent therapies promote trophic support for the paralyzed muscles, enhance axonal growth and also modulate the maladaptive plastic changes induced by the injury at the spinal level. We have previously demonstrated that an intensive protocol of treadmill running in rats reduces synaptic stripping on axotomized motoneurons, preserves their perineuronal nets and attenuates microglia reactivity. However, it is not clear through which mechanisms exercise is exerting these effects. Here we aimed to evaluate if descending noradrenergic projections play a role in these effects. Since descending noradrenergic projections from the locus coeruleus (LC) are markedly activated in stressful situations, as during intensive exercise, we destroyed the LC by administering the neurotoxin DPS-4 before injuring the sciatic nerve of adult rats. In animals with LC loss, an increasing intensity protocol of treadmill running was not able to prevent synaptic stripping on axotomized motoneurons and the reduction in the thickness of their perineuronal nets. In contrast, microglia reactivity was attenuated, thus indicating that the noradrenergic projections are important for some but not all the effects that exercise induces on the spinal cord after peripheral nerve injury. Animals submitted to treadmill training showed delayed muscle reinnervation, more evident if treated with DSP-4. However, we did not find differences in treated animals regarding the H/M amplitude ratio, which increased during the first stages of regeneration in all injured groups.

INTRODUCTION

Peripheral nerve injury (PNI) results in a combination of motor, sensory and autonomic impairments. After PNI, axons have the ability to regenerate, but the regenerative process is slow and functional recovery is often limited, due to unspecific reinnervation of the target organs and also to alterations at the central level. The disconnection between the neurons and their target organs is accompanied by a disorganization of the central circuitry, in part due to the massive stripping of central synapses that axotomized motoneurons suffer (Alvarez et al., 2010).

Activity-dependent therapies have been extensively used to improve axonal regeneration (Al-majed et al., 2000; Asensio-Pinilla et al., 2009; Meeteren et al., 1997; Sabatier et al., 2008), and to promote muscle reinnervation after sciatic nerve injury in rats (Asensio-Pinilla et al., 2009; Udina et al., 2011a). In general, when initiated during the denervation phase, moderate exercise training results in accelerated functional sensorimotor recovery (Gutmann and Jokoubek, 1963), whereas long term hyperactivity or forced exercise tend to have a detrimental effect (Herbison et al., 1974; Meeteren et al., 1997). Activity can also modulate the plastic changes observed after PNI, modulating hyperreflexia (Asensio-Pinilla et al., 2009; Udina et al., 2011a; Vivó et al., 2008) and ameliorating neuropathic pain (Cobianchi et al., 2010; Nam et al., 2001). Increased activity, by reducing excitability of sensory neurons and synaptic stripping of motoneurons, attenuates maladaptive plastic changes in the spinal cord and central pathways (Chapter 3; Modol et al., 2014).

Several studies have shown that the beneficial effects induced by exercise after spinal cord or PNIs are related with signaling of brain derived neurotrophic factor (BDNF) along with its specific receptor TrkB (Cobianchi et al., 2013; Gomez-Pinilla et al., 2012; Hutchinson et al., 2004a; Raffaella Molteni et al., 2004). In Chapter 4, we have tested how this pathway can mediate the effects of exercise in the central circuitry. On the other hand, in a previous study we found that the integrity of sensory inputs from the injured limb is important for the maintenance of motoneuron perineuronal nets (PNN) induced by treadmill exercise (Chapter 3). PNN restrict plasticity and stabilize synapses (Kwok et al., 2011), and thus, exercise, by activating

proprioceptive and cutaneous receptors facilitates preservation of central circuits after PNI. However, besides peripheral activation of muscles and sensory receptors, physical exercise is also activating a complex central circuitry related with locomotion and stress. Among it, the activation of the descending noradrenergic neurons of the locus coeruleus (LC) may play a role, since these projections inhibit nociceptive transmission (Millan, 2002; Pertovaara, 2006) and modulate excitability of motoneurons (Heckman et al., 2003) in the spinal cord. In addition, noradrenaline release influences microglial function, by suppressing production of pro-inflammatory cytokines and promoting anti-inflammatory mediators (Heneka et al., 2010; Jardanhazi-Kurutz et al., 2011). Interestingly, an intensive protocol of treadmill exercise is able to attenuate the activation of microglia observed in the spinal cord after PNI (Cobianchi et al., 2010). This anti-inflammatory effect may be related to the increased activation of LC descending projections induced by physical activity. However, the role of the LC in the activity-dependent-modulation of the spinal changes observed after axotomy has not been addressed yet. The LC can be chemically destroyed by using the neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), that selectively damages noradrenergic projections originating from the LC, inducing degeneration of noradrenergic terminals (Dooley et al., 1987; Dudley et al., 1990; Prieto and Giralt, 2001; Scullion et al., 2009). Therefore, we have evaluated the role of the LC on the modulation of physical exercise on the plastic changes that motoneurons suffer after axotomy by submitting to treadmill running control rats and rats that previously had suffered chemical ablation of the LC.

MATERIAL AND METHODS

Experimental design

Adult female Sprague Dawley rats (8 weeks old, 200–280 g) were housed with free access to food and water at room temperature of 22 ± 2 °C under a 12:12-h light–dark cycle. All experimental procedures were approved by the ethics committee of Universitat Autònoma de Barcelona and followed the guidelines of the European Commission on Animal Care (EU Directive 2010/63/EU). 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%).

One group of animals was used as control group and did not receive any treatment. Two group of animals received DSP-4 before the surgery, as described below. Animals of one of these groups were submitted to exercise 3 days after the surgery. A fourth group was submitted to the same protocol of exercise without receiving pharmacological treatment. Each group was divided in two subgroups: a subgroup of animals was euthanized 14 days after injury (short-term) to evaluate motoneurons changes and the other subgroup was euthanized 75 days after injury (long-term) in order to evaluate functional recovery (Table 1).

Retrograde labeling

To identify the motoneuron pools of tibialis anterior (TA) and gastrocnemius medialis (GM) muscles, retrograde tracing was applied one week before intervention. Bilaterally, two retrotracers, True Blue Chloride (TB, Setareh Biotech) and Fluorogold (FG, Fluorochrome), were used to identify both motoneuron pools. The corresponding muscle was exposed by making a small cut to the skin and then two injections (2.5 µl/injection) were distributed throughout the body of the muscle with a glass pipette using a Picospritzer.

Group	Follow-up	Study
1. Control group (AX)	Short-term	Mechanical nociceptive threshold measurement Changes around soma (n=4)
	Long-term	EMG (n=5)
2. DSP-4 administration group (DSP-4)	Short-term	Mechanical nociceptive threshold measurement Changes around soma (n=4)
	Long-term	EMG (n=5)
3. DSP-4 + Treadmill running (D-TR)	Short-term	Mechanical nociceptive threshold measurement Changes around soma (n=4)
	Long-term	EMG (n=5)
4. Treadmill running (TR)	Short-term	Mechanical nociceptive threshold measurement Changes around soma (n=4)
	Long-term	EMG (n=5)

Table 1. Experimental design. Groups and treatments applied. All animals were submitted to sciatic nerve section and repair by direct suture. Four experimental groups were defined depending on the treatment received. Control group of animals that did not receive any treatment. DSP-4 group, whose animals received DSP-4 four days before surgery. DSP-4+TR group, in which animals received DSP-4 before the surgery and were submitted to a high intensity protocol of treadmill running exercise for 2 weeks after the surgery. TR group, in which animals were submitted to exercise for 2 weeks after the surgery.

Surgical procedure

Animals where anesthetized and the sciatic nerve was exposed at the mid-thigh and cut by using microscissors. The proximal and distal stumps were rejoined with two epineural 10-0 sutures. Afterwards, muscle and skin were sutured in layers, iodine povidone was applied to the wound, and the rats were allowed to recover in a warm environment under close observation.

Noradrenergic depletion

DSP-4 (Sigma Aldrich) was dissolved in sterile NaCl 0.9% saline and delivered as a single i.p. dose of 50 mg/kg according to the work of Grzanna et al. (1989) within 10 min of preparation. DSP-4 was administrated 4 days before the injury to ensure LC destruction at the beginning of the treadmill protocol. Control animals received a

single i.p. injection of saline solution. The doses of DSP-4 used were devoid of adverse effects, and therefore rats did not require special care during follow up.

Treadmill training protocol

All animals submitted to training exercise were placed on the treadmill for 60 min twice a week prior to surgery in order to acclimatize them to a motor-driven rodent treadmill (Treadmill LE 8706, LETICA, Spain). During the training sessions, previous to surgery, shock grid intensity was set at 0.4 mA to provide a mild negative stimulus. The treadmill running (TR) protocol was started 3 days after surgery and was carried out during 2 weeks. TR consisted of one session of treadmill running 5 days/week 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 maximum speed of 30 cm/s for 60 min was reached during the final training session (Cobianchi et al., 2013).

Immunohistochemical analysis of locus coeruleus

After 4% paraformaldehyde in PBS perfusion, brains were removed, post-fixed for 4 h, cryoprotected in 30% sucrose, and stored at 4°C until use. Using an acrylic array of coronal brain matrix (Acrylic brain matrices Alto, for small rat coronal, 175-300gr) we obtain region of interest to analyze locus coeruleus. Samples were embedded in Tissue-Tek, serially cut (25 µm thickness) with a cryostat, and collected onto gelatin-coated glass slides. All sections were first blocked with 10% normal bovine serum for 1 h, followed by overnight incubation at 4°C with mouse anti tyrosine hydroxylase (1:500 Sigma). After washes, immunoreactive sites was revealed by using mouse-specific secondary antibodies conjugated to 488 Alexa Fluor (1:200, Invitrogen). After 2 h incubation at room temperature, the sections were thoroughly washed, mounted on slides, and cover-slipped with Fluoromount-G (SouthernBiotech). LC were localized and images captured with a scanning confocal microscope (LSM 700 Axio Observer, Carl Zeiss Objective Plan-Apochromatic 20x/0.8 Ph2 M27 dt=0.55mm).

Immunohistochemical analysis of spinal cord

Fourteen days after sciatic nerve injury, deeply anesthetized animals were transcardially perfused with 4% paraformaldehyde in PBS. The L3–L6 spinal cord segment was removed, post-fixed for 4 h, cryoprotected in 30% sucrose, and stored at 4°C until use. Samples were embedded in Tissue-Tek, serially cut (20 μm thickness) in the transverse plane with a cryostat, and collected onto gelatin-coated glass slides. All sections were first blocked with 10% normal bovine serum for 1 h, followed by overnight incubation at 4°C with combinations of primary antibodies: rabbit anti Synaptophysin (1:200, Covance), guinea pig anti VGlut1 (1:300, Millipore), guinea pig anti VGat (1:200, Synaptic Systems), Lectin from Wisteria Floribunda (1:100, Sigma), mouse anti GFAP (1:1000, Millipore), rabbit anti Iba1 (1:500, Wako) and mouse anti tyrosine hydroxylase (1:500 Sigma). After washes, immunoreactive sites were revealed by using species-specific secondary antibodies conjugated to 488 Alexa Fluor (1:200, Invitrogen), 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 40×/1,3 Oil DIC M27).

Image analysis, processing and regression analysis from motoneurons labeling quantification were 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 a perimeter of 5μm thickness surrounding the motoneuron soma (Arbat-Plana et al., 2015). For each animal, 10 to 15 motoneurons of each pool and each side were analyzed.

For spinal cord TH immunolabeling, images of ventral horn, specifically the region were motoneurons were identified by retrotracer, were acquired with a fluorescence microscope Olympus DP73 (40x; 1800x2400 pixels).

Electrophysiology tests

For the long term follow-up, motor reinnervation and H reflex were assessed by means of nerve conduction tests, performed at 30, 45, 60 and 75 days after surgery, using an electromyography apparatus (Synergy Medelec, Viasys HealthCare). Electrophysiological evaluation was performed under ketamine/xylazine anesthesia. During the test, the rat body temperature was kept constant between 34 and 36°C by means of a thermostated flat coil. The sciatic nerve was stimulated by two needle electrodes percutaneously inserted at the sciatic notch, applying single rectangular pulses of 0.1 ms duration up to the voltage required to obtain a maximal evoked response. The compound muscle action potentials (CMAP) evoked by stimulation of motor nerve fibers were recorded from the TA, GM and plantar (PL) muscles with microneedle electrodes. All potentials were amplified and displayed at the appropriate settings for measurements of the onset latency and the amplitude from baseline to the maximal negative peak of the direct M wave and the reflex H wave. The maximal H/M amplitude ratio was calculated for each muscle tested. The H wave (the electrophysiological equivalent of the stretch reflex) suffers a rate dependent depression (RDD) that is useful to corroborate that the recorded wave is truly the H wave. It is important to note that at early stages of regeneration, characterized by polyphasic CMAPs in the reinnervated muscles, etha H wave can be masked. Thus, to ensure that the selected wave was really the H wave, repeat stimulation was applied at increasing frequencies (0.3, 1, 3, 5, 10, 15, 20, 30 pps). The ratio between the amplitude of the last and the first H wave recorded for the different protocols was evaluated, in order to evaluate the RDD.

Evaluation of mechanical nociceptive threshold

Seven days before surgery, all the animals were habituated to the experimental device, by leaving them to explore the apparatus for 20 min and then starting the test for recording baseline nociceptive thresholds. The nociceptive behavior tests were performed on both hind paws before and at different days after

injury. In postoperative tests, the experimenter was blind to rat assignment to the different groups.

Sensitivity to mechanical stimulus was measured by means of an electronic Von Frey algesimeter (Bioseb, Chaville, France). Rats were placed on a wire net platform in plastic chambers 10 min before the experiment for habituation. A non-noxious pointed probe was gently applied to medial and lateral plantar paw area, and then slowly increasing the pressure. The threshold was expressed as the force (expressed in grams) at which rats withdrew the paw in response to the stimulus. A cutoff force was set at 40 grams, when the stimulus lifted the paw without response. Threshold was calculated as the mean of three measurements per test site, with 3 min interval between each measurement. Algesimetry tests were performed during the morning, while TR sessions were carried out during the afternoon.

Statistical analysis

For quantitative variables, normality was assessed by Shapiro-Wilk test (Royston, 1993). For variables with a normal distribution 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 statistical analyses. A nested design ANOVA test was used in order to determine if the variability was due to differences between the different motoneurons or between animals in each group. Statistical analysis of mechanical threshold was made by two-way ANOVA with group and time after injury as factors, followed by Bonferroni post hoc comparisons. A value of $p<0.05$ was considered significant. Data are presented as mean±SEM.

RESULTS

DSP-4 was administrated to destroy the LC and thus, to evaluate the effects of noradrenaline descending projections on the spinal changes mediated by TR during 14 days following PNI. Firstly, we verify the destruction of LC after DSP-4 administration using Tyrosine Hydroxylase antibody (TH), which is a useful marker for

dopaminergic and noradrenergic neurons. All of the animals with DSP-4 injection had a complete destruction of LC (Fig 1).

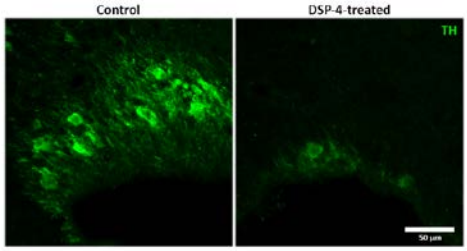


Fig 1. LC section in a control and DSP-4 treated animals. TH immunostaining (green) to verify the destruction of LC after DSP-4 treatment.

The loss of noradrenergic descendent projections was also corroborated in the spinal cord after DSP-4 administration by TH immunostaining in the area with retrotracer labeling, to ensure that there was not noradrenaline at the region of interest. All of the animals with DSP-4 injection did not show any presence of TH immunostaining at the end of the follow-up (Fig 2).

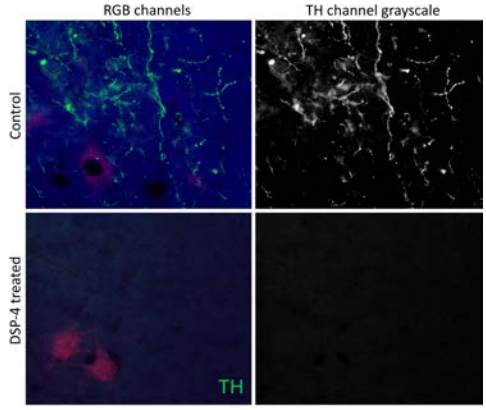


Fig 2. SC section in a control and DSP-4 treated animals. TH immunostaining (green) to verify the destruction noradrenaline descendent projections after DSP-4 treatment.

Effects of DSP-4 administration on central changes after axotomy

Synaptophysin (Syn) immunolabeling, a general marker for synapses, showed that the number of synaptic coverage of motoneurons was reduced after sciatic nerve injury, whereas TR partially preserved that loss (77±3 Syn/µm^2 and 97± 3 Syn/µm^2 in

AX animals versus 89±2 Syn/µm^2 and 104±4 Syn/µm^2 in TA and GM, respectively, in trained animals, p<0.001). No significant differences were found between the group of animals treated with the DSP-4 compared to control injured animals (83±3 Syn/µm^2 in both TA and GM). DSP-4 administration combined with TR induced a marked decrease of synapses labeling on injured motoneurons compared to AX (63±5 Syn/µm^2 in TA and 71±10 Syn/µm^2 in GM, p<0.005) (Figs 3A, 5).

Excitatory synapses were analyzed using VGlut1. Axotomized TA and GM motoneurons had a density of about 40±2 per µm^2, less than half than in intact motoneurons. After TR, the loss of excitatory synapses was also attenuated (60±4 VGlut1/µm^2 in both TA and GM, p<0.001). Animals treated with DSP-4 did not show significant differences compared to injured control animals (42±5 VGlu1/µm^2). However, animals treated with DSP-4 submitted to TR showed a significant decrease of VGlut1 contacts on motoneurons in both TA and GM pools compared to AX and TR animals (30±3 VGlut1/µm^2, p<0.001) (Figs 3B, 5).

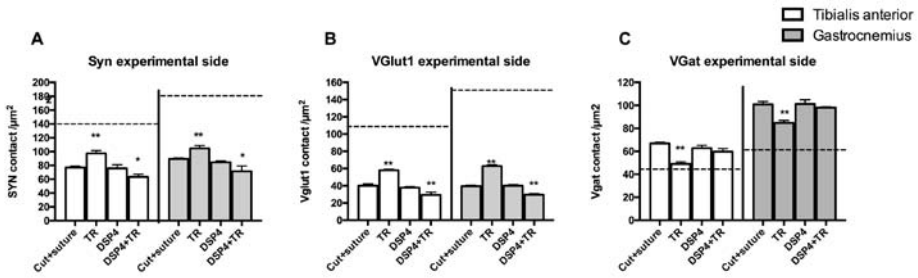


Fig 3. Quantitative analysis of synaptic stripping and excitatory/inhibitory synapses after axotomy in animals submitted to treadmill and treated with DSP-4. Evaluation of Synaptophysin (A), VGlut1 (B) and VGat (C) in TA (white bars) and GM (grey bars). Horizontal dotted lines indicate the average of motoneuron labeling in the non-injured side of both TA and GA motoneurons. Data are expressed as mean ± SEM, *p<0.05, **p<0.01.

The reduction of excitatory synapses suffered by axotomized motoneurons was accompanied by an increase of VGat labeled inhibitory synapses (67±1 VGat/µm^2 and 100±3 VGat/µm^2 in TA and GM, respectively). TR animals, showed a lower increase in inhibitory synapses after axotomy (49±2 VGat/µm^2 and 85±2 VGat/µm^2 in TA and GM, p<0.001) than controls. In this case, no significant differences were found between DSP-4 and DSP-4-TR animals compared to control animals (Figs 3C, 5).

Wisteria floribunda immunoreactivity was used to analyze PNN. Axotomized motoneurons had a reduction in the amount of PNN (52±5 PNN/μm^2 in AX group compared to 150±21 PNN/μm^2 in intact rats) that was partially prevented if animals were submitted to exercise (75±4 PNN/μm^2; $p<0.001$ vs AX group). We observed that PNN immunoreactivity in injured motoneurons was lower in rats receiving DSP-4 (35±1 PNN/μm^2 and 42±2 PNN/μm^2 in TA and GM, respectively) than in only injured animals ($p<0.001$). DSP-4-TR animals had also a low density of PNN surrounding motoneurons (35±5 PNN/μm^2 in both TA and GM). Interestingly, in both groups of animals treated with DSP-4, GM motoneurons at the contralateral side showed a reduced PNN density (145±18 PNN/μm^2 in TA and 172±8 PNN/μm^2 in GM) compared to those of AX animals (139±2 PNN/μm^2 in TA and 182±6 PNN/μm^2, in DSP-4) (Figs 4,5).

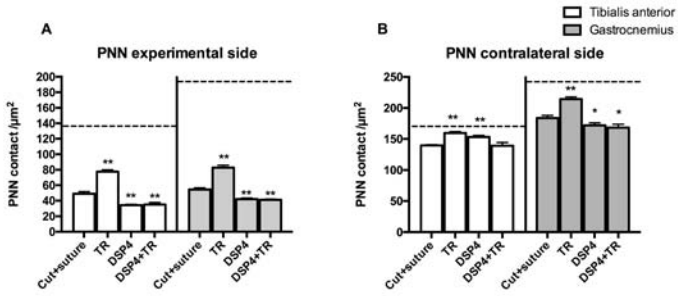


Fig 4. Quantitative analysis of PNN after axotomy in animals submitted to treadmill and treated with DSP-4 in both experimental side (A) and contralateral side (B). Horizontal dotted lines indicate the mean of motoneuron labeling in the non-injured side of both TA and GA motoneurons. Data are expressed as mean ± SEM, *p<0.05, **p<0.01.

Astroglial reactivity, estimated by measuring the density of GFAP immunolabeling surrounding motoneurons, was significantly increased in animals submitted to TR (262± 4 GFAP/μm^2) compared to the control injured group (5±2 GFAP/μm^2, $p<0.0001$). Astroglial reactivity was also increased in both groups of animals treated with DSP-4 (271±8 GFAP/μm^2) similarly to TR only animals. Thus, administration of DSP-4 did not affect the modulation of astroglial reactivity induced by exercise (Fig 5).

Iba1 immunostaining around axotomized motoneurons was used to evaluate microglial reactivity. Fourteen days after nerve injury, there was a marked increase of

microglia labeling (80±20 Iba1/µm^2) in control rats. DSP-4 administration significantly increased microglial reactivity (321±12 Iba1/µm^2), that also presented a phagocytic phenotype (Fig 4). Exercise drastically reduced microglia reactivity around injured motoneurons (49±4 Iba1/µm^2), and similar low activation of microglia was observed in animals treated with DSP-4 and also submitted to exercise (51±3 Iba1/µm^2), thus indicating that the modulation of microglia by exercise was not affected with the loss of descending noradrenergic projections.

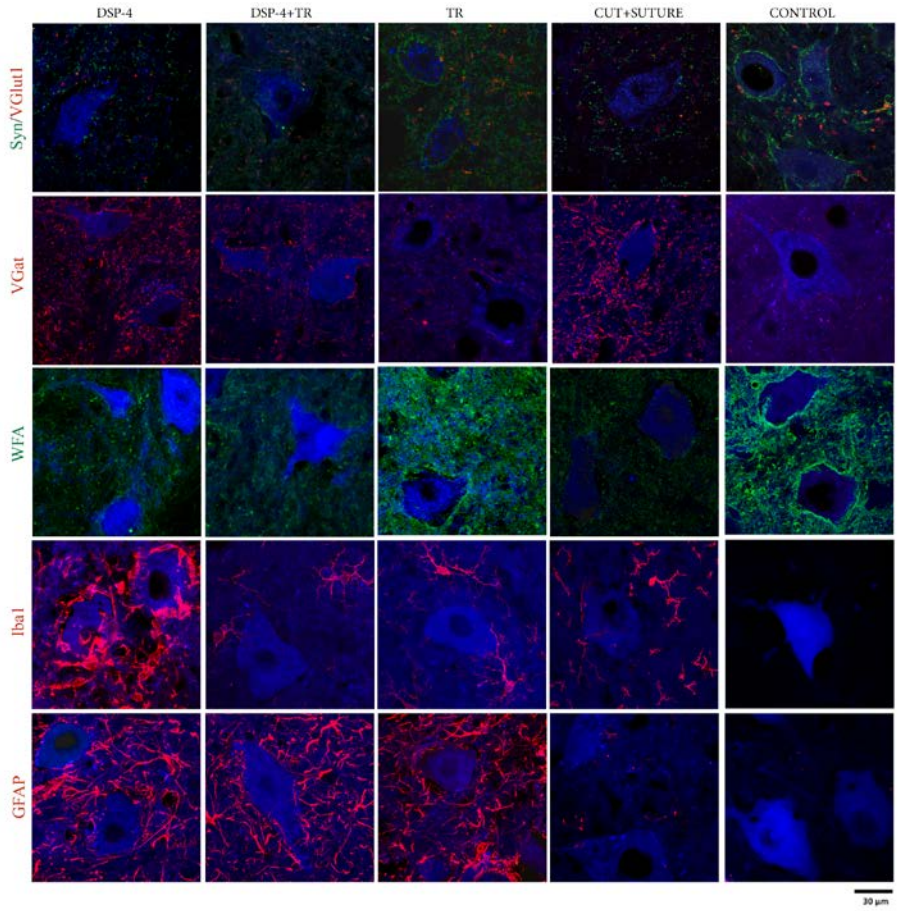


Fig 5. Evaluation of excitatory and inhibitory synapses, PNN, astroglia and microglia reactivity surrounding axotomized motoneurons of animals submitted to treadmill or/and DSP-4 treatment. All immunostainings were evaluated in confocal images of spinal cord regions containing back-labeled motoneurons (blue) of TA and GM muscles after nerve cut repaired with direct suture in the different groups of animals.

Muscle reinnervation

Electrophysiological studies were performed to evaluate motor reinnervation during 75 days after sciatic nerve injury. The M wave values of the muscles at the contralateral side were similar in all animals. At the injured side, all the groups showed an increase in the amplitude of the M wave of the muscles tested along time, but with different progression.

The groups of animals that followed TR had lower M wave amplitude of the GM muscle at 40 and 60 days than the untreated group. However, at the end of follow up, the TR group achieved similar levels of reinnervation than the control, whereas the D-TR group remained with lower levels of reinnervation in both GM and TA muscles (Fig. 6). The DSP-4 group followed a similar progression of reinnervation than the control group. Reinnervation of the PL muscles started in AX and DSP-4 groups at 45 dpi, and at 60 dpi in TR group. In contrast, in the D-TR group, we did not record M waves in the PL muscles at the end of the follow up (data not shown), further indicating that motor regeneration was hampered by the combination of both treatments (Fig 6).

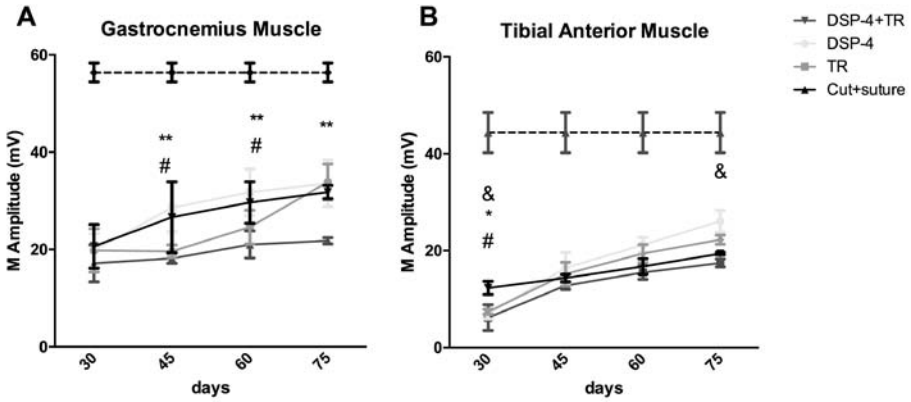


Fig 6. Electrophysiological tests performed. Results of the M wave recorded in GM (A) and TA (B) muscles at 30, 45, 60 and 75 dpi. Horizontal dotted lines indicate the average of CMAP in the non-injured side of both TA and GA motoneurons. Data are expressed as mean ± SEM, *p<0.05, **p<0. * AXs vs. DSP-4+TR; # AX vs TR; & DSP-4 vs TR.

Regarding the recovery of the H wave, there was an increase of the *H/M* ratio after sciatic nerve injury, indicative of hyperreflexia, that followed a similar course to attain close to normal values at 75 days, without significant differences between groups (Fig. 7). In the injured side, the RDD rate was increased during the study, but again no differences between groups were observed.

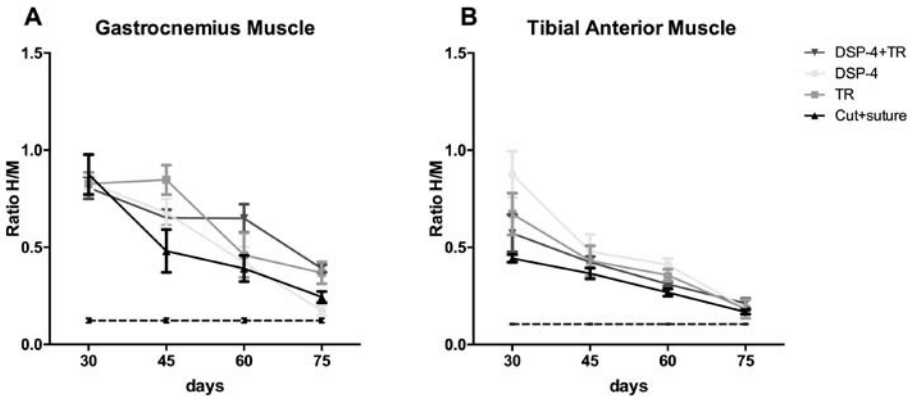


Fig 7. Plots of the H/M ratio (ratio between the amplitude of the H reflex wave and the amplitude of the direct M wave after electrical stimulation of the nerve). Results recorded in GA(A) and TA(B) muscles at 30, 45, 60 and 75 dpi. Horizontal dotted lines indicate the average of H/M ratio in the non-injured side of both TA and GA motoneurons. Data are expressed as mean ± SEM.

Evaluation of mechanical nociceptive threshold

Sciatic nerve injury induced a fast decrease of withdrawal threshold in the medial injured paw, innervated by the saphenous nerve, rapidly lowering to 53±7%; of contralateral at all days postinjury respectively as showed by AX control group (Fig 8A), reflecting a state of mechanical allodynia due to collateral sprouting of the intact terminals from the saphenous nerve. The group of animals submitted to TR had a significant increase in the withdrawal threshold of the injured paw compared with the control group, at all analyzed times postinjury (67±3%; $p<0.001$ in AX vs. TR groups).

In contrast, when animals treated with DSP-4 were submitted to exercise, withdrawal threshold was scientifically higher than the one in AX group at 7 and 14 dpi ($p>0.01$), and similar to TR. However, from 21 dpi to 40 dpi, this increase was lost and values were similar to AX group.

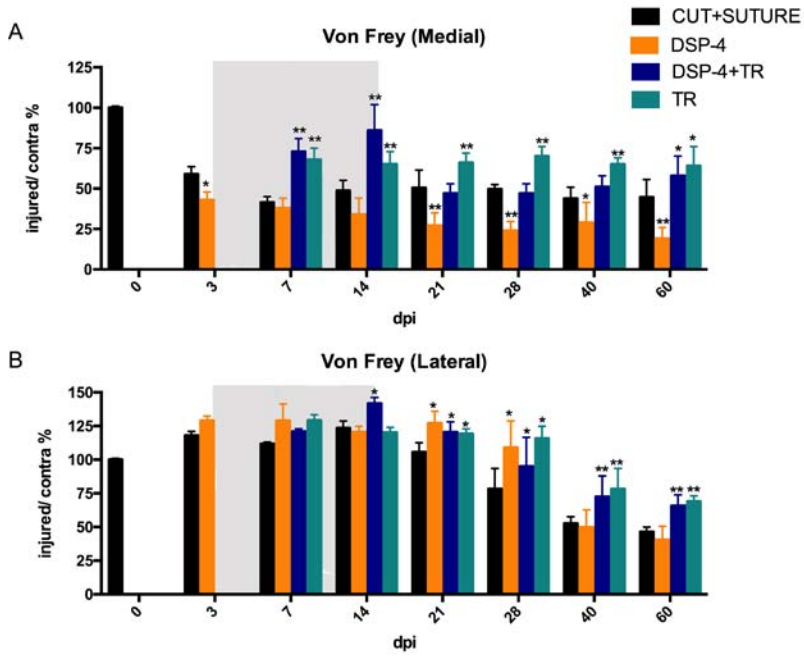


Fig 8. Mechanical algesimetry test results on animals submitted to different treatments. Values are expressed as the % ratio between the threshold force ipsilateral versus contralateral to the injured paw at different days post injury (dpi). **A.** Percentage from medial hind paw, which is innervated by saphenous nerve. **B.** Percentage from medial hind paw area, which is innervated by tibial and sural nerves (branches/fascicles of sciatic nerve). Gray area shows period of treadmill running application. Significant statistical comparisons are represented compared to axotomized group. Data are expressed as mean ± SEM, *p<0.05, **p<0.01.

Regarding, withdrawal threshold in the lateral injured paw, innervated by the sciatic nerve, a higher withdrawal responses were found at 3, 7, 14 and 21 dpi in the sciatic lateral test site, reaching to 117±7% of contralateral, reflecting a loss of mechanical sensitive. Due to denervation from 21 dpi, withdrawal threshold progressively decreased, reaching 45% from contralateral at 60 dpi and reflecting a course from hypoesthesia to mechanical hyperalgesia to 45% at 60 dpi (Fig 8B).

Administration of DSP-4, TR exercise and both combined also resulted in an increase in the withdrawal threshold increase from 7 dpi to 21 dpi. At these times post injury, the group of animals treated with DSP-4 showed a much higher hypoesthesia compared to only axotomized ones ($p<0.005$ at 14 and 21 dpi). Moreover, animals submitted to TR and also treated with DSP-4 showed a higher increase of percentage

compared to AX ($p<0.05$ at 21 dpi). At 28 dpi, all these groups showed significant higher values compared to AX ($p<0.05$). However, at 40 and 60 dpi whereas DSP-4 obtained similar values than AX, both TR and D-TR group remained significantly higher ($p>0.01$). It is important to note that D-TR group showed an earlier withdrawal threshold decrease compared to only trained ones, reproducing a state of a previous mechanical allodynia (Fig 8B).

DISCUSSION

The results of this study corroborate that a high intensity protocol of treadmill running applied during 2 weeks after sciatic nerve injury was able to attenuate synaptic stripping and PNN loss around the axotomized motoneurons (Arbat-Plana et al., 2015). In this work we aimed to analyze if the descending noradrenergic projections to the spinal cord, that participate in modulation of nociceptive transmission (Millan, 2002; Pertovaara, 2006) and of excitability of spinal motoneurons (Heckman et al., 2003), play a role in these effects of the exercise protocol. Disruption of the LC, the main source of descending noradrenergic projections to the spinal cord (Grzanna and Fritschy, 1991), by administration of DSP-4 before performing the sciatic nerve injury did not affect synaptic stripping on spinal motoneurons after injury. In contrast, when these animals were submitted to TR, the preventing effects of exercise on synaptic stripping were lost. In fact, they even showed more marked synaptic stripping and larger loss of VGlut1 synaptic contacts than injured control rats. Loss of the noradrenergic projections also blocked the ability of TR exercise to revert the reduction in PNN. These results support the hypothesis that the increasing intensity TR protocol promotes activity of descending noradrenergic inputs to the spinal cord, which play a role on the maintenance of synapses in axotomized motoneurons. Thus, loss of these projections reduces the amount of inputs that spinal motoneurons receive during treadmill running, and influences the plastic changes of synaptic contacts.

It is important to take into account that descending noradrenergic projections have a modulatory effect on the excitability of motoneurons, facilitating their

activation when receiving other synaptic inputs (Heckman et al., 2008). Noradrenaline acts by facilitating persistent inward currents (PICs) in the dendrites, that interact with and amplify ionotropic synaptic inputs (Heckman et al., 2008, 2003; Hultborn et al., 2004) Perrier and Delgado-Lezama, 2005). When activated, PICs amplify synaptic inputs and are essential for normal repetitive firing (Heckman et al., 2003; Lee and Heckman, 1999), for the production and facilitation of movement. Thus, the loss of noradrenergic projections can reduce the effects of other synaptic inputs on motoneurons and, therefore, limit the effects of increased activity induced by exercise. It is interesting to note that loss of descending projections also had effects on the PNN of uninjured motoneurons, suggesting a widespread effect.

Besides its ability to increase activity of the injured circuits, TR exercise also modulates the inflammatory response observed in the spinal cord after PNI. Interestingly, noradrenaline has anti-inflammatory effects in the periphery (Kohm and Sanders, 1999; Sanders and Straub, 2002). The loss of noradrenergic descending projections in animals treated with DSP-4 increased microglia reactivity around axotomized motoneurons compared to untreated animals. In contrast, animals forced to run in the treadmill downregulated microglia reactivity (Cobianchi et al., 2013). However, this anti-inflammatory effect of exercise at the spinal level seems independent of the noradrenergic system, since similar attenuation of microglia reactivity was observed in exercised animals after LC depletion. Recent works have shown that the inflammatory response is important to switch the peripheral sensory neurons from a neurotransmitter state to a pro-regenerative state (Frey et al., 2008; Liberto et al., 2004; Niemi et al., 2013). Similarly, reactive microglia might influence the regenerative program in axotomized motoneurons. The loss of noradrenaline, a known anti-inflammatory (Heneka et al., 2010), by increasing microglia reactivity around motoneurons might facilitate faster axonal regeneration and thus, explain the increased motor reinnervation observed in the DSP-4 group at early stages. On the contrary, TR exercise by itself attenuated the inflammatory response and delayed muscle reinnervation. That could explain the lower amplitude of CMAPs observed in exercised animals during the first 8 weeks. Indeed, the effects of exercise on

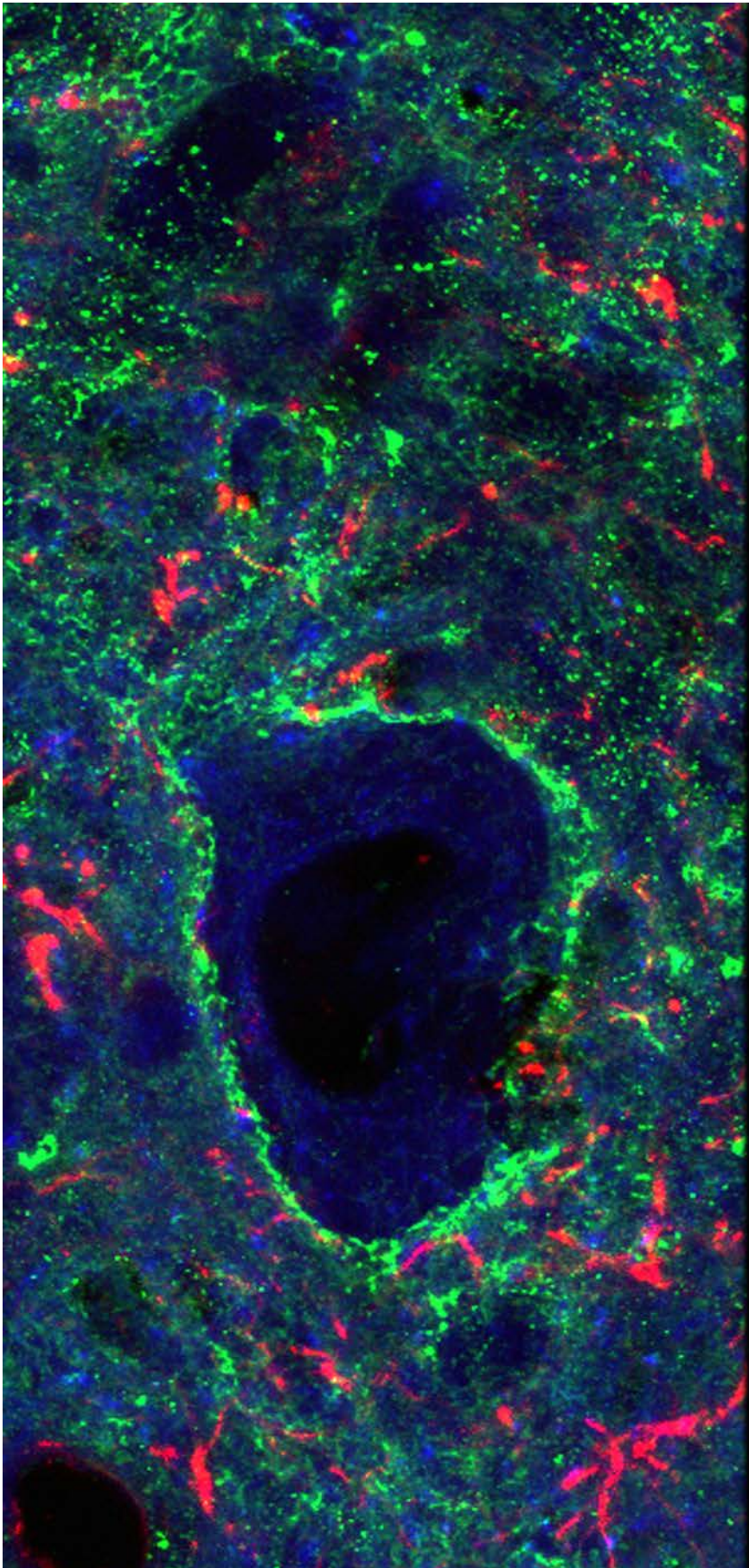
regeneration and muscle reinnervation are variable, depending upon intensity and duration of the protocol, and the period during which it is applied after the injury (Udina et al., 2011a; Gordon and English, 2016). It has been hypothesized that moderate exercise training enhances functional sensorimotor recovery, whereas forced intense exercise, such as the increasing intensity TR protocol applied here, may have a detrimental effect. In our study we also observed that at later stages, several weeks after stopping TR training, reinnervation improved. However, in exercised animals treated with DSP-4 this late increase of reinnervation was not observed, indicating that noradrenergic pathways are somehow mediating the effects of exercise on nerve regeneration.

It is interesting to note that that low intensity TR protocols, although being more effective promoting nerve regeneration and muscle reinnervation (Udina et al., 2011b), have limited effects on the preservation of synapses and PNN on axotomized motoneurons (Arbat-Plana et al., 2015), thus indicating that the protocol that facilitates regeneration differs from the one that favors maintenance of spinal circuitry. In fact, the increasing intensity TR protocol used in this study failed to modulate the hyperreflexia observed after PNI. In contrast, previous papers show that a milder intensity protocol or electrical stimulation of the injured nerve (Asensio-Pinilla et al., 2009; Udina et al., 2011b; Vivó et al., 2008) attenuated such hyperreflexia. This finding is surprising, since we previously found that the same increasing intensity TR training was more effective preserving proprioceptive synapses on axotomized motoneurons compared with the low intensity one (Arbat-Plana et al., 2015). On the other hand, one hour of 20 Hz electrical stimulation of the injured sciatic nerve, a pattern that enhances motor axon regeneration (Al-Majed et al., 2000; Asensio-Pinilla et al., 2009; Cobianchi et al., 2013), had no effects on preservation of synaptic contacts on the injured motoneurons (non published data). The functional state of the stretch reflex circuit was assessed by means of its electrophysiological equivalent, the H wave. The H/M amplitude ratio has been extensively used in the literature to evaluate spinal excitability. When the spinal reflex response is facilitated the H/M ratio increases, whereas it decreases when excitability is reduced (Burke et al., 1999; Thomson et al.,

1992). After peripheral nerve injury, at early stages of muscle reinnervation there is an important increase of the H/M ratio, indicating hyperexcitability, returning to normal values as muscle reinnervation is consolidated (Valero-Cabré and Navarro, 2001). In our study we observed the same changes along time, without differences between experimental groups. Nevertheless, it is not clear if the recovery of electrically evoked monosynaptic EPSPs from sensory afferents onto motoneurons following PNI actually represents the recovery of the stretch reflex (Bullinger et al., 2011; Cope et al., 1994; Haftel et al., 2005). The lack of recovery of the functional stretch reflex can be due to the permanent loss of IA afferent/VGlut1 synapses onto injured motoneurons even when they reinnervate the muscle (Alvarez et al., 2010; Rotterman et al., 2014). Therefore, modulation of the H/M ratio does not guarantee a better function of the stretch reflex and thus, functional evaluation of the complete reflex circuit would be needed in order to fully understand the relevance of proprioceptive synaptic preservation by exercise in functional recovery after PNI.

In conclusion, descending noradrenergic projections appear to play a complex role on the effects of exercise on injured motoneurons. On the one hand, they mediate the ability of physical exercise to activate motoneurons, and contribute to the maintenance of the normal synaptic inputs. In contrast, these projections do not participate in the anti-inflammatory effect of TR at the spinal level. Interestingly, the sole elimination of LC projections in injured animals not submitted to exercise markedly increased microglia reactivity and improved early muscle reinnervation. In contrast, TR exercise, by attenuating microglia reactivity, would delay motor regeneration and muscle reinnervation. However, this reduced microglia reactivity mediates other positive effects, such as the attenuation of synaptic stripping, and amelioration of neuropathic pain (Cobianchi et al., 2010; López-Álvarez et al., 2015). As expected, destruction of the LC increased the mechanical hyperalgesia developed in medial paw area after sciatic nerve injury, whereas animals without LC submitted to exercise have less marked responses to pain in the denervated areas compared to non-exercised ones. This hyperalgesia observed at medial paw area is due to the collateral sprouting of the nerve terminals of the intact saphenous nerve, that also

innervates this part of the paw, and it is developed at early stages after sciatic nerve injury. In contrast, the lateral part of the paw, territory of the sciatic nerve, will show elevated withdrawal thresholds to pain until the first injured axons are able to reinnervate this region. Then, hyperalgesia can also be observed. Exercise was attenuating both early hyperalgesia at medial site and late hyperalgesia at lateral side



General discussion

Peripheral nerves are frequently injured, and at least one nerve is damaged in almost 5% of trauma patients (Ciaramitaro et al., 2010). Despite the ability of peripheral neurons to regenerate, only 10% of patients recover movement in the injured limb (Scholz et al., 2009). This poor functional outcome is usually attributed to the unspecific regeneration of damaged axons. However, other factors can also interfere with the functional outcome of these lesions, among them the changes that the spinal circuits suffer after PNI. Nevertheless, not much attention has been paid in these changes and their role in the functional recovery after nerve injuries, even when there is plenty of studies, mainly from the group of Cope and Alvarez, that describe in detail the poor recovery of the stretch reflex after PNI. Moreover, few studies have evaluated strategies that could modulate these changes and, thus, improve functional recovery after PNI.

Thus, the aim of this thesis was to characterize the spinal changes that are induced around MN after sciatic nerve injuries in an experimental model. We studied these changes in adult and also in postnatal animals. As a second step, we wanted to evaluate strategies that could modulate these changes and, eventually, facilitate functional recovery. We mainly focused on activity dependent therapies, specifically physical exercise. We evaluated the effects of different protocols and types of exercise and we also tested potential mechanism of action.

Characterization of the spinal changes around motoneurons induced by PNI

We firstly evaluate the central changes that motoneurons suffer after axotomy. As previously described (Alvarez et al., 2011; Rotterman et al., 2014) we also observed a marked decrease in proximal glutamatergic synapses, with a maximum peak at 2 weeks post-axotomy, which was partially reversed with time. This decrease was accompanied by an increase in Gephyrin immunoreactivity and a disintegration of PNN surrounding the motoneurons. On the other hand, the most important activation of microglia was 1 week after injury, while at 4 and 8 weeks after injury we observed a marked increase of astroglia reaction (Chapter 1).

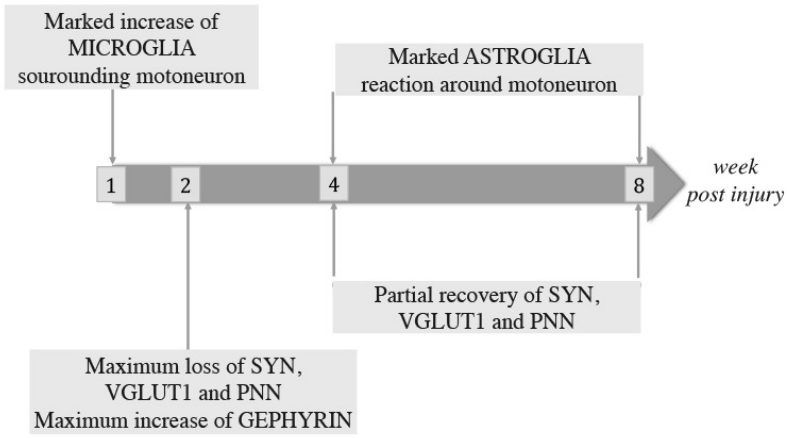


Fig 1. Timeline of central changes surrounding motoneurons after PNI.

It is well established that muscle reinnervation following sciatic nerve transection does not occur until at least 4 weeks after axotomy in adult rats (Lago and Navarro, 2006; Navarro et al., 2007). Within this time period, a selective stripping and a preferential depression of excitatory potentials indicates a good correlation between physiological and morphological aspects of the reaction (Blinzinger and Kreutzberg, 1968; B. Sumner, 1975). Moreover, after axotomy of spinal motoneurons, a larger number of excitatory glutamatergic synaptic terminals were removed from the soma and proximal dendrites compared to inhibitory synapses (Lindå et al., 2000). This could be interpreted as a protective mechanism from excitotoxicity after peripheral injury. However, the reformation of synapses onto the motoneuron cell body may lead to unnecessary plastic changes in synaptic input composition around the soma (Oliveira et al., 2004). Thus, synaptic stripping may serve as a neuroprotective and may contribute to poor functional recovery following peripheral nerve injury. In fact, in parallel to the loss of excitatory synapses loss we observe an increase of inhibitory synapses. Moreover, VGlut1 contacts never completely recover after injury and therefore there is a permanent disconnection between Ia afferents

and MNs (Alvarez et al., 2011) and a consequent loss of the functional stretch reflex (Haftel et al., 2005).

Synaptic stripping and glia reactivity in postnatal animals

One of the goals of this work was to understand the mechanisms underlying the differences that PNI provokes in MNs of young and old. The time course for synaptic stripping and subsequent synapse recovery after peripheral neve injury has not been extensively studied. It is know that in cat motoneurons, the progression of synapsis loss increases from 3 weeks, 6 weeks, to 12 weeks (Brännström and Kellerth, 1998) whereas in mice, synaptic stripping can be seen as soon as one week after axotomy (Payés et al., 2008); thus, time of onset and recovery are diverse in different animal models. In rat, we found that while neonatal animals had the most important decrease of synapses and 1 week post injury (Chapter 2), in adults this decrease is observed 2 weeks after axotomy (Chapter 1). Moreover, in neonatal model we observed a significant increase of microglia and astroglia around MNs at 7 dpi. In contrast, in adult model there are not astroglia activation until 4 weeks post injury. Astrocytic activation was more robust than microglial activation in young animals and displayed a markedly different time course post injury from that seen in the adult (Vega-Avelaira et al., 2007), consequently there is a differential development of the glial response to nerve injury which may explain the higher and earlier glia activation in young animals.

Activity dependent therapies to modulate spinal changes after PNI

We evaluated different strategies to modulate the spinal changes induced by PNI, with the aim to facilitate functional recovery. Although synaptic stripping has been related with the loss of neurotrophic support by motoneurons after the injury, application of neurotrophins, such as BDNF and NT-3, at the proximal stump, has no effects on the spinal changes observed after PNI. We therefore tested different activity dependent therapies. 1 h of electrical stimulation of the proximal injured nerve had no effects in central changes after injury. In contrast, different protocols of

exercise allowed us to obtain more encouraging results. In recent years, there has been a growing interest in evaluating the effects of physical exercise on various neurological conditions, including PNI (English et al., 2011; Hutchinson et al., 2004a; Lau et al., 2011). We studied the effects of different protocols of voluntary wheel training, forced treadmill running and passive bicycle exercise on central changes after PNI. Our data indicated that is not so important the type of exercise but the intensity (Fig 2).

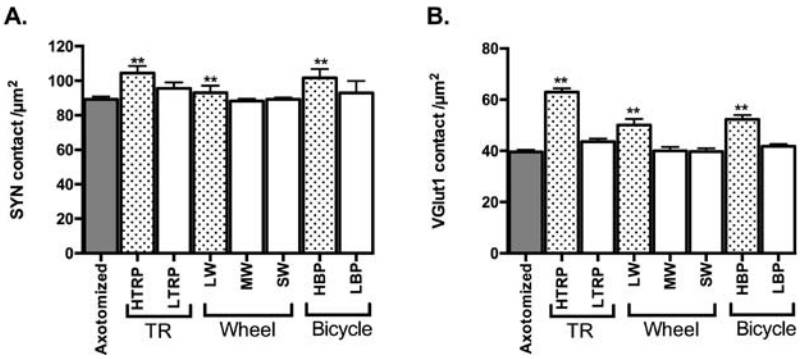


Fig 2. Effects of different protocols evaluated. Evaluation of Synaptophysin (A) and VGlut1(B) immunostaining in GM after different training protocols (white bars) or just axotomy repaired with direct suture (gray bars) in back-labeledmotoneurons from GM. All high intensity exercises (dot plots) are able to partially revers the Syn and VGlut1 loss after axotomy. Data are expressed as mean±SEM, *p> 0.05, **p> 0.01 vs axotomized.

High intensity training partially prevented the loss of Vlgut1 synapses and prevented the increase of VGat synaptic coverage. Although prevention of the loss is the most feasible explanation, exercise could also be mediating formation of new synapses or enlargement of retained synaptic terminals. Therefore, the effect can be termed restorative but the exact mechanism of the effect remains unknown.

As we said previously, the more promising results were obtained when animals were submitted to high intensity exercise. Although low intensity forced exercise in the form of treadmill running induced a slightly preservation of synapses and PNN, the most important changes were observed after HTRP and, only in the group of voluntary and passive exercise that run more distances, similar recoveries than HTTP animals were observed. Therefore, probably the effects of exercise seem

intensity dependent. In fact, different studies showed that volume and intensity modulate the effects of exercise (English et al., 2011; Sabatier et al., 2008). Moreover, stress-related mechanisms could influence the differences between low intensity protocols. Forced exercise, even at low intensity is stressful for animals, whereas voluntary or passive exercise, at least if not too intense, are probably not activating stress-related signals.

As exercise in form of treadmill training has been shown to enhance axon regeneration after PNI (Sabatier et al., 2008) without an increase in the misdirection of regenerating axons (English et al., 2011), it is a promising strategy to enhance functional recovery. The central plastic changes that are induced by peripheral axotomy (Alvarez et al., 2010) partially due to reduction of afferent inputs are likely an important contributor to the poor functional recovery found following axotomy (Frostick et al., 1998). The evidence for a restorative/protective effect of high intensity protocols of exercise on synaptic stripping and PNN might indicate a possible treatment to enhance recovery after PNI. Further investigations by exercise on the correlation between synaptic and PNN preservation and functional recovery are needed.

Spinal PNN after PNI and how can we modulated by physical exercise

Besides the synaptic stripping that motoneurons suffer after axotomy, and that has been extensively described and evaluated in the literature, we also wanted to evaluate the fate of PNN around these motoneurons. These nets are critical for the stability of the neural circuits and are involved in synaptic maturation and stabilization (Pizzorusso et al., 2002). Different studies showed that increased activity facilitates neuronal growth and facilitating plastic restructuring of the network through down-regulation of PNN (Corvetti and Rossi, 2005; Foscarin et al., 2011). However, PNN have been extensively evaluated in the brain and cerebellum. A large number of studies showed that the formation and maintenance of PNN are influenced by the activity of sensory afferents, with increased activity reducing PNN in different brain areas (Carulli et al., 2013; Lander et al., 1997; McRae et al., 2007).

In contrast, there are few studies that focus on spinal PNN, and how activity could modulate these. Marginally, the group of Fawcett described that after spinal cord injury, physical rehabilitation increased the thickness of spinal PNN (Wang et al., 2011). This finding was quite surprising, since it was assumed that rehabilitation, by increasing plasticity, would facilitate functional recovery after spinal cord injury. In contrast, rehabilitation was increasing PNN and thus, limiting plasticity and stabilizing neural circuits. In fact, the same group has multiple studies showing that application of Chondroitinase ABC in SCI models, by degrading PNN, facilitates functional recovery (García-Alías and Fawcett, 2012; García-Alías et al., 2011).

Our results corroborated the findings of Wang et al, 2011, since we also observed that exercise was increasing PNN of intact MNs, and partially preventing the reduction of PNN in axotomized motoneurons (Chapter 3; Arbat-Plana et al., 2015). In parallel to the publication of this study, Smith et al also published another paper that showed that activity was increasing spinal PNN (Smith et al., 2015).

Thus, it seems clear that spinal PNN are differently affected by activity when compared to PNN from brain and cerebellum. This also indicates that the "plasticity" that rehabilitation/exercise is promoting in the nervous system can be different in the spinal cord or the brain. In fact, it seems that in the spinal cord, activity reinforces the circuitry that it is already stablished and promotes their preservation. This makes sense, since spinal circuits are reflex circuits, and any kind of plasticity could interfere with their function. In contrast, cortical regions and cerebellum have more complex circuits, that have the ability to adapt to experience and thus, plasticity would favor their functionality.

In fact, we observed that after PNI, there was a reduction in the amount of PNN around axotomized MN. Reduction of PNN together with the synaptic stripping that MN suffer after PNI can explain the disorganization of the spinal circuits and the loss of function of these circuits. Since physical exercise is partially preventing synaptic stripping and loss of PNN, it would facilitate preservation of the spinal circuitry and, thus, their functionality, favoring better functional recovery. It is interesting to note that we found that a protocol of high intensity TR exercise was able to partially

preserve PNN after PNI and increased PNN around intact MN. Intensity of the exercise seems a key element in the effects on PNN. To evaluate if the effects of exercise on PNN are due to increased depend on increased input activity mediated by sensory afferents, we evaluated the amount of PNN in intact motoneurons after performing a multiple rhyzotomy to interrupt the sensory inputs of the hind limb. As expected, we observed a partial loss of PNN, that was not reversed when animals were forced to run on the treadmill.

How physical exercise can modulate the spinal changes induced by PNI

It has been proposed that neurotrophic factors may mediate the effects of exercise after PNI (Cobianchi et al., 2016; Wilhelm et al., 2012). Treadmill training increases the expression of neurotrophins such as BDNF and NT-3 (Gómez-Pinilla et al., 2001). BDNF is a particularly activity-dependent neurotrophic factor, making it an excellent candidate for mediating protective effects of exercise (Al-majed et al., 2000; Ding et al., 2011; Wilhelm et al., 2012).

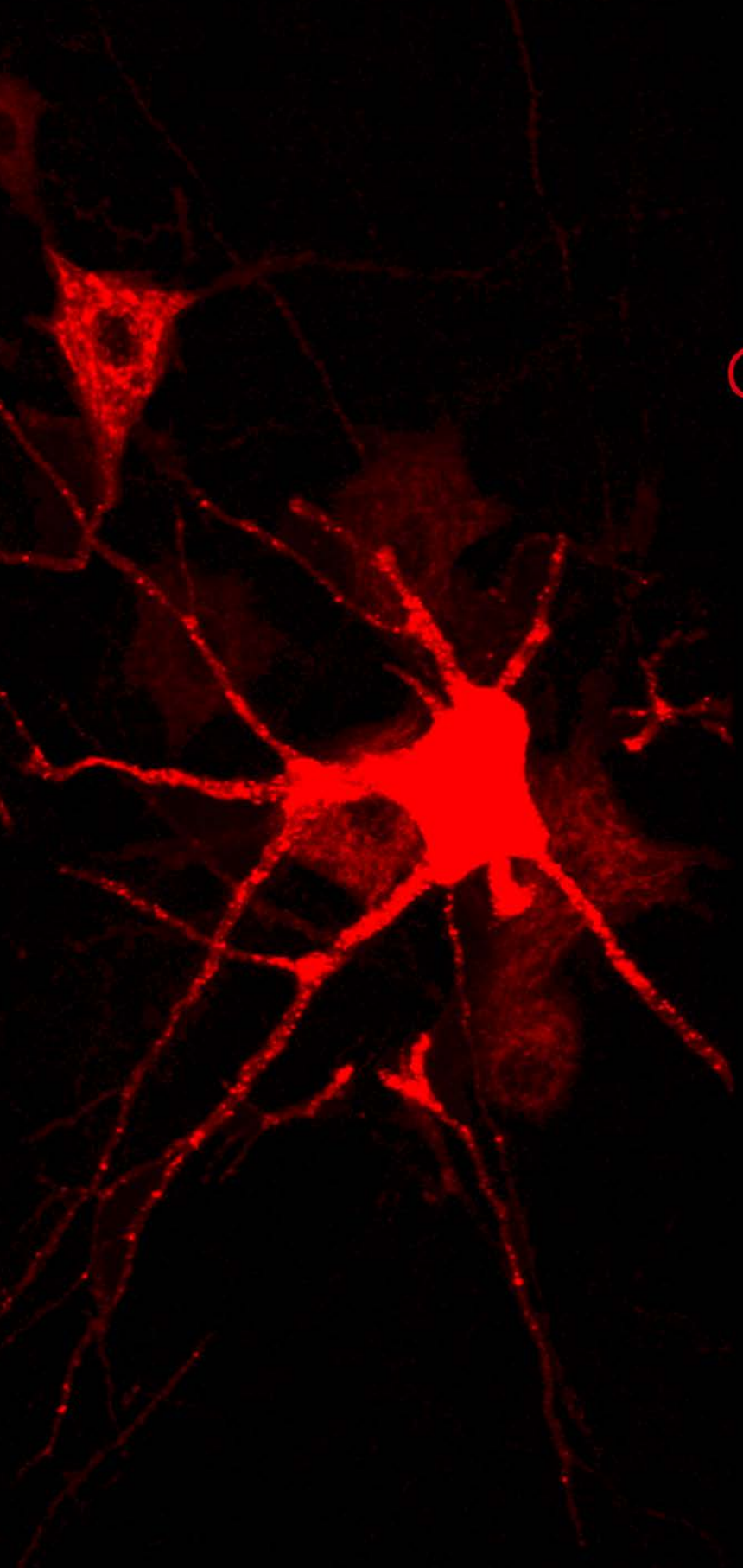
It has been suggested that a transient dose-response relationship exists between exercise intensity and BDNF production in response to exercise in various brain areas including the hippocampus, cerebral cortex, cerebellum and spinal cord (Gómez-Pinilla et al., 2002, 2001; Neeper et al., 1996, 1995). In chapter 4, we have evaluated if systemic administration of a TrkB analog (7,8-DHF) can mimic the partial recovery observed after treadmill exercise following PNI in synaptic stripping. Moreover, we have studied if an antagonist of TrkB administration blocked the positive effects induced by treadmill running. The results of this study revealed that a low dose of 7,8-DHF was able to favored the maintenance of synapses in axotomized motoneurons, whereas a high dose caused a further loss of excitatory synapses and a significant increase of inhibitory synapses in both injured and contralateral sides. Thus, these findings are consistent with previous studies (Ferreira et al., 2011; Krakowiak et al., 2015; Ying et al., 2005) that point that the positive effects of TR depend on specific levels of BDNF and an increased activation of TrkB does not potentiate these benefits. Our results also indicate that not all the improvements observed after exercise are

due dependent on BDNF/TrKB pathway, suggesting that other processes play a role in changes observed after exercise in central circuitry. Consequently, in chapter 5 we evaluated the role of NA descendent projections in the effects mediated by exercise. We destroy the LC by DSP-4 injection, and we observed that the positive effects of exercise on synaptic stripping were partially lost. Therefore, we found that physical exercise is promoting activity of the descending noradrenergic inputs to the spinal motoneurons, and thus, loss of this projections would reduce the amount of inputs that spinal motoneurons receive during treadmill running.

We also evaluate the mechanisms involved in the maintenance of PNN by exercise following PNI. In chapter 4, we found that maintenance of PNN can be mediated by blockade of TrkB activation. Moreover, exercise was not able to partially preserve PNN thickness after injury and contralateral intact MNs also had reduced PNN. Thus, even when MNs still received segmentary sensory inputs, PNN were reduced. It is important to take into account that NA projections are modulating excitability of MNs (Wohlberg et al., 1986) and thus, its loss limits the ability of physical exercise to increase MN activity and therefore, also reduces its positive effects.

Besides its ability to increase activity of the injured circuits, exercise is also able to modulate the inflammatory response observed at the spinal cord after PNI. In fact, the intensity of exercise was related to the degree of attenuation of microglia reactivity, since low intensity treadmill had no effect on microglia activity. In our study, reduction in microglia activation at early time points was associated with less severe synaptic stripping observed in the animals that were running at high intensity. However, when voluntary and passive exercise were applied we observed a higher activation of microglia in these animals that runs less or low distances. In contrast to treadmill running, which is a forced exercise, it is possible that wheel and bicycle exercise generate less stress than treadmill running. Therefore, the protocols of exercise that generates less stress, such as less distance in wheel or low velocity in bike, would had a limited capacity to modulate glia activity. As expected, animals treated with DSP-4 showed an increased amount of microglia reactivity, being these microglia more reactive than the one observed in injured motoneurons of untreated

animals. However, similar attenuation of microglia reactivity was observed in exercised animals after LC depletion. This fact indicates that exercise is modulating the inflammatory response in the spinal cord by mechanism not related with the activation of noradrenergic projections. Thus, further studies to investigate the mechanism through which exercise modulates glia activity after PNI are still needed.



Conclusions

1. After a peripheral nerve injury, there are important changes at the spinal level around axotomized motoneurons in adult rats.
    1.1. Motoneurons suffer a synaptic stripping and a loss of perineuronal nets that peaks at 2 weeks, and it is reversed between 4 and 8 weeks.
    1.2. Motoneuron synaptic stripping consist in a reduction of immunolabeling for synapses (Synaptophysin+), mainly excitatory (VGlut1+), that it is accompanied with an increase of inhibitory ones (Gephirin+).
    1.3. Microglia reactivity around motoneurons is maximum at 1 week post injury, whereas astroglia activation is observed 4 weeks post injury.

2. A peripheral nerve injury in 10 days old postnatal rats also induces important changes in motoneurons.
    2.1. The most important decrease of synapses in motoneurons is observed already at 1 week post injury. Decrease in VGlut1 density is greater than for synaptophysin, and there is an increased immunolabeling for inhibitory VGat synapses.
    2.2. Both microglia and astroglia are increased around axotomized motoneurons at 1 week post injury, thus indicating that the pattern of glia reactivity in the spinal cord after peripheral nerve injury in postnatal animals is different than in the adults.
    2.3. In the proximal dendrites of axotomized motoneurons there is around 50% loss of VGlut1 contacts, that it is not recovered after 2 months.
    2.4. A nerve crush at 10 days postnatal rats does not limit muscle reinnervation of proximal muscles, since there is a complete recovery of CMAP amplitudes at 2 months.
    2.5. Although there is a permanent loss of VGlut1 synapses, the H wave, the electrophysiological equivalent of the stretch reflex, is normalized 2 months after PNI.

3. Application of different protocols of physical exercise after a peripheral nerve injury is able to partially revert some of the central changes observed after axotomy.
    3.1. Forced running in a treadmill, passive cycling in a motorized bike and free running in a wheel are able to reduce synaptic stripping and loss of glutamatergic synapses and destructuring of PNN when applied at high intensity for 2 weeks after the injury.
    3.2. There is a high activation of microglia after low intensity protocols of voluntary and passive exercise, whereas high intensity exercise significantly reduces microglia reactivity. Moreover, there is a marked increase in astrocytes in all trained animals; that suggests that astrocyte activation is independent of microglia activation.
    3.3. Forced treadmill running at high intensity for two weeks increases the thickness of perineuronal nets around non injured motoneurons.

4. Homolateral afferent inputs to motoneurons are important for the maintenance of perineuronal nets.
    4.1. A rhyzotomy of dorsal roots of the same hindlimb, that suppress homolateral afferent inputs to motoneurons, blocks the positive effects of forced treadmill running on perineuronal nets.

5. Pharmacological activation of TrkB receptor is not able to mimic all the positive effects of treadmill exercise following peripheral nerve injury. Thus, the complex pattern of activation induced by specific regimes of physical exercise better modulate the maladaptive plastic changes induced by PNI than systemic modulation of TrkB.
    5.1. High activation of TrkB does not mimic the effects of TR on synaptic maintenance in axotomized motoneurons, and even can induces deleterious effects on synaptic content when administered at high doses. However, TrkB inactivation impedes some of the effects exerted by

exercise, thus suggesting that the positive effects of TR depend on specific levels of BDNF and increased activation of TrkB does not potentiate these effects.

5.2. There is an unspecific modulation of glial reactivity after activation/inactivation of TrkB.

5.3. Pharmacological blockade of TrkB receptor by ANA-12 administration increases PNN density around axotomized motoneurons, suggesting that maintenance of PNN induced by exercise can be mediated by blockade of TrkB activation, probably on glial cells.

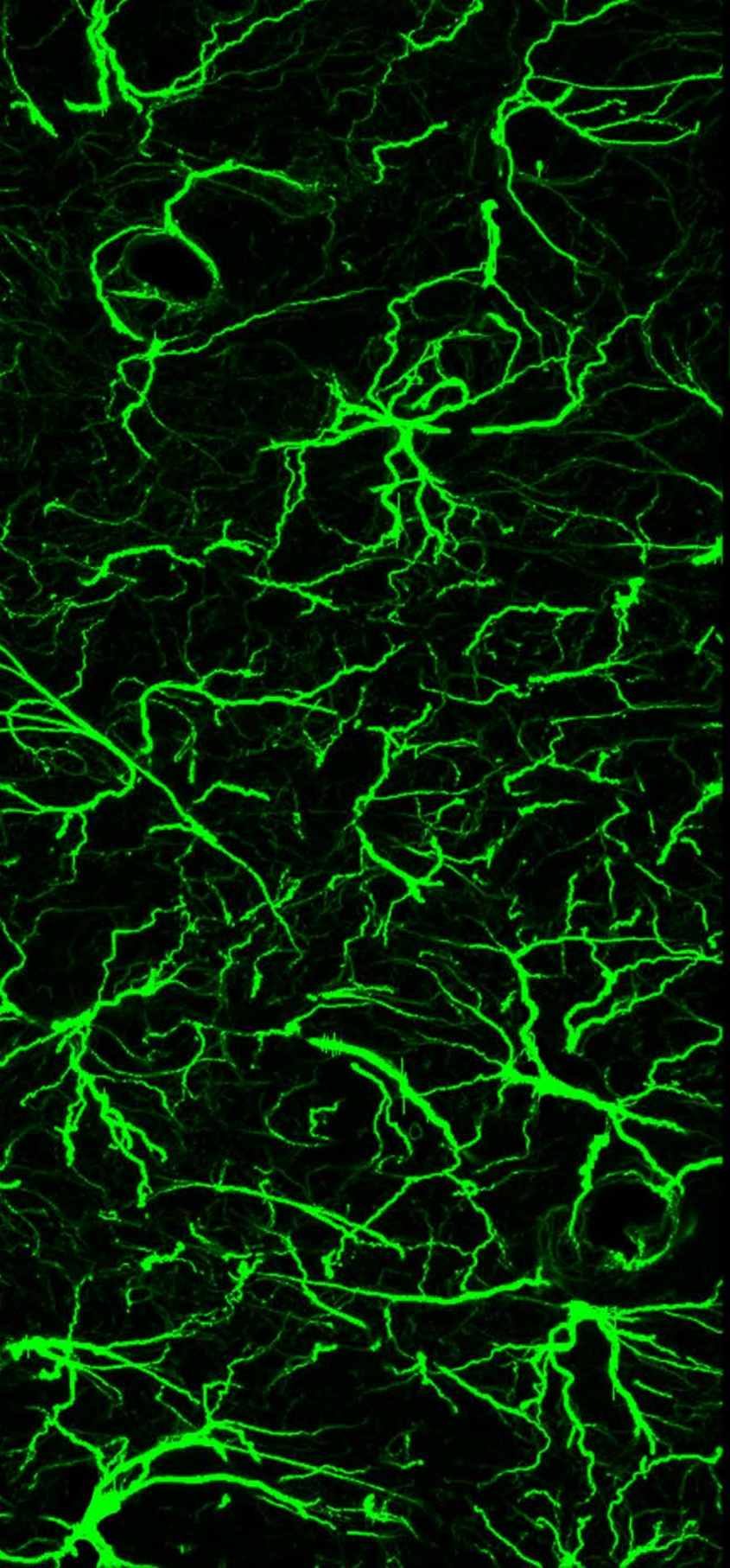
6. The changes observed after treadmill training could be partially mediated by activation of descendent noradrenergic projections to the spinal cord. However, the modulation of microglia induced by exercise in not dependent on noradrenergic inputs.

6.1. Destruction of the LC by DSP-4 administration does not affect the synaptic stripping that motoneurons suffer after injury. In contrast, if these animals are submitted to TR, the positive effects of exercise on synaptic stripping are lost and even suffer a more marked synaptic stripping and VGlut1 loss than injured animals.

6.2. The loss of noradrenergic descendent projections in animals treated with DSP-4 increases the amount of microglia around axotomized motoneurons, whereas an attenuation of microglia reactivity is observed if these animals are submitted to exercise after LC depletion, thus indicating that the anti-inflammatory effect of exercise at the spinal level is independent of activation of the noradrenergic projections.

6.3. Animals without noradrenergic descendent projections show a faster and higher muscle reinnervation than exercised animals at early stages of regeneration, but also an increased mechanical hyperalgesia at the denervated paw, probably due to the increased microglia reactivity at the spinal level. In contrast, when these animals are submitted to exercise,

and microglia reactivity is almost absent, muscle reinnervation at early stages is lower and mechanical hyperalgesia of the denervated areas is attenuated as in exercised animals.



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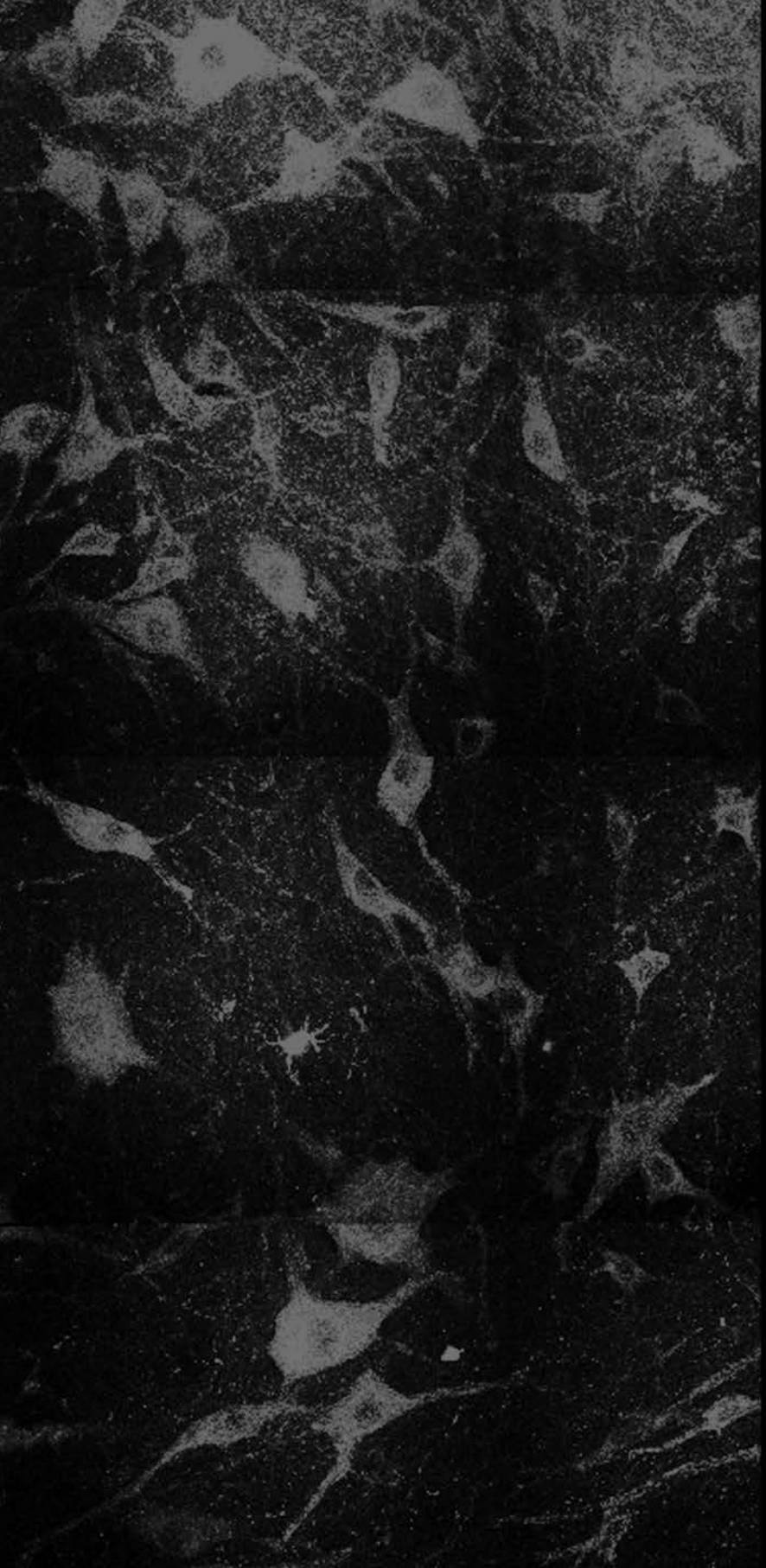

Annexes

Published manuscripts from the work of this thesis:

Peer reviewed publications

6. Arbat-Plana A, Torres-Espín A, Navarro X, Udina E. 2015. **Activity dependent therapies modulate the spinal changes that motoneurons suffer after a peripheral nerve injury.** Experimental neurology 263, 293-305.

Under revision

7. Arbat-Plana A, Cobianchi S, Herrando-Grabulosa M, Navarro X, Udina E. **Endogenous modulation of TrkB signaling by treadmill exercise after peripheral nerve injury.**
8. Arbat-Plana A, Navarro X, Udina E. **Effects of physical exercise and noradrenergic descending inputs on changes in spinal motoneurons after peripheral nerve injur**y.

In preparation:

9. **Effects of passive, active and voluntary exercise in the spinal circuitry after a peripheral nerve injury.**
10. **Characterization of the dendritic arbor and the spinal changes that postnatal motoneuron suffer after peripheral nerve injury.** *Study in collaboration with Dr. Alvarez (Alvarez group), partially realized in Emory University School of Medicine, Atlanta (EUA) during a 4 month stage.*

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Regular Article

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 not only 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, unspecific reinnervation and the limited reconnection of the spinal circuits impair functional recovery. The aim of this study was to describe the changes that axotomized motoneurons suffer after peripheral nerve injury and how activity-dependent therapies and neurotrophic factors 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 reversed with time. This decrease was accompanied by an increase in gephyrin immunoreactivity and a disintegration of perineuronal nets (PNNs) surrounding the motoneurons. Direct application of neurotrophins at the proximal stump was not able to reverse these effects. In contrast, activity-dependent treatment, in the form of treadmill running, reduced the observed destructuring 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 reverse 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.

Introduction

Rehabilitation is one of the cornerstones of the treatment of injuries of the nervous system. It is assumed that repeated activity will 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 promotes neurite outgrowth and functional plasticity (Ghiani et al., 2007; Rampon et al., 2000; Sale et al., 2007; Vaynman and Gomez-Pinilla, 2005). 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 note the importance of a specific rehabilitation therapy to improve functional recovery after neural 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; Van Meeteren et al., 1997; Sabatier et al., 2008). However, just how these therapies can influence the plasticity of the central circuits where spinal motoneurons are involved is not clear. It is known 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 modulating 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 behavior 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 massive stripping of their central

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synapses, related to the loss of trophic support from the muscle. Brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) are two key neurotrophins that regulate the synaptic plasticity, formation and density of synaptic innervation of motoneurons and, when exogenously delivered, are able to prevent/reverse the synaptic stripping suffered by axotomized motoneurons (Davis-López de Carrizosa et al., 2009; Novikova et al., 2000).

When neurons regenerate and reinnervate target organs, they partially recover their synaptic arbor, but in contrast to other excitatory and inhibitory inputs, muscle spindle Ia excitatory synapses, among the most affected by synaptic stripping, never recover baseline levels, even when the muscle spindle and the muscle are correctly reinnervated (Alvarez et al., 2011; Haftel et al., 2005). This reduced connectivity may 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 not only 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, which can be detrimental to 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 neurotrophic support and activity in the neural circuits after lesions can be a key-point in reducing the plastic changes that neurons suffer due to the loss of synaptic and neurotrophic inputs. Therefore, supply of trophic factors and appropriate training and/or provision of afferent inputs to spinal neurons may help to prevent these changes. The aim of this study was to analyze the changes that axotomized motoneurons suffer after peripheral nerve injury and how the addition of trophic factors into the injured stump and provision of activity-dependent strategies modulate these events. A better understanding of these changes would make it easier to determine 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 cycle. 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 1 week before any intervention. Bilaterally, two retrotracers, True Blue Chloride (TB, Setareh Biotech) and Fluorogold (FG, fluorochrome), were applied to identify both motoneuron pools in the same animal. 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 with a 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 the mid-thigh and cut by using microscissors. In a first group of animals (n = 16), the transection was not repaired. In another group of animals (n = 16), the proximal and distal stumps were rejoined with two epineural sutures. Afterwards, muscles and skin were sutured in layers, iodine povidone was applied to the wound, and the 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 condition) we applied BDNF, 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

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 repaired by direct suture. Animals were sacrificed at 4 different times post-injury. 2. Influence of trophic support on the synaptic changes. BDNF, NT3 or a mixture of the two neurotrophic factors was applied at the injury site 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.

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

mix of 8.5 μl collagen type I (Corning), 2.5 μl MEM (GIBCO 10×), and 0.10 μl bicarbonate. The collagen matrix was enriched with 14 μl of phosphate-buffered saline (PBS, 0.1 M) containing 2 ng/μl of BDNF, NT3 or a mixture of both (Ionova). 14 μl of PBS was added to the tubes of the control side. The homogenous solution was carefully pipetted onto the mid-zone of 8 mm-long silicone tubes (2.0 mm i.d.), and the tubes were incubated overnight at 37 °C. The sciatic nerve was exposed mid-thigh and sectioned as described above. The right sciatic nerve was repaired with tubes containing a collagen matrix with neurotrophins, whereas the left side was repaired with tubes containing control collagen matrices. 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 carried out during 1 and 2 weeks, involving four different groups of animals (4 rats per group and condition). All animals were placed on the treadmill for 60 min 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 one 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 maximum speed of 30 cm/s for 60 min was reached 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 cm/s in two sessions of 30 min each with 10 minute resting period, daily during all the follow-up.

Suppression of homolateral sensory inputs

In order to evaluate the role of segmental sensory inputs from the homolateral hindlimb, we performed unilateral preganglionic rhizotomy of L3 to L6 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. Post-operative analgesia was provided with buprenorphine (0.05 mg/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 may 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 PBS. The L3–L6 spinal cord segment was removed, post-fixed for 24 h, cryoprotected in 30% sucrose, and stored at 4 °C until use. Samples were embedded in Tissue-Tek, 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 1 h, followed by overnight incubation at 4 °C with combinations of primary antibodies (see Table 2). After washes, immunoreactive sites were revealed by using species-specific secondary antibodies conjugated to 488 Alexa Fluor (1:200, Invitrogen), 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 40×/1,3 Oil DIC M27).

Image analysis and processing, and regression analysis were 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 a perimeter of 5 μm thickness surrounding the soma. This 5 μm-thick perimeter covers the synaptic area surrounding the neuron and limits the overlapping with synapses of neighboring 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 with the 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 with the Kruskal–Wallis test. SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. A nested design ANOVA test was used in order to determine whether 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 (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 were affected by the treatment, absolute values were also used. For the relevant values, experimental power was estimated using R version 3.1.1 (free software).

Table 2
Primary antibodies used in this study.

Antigen	Immunogen	Host type	Working dilution	Manufacture
Vglut1	Synthetic peptide from rat Vglut1	Guinea pig polyclonal	1:300	Millipore Ref AB5905
Perineuronal nets	Lectin from *W. 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 Iba1 synthetic peptide	Rabbit polyclonal	1:500	Wako
Synaptophysin	C-terminus of human synaptophysin	Rabbit polyclonal	1:500	Invitrogen

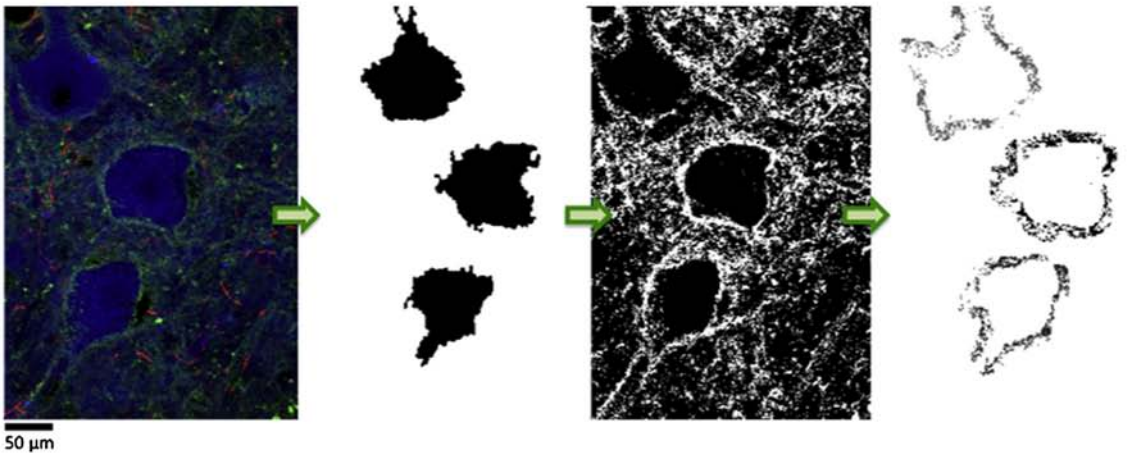

Fig. 1. Analysis of the amount of immunostaining surrounding motoneurons. Automatic selection of the somata of back-labeled motoneurons, detection of the immunostaining using a fix threshold for every staining, determination of a circular perimeter area surrounding the soma of a 5 μm thickness, and measurement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

Results

Measurement of the size of motoneuron somas indicated that TA motoneurons had slightly lower areas (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 5 μm perimeter surrounding the motoneuron soma, the average density of synaptophysin ranged from 130 to 160 μm^2 in TA and from 170 to 200 μm^2 in GM motoneuron pool. Vglut1 density was also similar in all the neurons, ranging from 100 to130 μm^2 for TA and from 140 to170 μm^2 for GM motoneurons. In TA motoneurons, gephyrin density ranged from 35 to 45 μm^2 and in GM ones from 50 to 70 μm^2. 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. The experimental power estimated for the relevant data (Syn, Vglut and PNN measurements) was about 0.8 in the different experiments.

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 in synaptophysin density, with a maximum reduction at 2 weeks, and a 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 to those for controls. 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 greater 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 Vglut1 levels still 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 post-injury ($P < 0.05$) compared to the intact contralateral motoneurons. Again, the maximum changes were observed at 2 weeks, with a peak increase of 42 ± 6% in TA and 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 destructuring (Fig. 4). When assessing the immunoreactivity of *Wisteria floribunda*, which specifically labels for PNN, in a 5 μm-wide perimeter surrounding the motoneuron, we observed a significant decrease in 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, with 7 ± 1% in TA motoneurons and 20 ± 2% in GM motoneurons at 8 weeks.

To evaluate the role of glial cells in the stripping process, we also performed immunolabeling against Iba1 to identify microglia and against GFAP to label astrocytes. In non-injured motoneurons, the presence of glial cells close to the soma was minimal, but 1 week after axotomy we observed a marked increase in microglia immunolabeling (280 ± 20 Iba1/μm^2, $P < 0.01$), which decreased at 2 weeks post-injury (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 markedly at 8 weeks post-injury (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 whether the presence of the distal segment, thus favoring axonal regeneration, affects 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

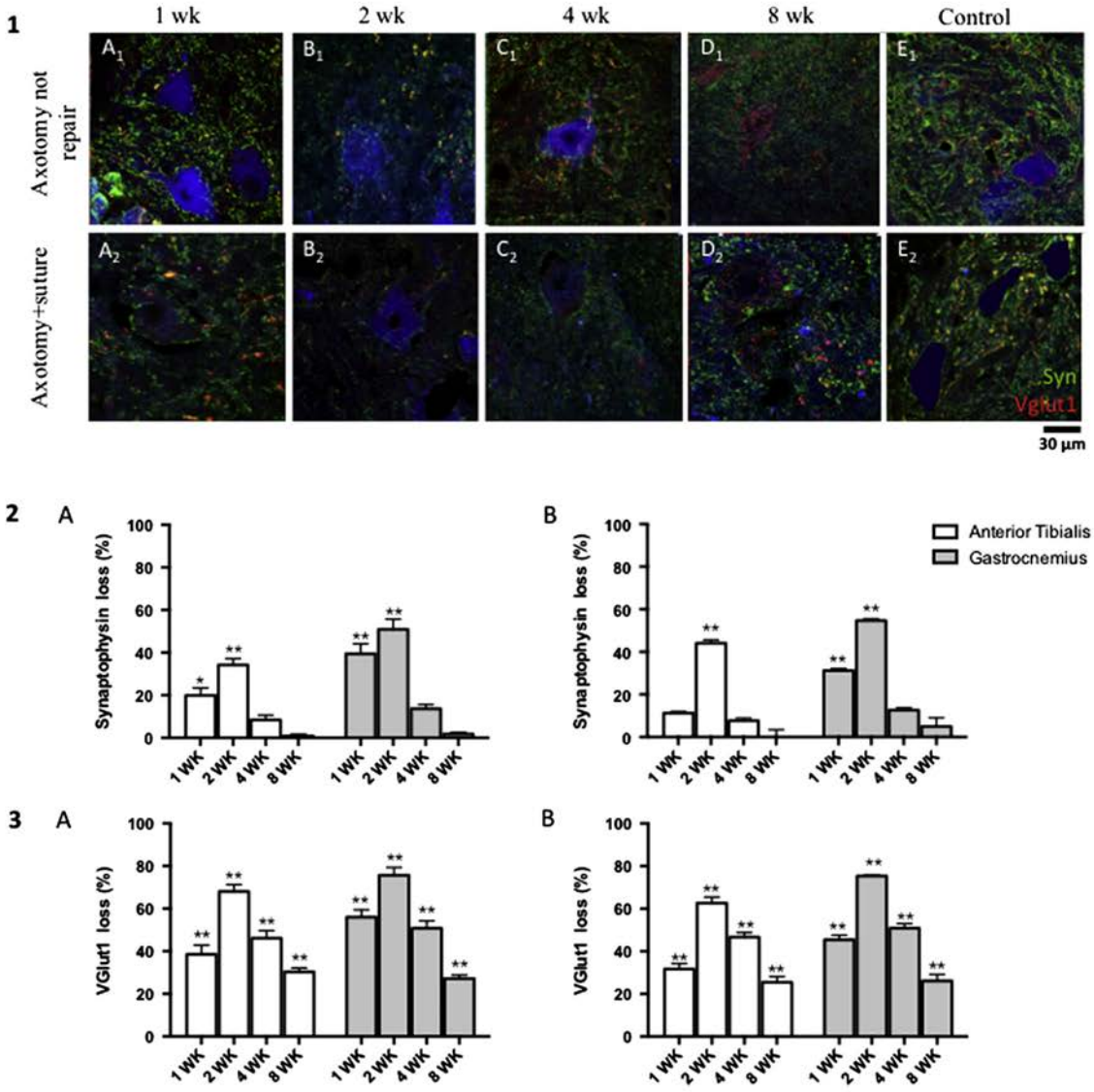

Fig. 2. Evaluation of synaptic stripping after axotomy. 1. Synaptophysin (green) and Vglut1 (red) immunostaining were evaluated in confocal images of spinal cord regions containing back-labeled motoneurons (blue) from TA and GM at 1 week (A), 2 weeks (B), 4 weeks (C), and 8 weeks (D) after nerve cut (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 back-labeled motoneurons from TA (white bars) and GM (gray bars). Vglut1 and synaptophysin reach a low point of depletion 2 weeks after injury in both motor pools, and then recover with time. In contrast to synaptophysin, Vglut1 never returns to baseline levels. Changes are slightly more marked in motoneurons from GM than from TA (data are expressed as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$).

suturing the proximal and distal stumps. There were no significant differences between animals with axotomy only and those with axotomy and repair (Figs. 2–4).

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

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

At 1 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 for synaptophysin labeling in GM motoneurons

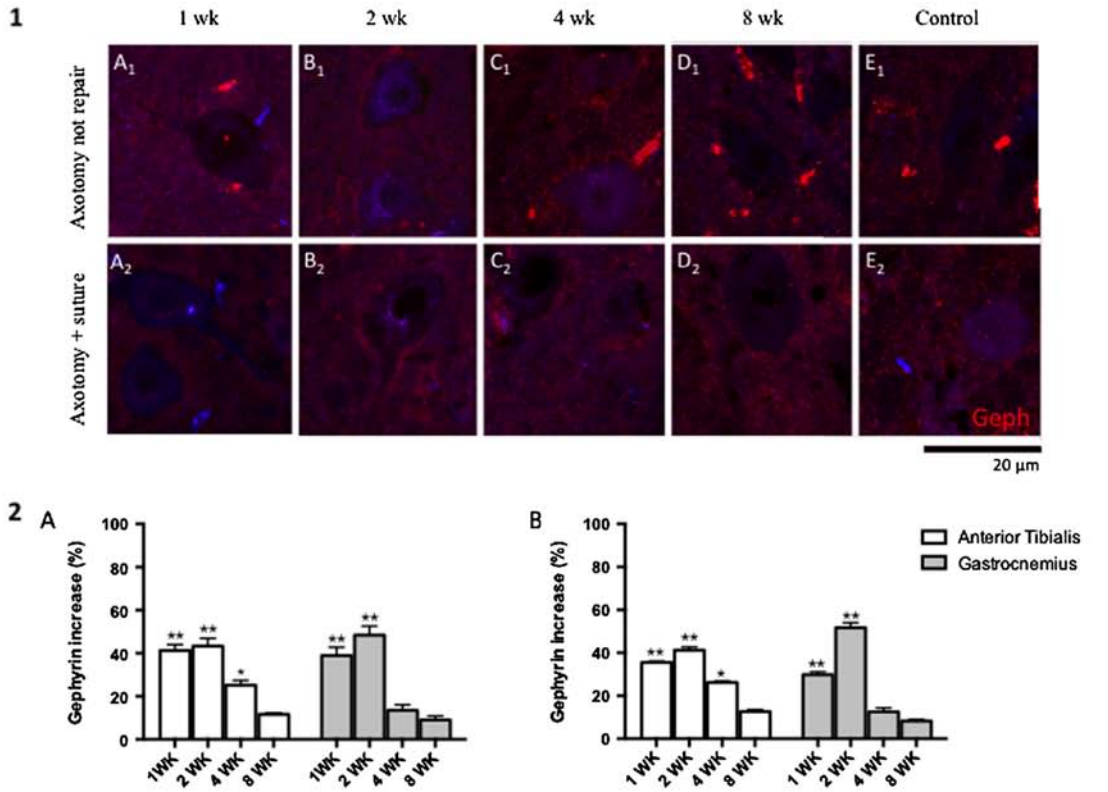

Fig. 3. Gephyrin staining in axotomized motoneurons. 1. Confocal images of spinal cord regions stained against gephyrin at 1 week (A), 2 weeks (B), 4 weeks (C), and 8 weeks (D). Back-labeled motoneurons from TA and GM in blue, gephyrin in red. Control motoneurons (E_1, E_2) show less immunoreactivity to gephyrin, whereas after axotomy, there is an increase with 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 back-labeled motoneurons from TA (white bars) and GM (gray bars). Repair of the nerve does not change the evolution of synaptic stripping, with a maximum increase in gephyrin at 2 weeks. Changes are slightly more marked in motoneurons from GM than from TA (data are expressed as mean ± SEM, $^*P < 0.05$, $^{**}P < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

1 week post-injury ($P = 0.025$, Fig. 5). In contrast, NT3 application did not affect the changes induced by axotomy on synaptophysin, Vglut1, and gephyrin immunolabeling surrounding the motoneurons. Neither the application of BDNF nor NT3 affected the reduction in PNN immunoreactivity observed after axotomy (Fig. 5.4).

Application of a mixture of BDNF and NT3 had no noticeable effect on synaptophysin, Vglut1, gephyrin and PNN labeling after axotomy (data not shown).

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

In order to learn whether 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. 6.1). The Vglut1 decrease observed after axotomy was also partially reduced with HTRP already at 1 week post-lesion in both motoneuron pools, and was only 50 ± 5% at 2 weeks, in contrast to the 71 ± 6% observed in non-trained animals (Fig. 6.2). Gephyrin increase was partially reduced by HTRP, at 1 and 2 weeks for GM motoneurons (Fig. 6.3). In contrast, the low intensity treadmill exercise (LTRP) was not able to reverse the synaptic stripping changes observed after axotomy (Fig. 6).

When analyzing PNN immunoreactivity surrounding the motoneurons, we found that both exercise protocols reduced the decrease observed 2 weeks after axotomy by about 20%. Moreover, the high intensity protocol (HTRP) prevented this decrease already at 1 week (Fig 7.1). Unexpectedly, we observed that PNN immunoreactivity surrounding the contralateral intact motoneurons of intensively trained animals was greater in non-exercised rats (Fig. 7).

At 1 week, TA motoneurons in LTRP animals had a 35 ± 4% PNN loss, whereas in GM motoneurons the reduction was 42 ± 1%. At 2 weeks, levels were 54 ± 3% for both pools of motoneurons. In the HTRP group, 1 week after axotomy, TA motoneurons had a 22 ± 2% reduction in PNN, whereas this fall reached 34 ± 2% in GM motoneurons. At 2 weeks, the reduction was 51 ± 2% in TA motoneurons and 61 ± 2% in GM ones. In fact, GM motoneurons of animals receiving HTRP for 2 weeks showed a significant increase in PNN staining compared to motoneurons from untrained animals. Therefore, we also expressed the amount of PNN immunoreactivity as absolute values (Fig. 7).

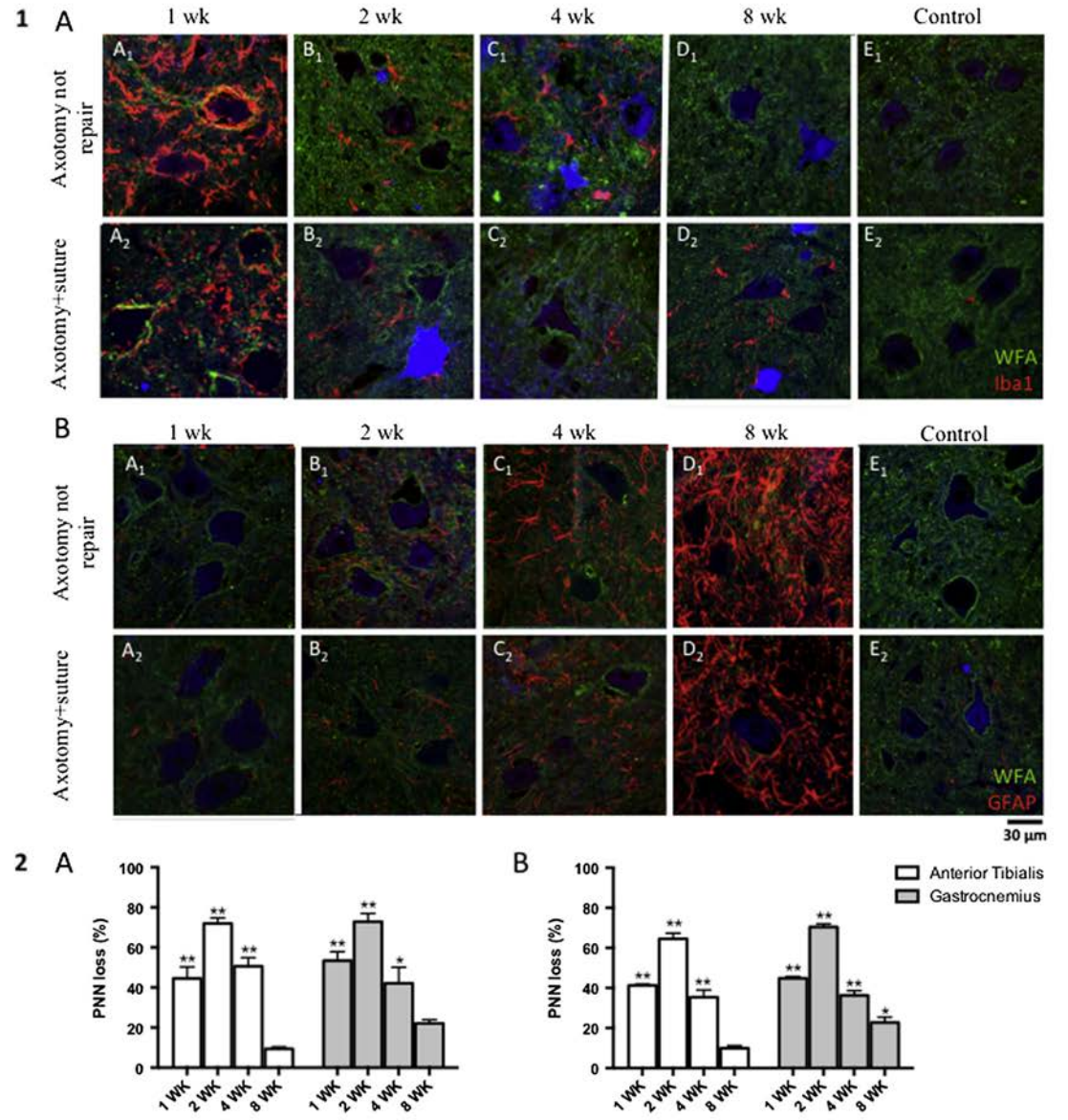

Fig. 4. Glia and PNNs surrounding axotomized motoneurons. 1. Confocal images of spinal cord regions stained for microglia or astroglia and PNNs 1 week (A), 2 weeks (B), 4 weeks (C), and 8 weeks (D) after injury 1A. Back-labeled motoneurons from TA and GM in blue, PNNs (*W. floribunda*) in green and microglia (Iba1, panel A), and astrocytes (GFAP, panel B) in red. Control motoneurons (E_1, E_2) have no glial processes around their somas, and a strong density of PNN surrounding them. 2. Quantitative analysis of motoneuron PNN after axotomy. Evaluation of *W. floribunda* staining at 1 week, 2 weeks, 4 weeks, and 8 weeks after axotomy (A) or axotomy repaired with direct suture (B) in back-labeled motoneurons from TA (white bars) and GM (gray bars). Repair of the nerve does not change the evolution of PNN destructuring, with a maximum loss of staining at 2 weeks. There is a marked increase in microglial processes around axotomized motoneurons 1 week after injury, which decreases with time; whereas astroglia processes around axotomized motoneurons increased at 8 weeks after injury, concomitant with the recovery of PNN staining (data are expressed as mean ± SEM, $^*P < 0.05$, $^{**}P < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HTRP also modulated glial activation. Animals submitted to HTRP showed reduced microglia reactivity compared to non-trained animals. There was lower immunoreactivity to Iba1 surrounding axotomized motoneurons in exercised rats (60 ± 10 Iba1/µm^2, $P < 0.05$) than in non-trained animals. In contrast, 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, $P < 0.01$, Fig. 7.1). For the low intensity protocol (LTRP) no changes in astroglia or microglia immunoreactivity between exercised and non-trained animals were observed.

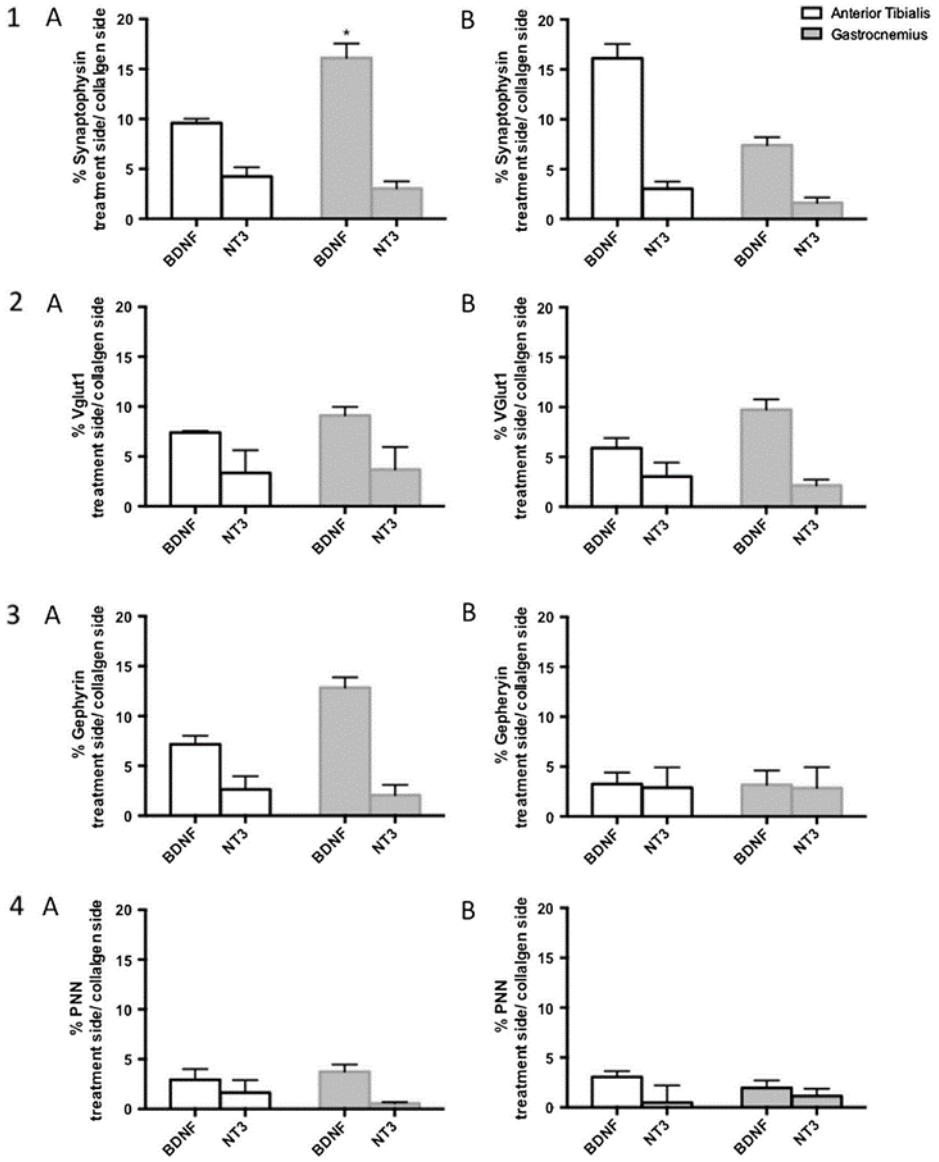

Fig. 5. Quantitative analysis of synaptic stripping after axotomy and application of neurotrophic factors. Evaluation of synaptophysin (1), Vglut1 (2), gephyrin (3) and PNN (4) staining 1 week (A) and 2 weeks (B) after repairing the sciatic nerve with a tube containing a collagen matrix with BDNF or NT3, expressed as percentage versus contralateral side, which was repaired with a tube with collagen matrix. BDNF application shows a trend toward reduction of synaptic stripping, although it is not significant. NT3 application has no effect (data are expressed as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$).

Influence of homolateral hindlimb sensory inputs on PNN

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

As expected, disruption of peripheral sensory inputs 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

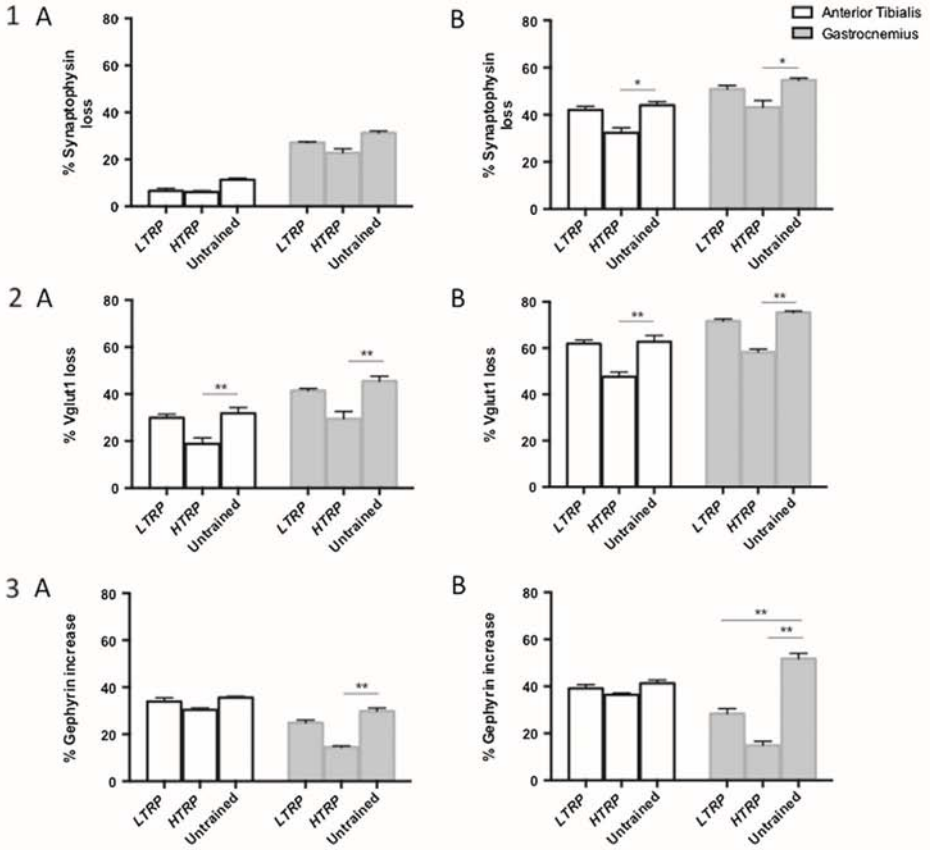

Fig. 6. Quantitative analysis of synaptic stripping after axotomy in animals submitted to different protocols of treadmill. Two protocols were used, a low intensity treadmill running program (LTPR) and a high intensity program (HTRP), and compared to untrained animals. Evaluation of synaptophysin (1), 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.

differences were observed surrounding the axotomized motoneurons versus the intact ones. Animals exercised in the treadmill showed similar values to non-trained ones.

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

Dorsal root lesion did not induce 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 in 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 on the contralateral side a significant increase in 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 also observed a marked decrease in proximal glutamatergic synapses, with a maximum peak at 2 weeks post-axotomy, which was partially reversed with time. This decrease was accompanied by an increase in gephyrin immunoreactivity and a disintegration of PNN surrounding the motoneurons. Application of neurotrophins at the proximal stump was not able to reverse these effects. In contrast, activity-dependent therapy, such as treadmill running, reduced the destructuring 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., 2010, 2011), we specifically evaluated two motoneuron pools, the extensor GM and the flexor TA, both innervated by the sciatic nerve, and we compared the changes induced by axotomy with the contralateral intact side. In fact, we observed greater synaptic depletion in axotomized GM motoneurons. For synaptic characterization, we used an immunohistochemical approach to identify different synaptic populations. Although these are indirect markers of innervation, they normally correlate well with the presence of terminals measured by electron microscopy (Rask-Andersen et al., 2000; von Krosigk et al., 1992).

The synaptic stripping that motoneurons suffer after axotomy is a well-known phenomenon that has been related with a shifting state

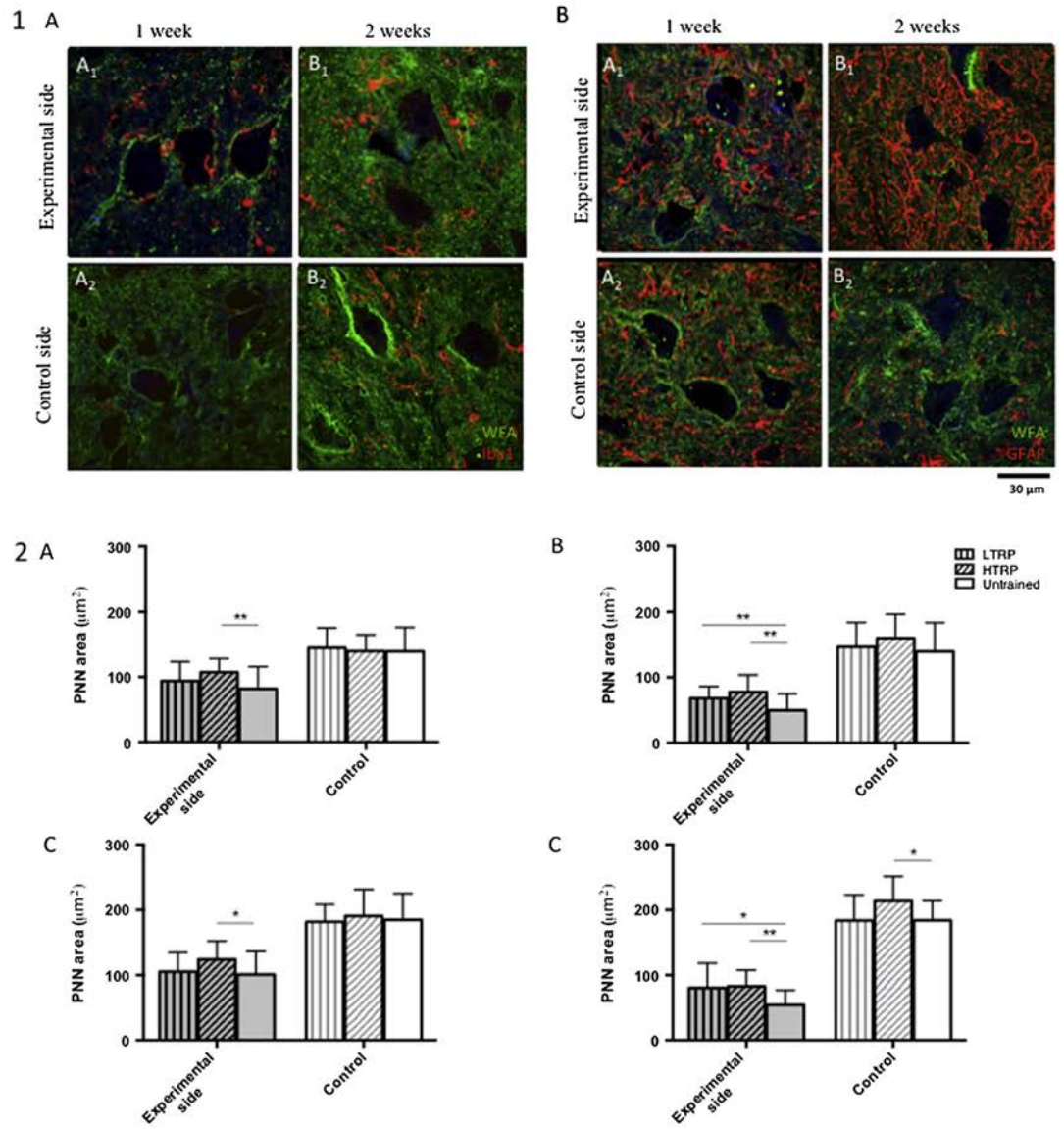

Fig. 7. Glia and PNNs surrounding axotomized motoneurons in animals submitted to different treadmill protocols. 1. Confocal images of spinal cord regions stained for microglia (A), astrocytes (B) and PNNs (A, B) 1 week and 2 weeks after injury in HTRP animals. Back-labeled motoneurons from TA and GM muscle in blue, microglia (Iba1) and astrocytes (GFAP) in red, and PNNs (*W. floribunda*) in green. 2. Evaluation of PNN staining in animals submitted to low intensity treadmill (LTPR, vertical dashed bar), high intensity treadmill (HTRP, dashed bar), and untrained (solid bar) in TA (1) and GM (2) motoneurons axotomized for 1 week (2A, 2B) or 2 weeks (2C, 2B) with cut and suture of the sciatic nerve. Data are expressed in absolute values, since the contralateral non-injured side was also submitted to the treatment. Exercise reduced the loss of motoneuron PNN observed after injury, with the effect being more marked with the more intense protocol. At 2 weeks, animals submitted to HTRP also showed increased PNN immunostaining surrounding MN of the contralateral non-injured side (data are expressed as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of the neurons from one related to distribution of information to one where mechanisms for survival and repair are warranted (Aldskogius and Svensson, 1993). This synaptic detachment has been linked with the rapid activation of microglia, which withdraw their processes, proliferate, and migrate toward the axotomized motoneuron cell body (Greaber et al., 1993). In our study, we found a marked increase in microglial processes surrounding 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 lowest point at 2 weeks. This reduction may be partially attributed to the loss of glutamatergic synapses, among the synapses most affected by stripping after injury (Alvarez et al., 2011; Novikova et al., 2000). Vglut1 is a specific marker for Ia sensory afferents from the muscle spindle (Todd et al., 2003), a key element in

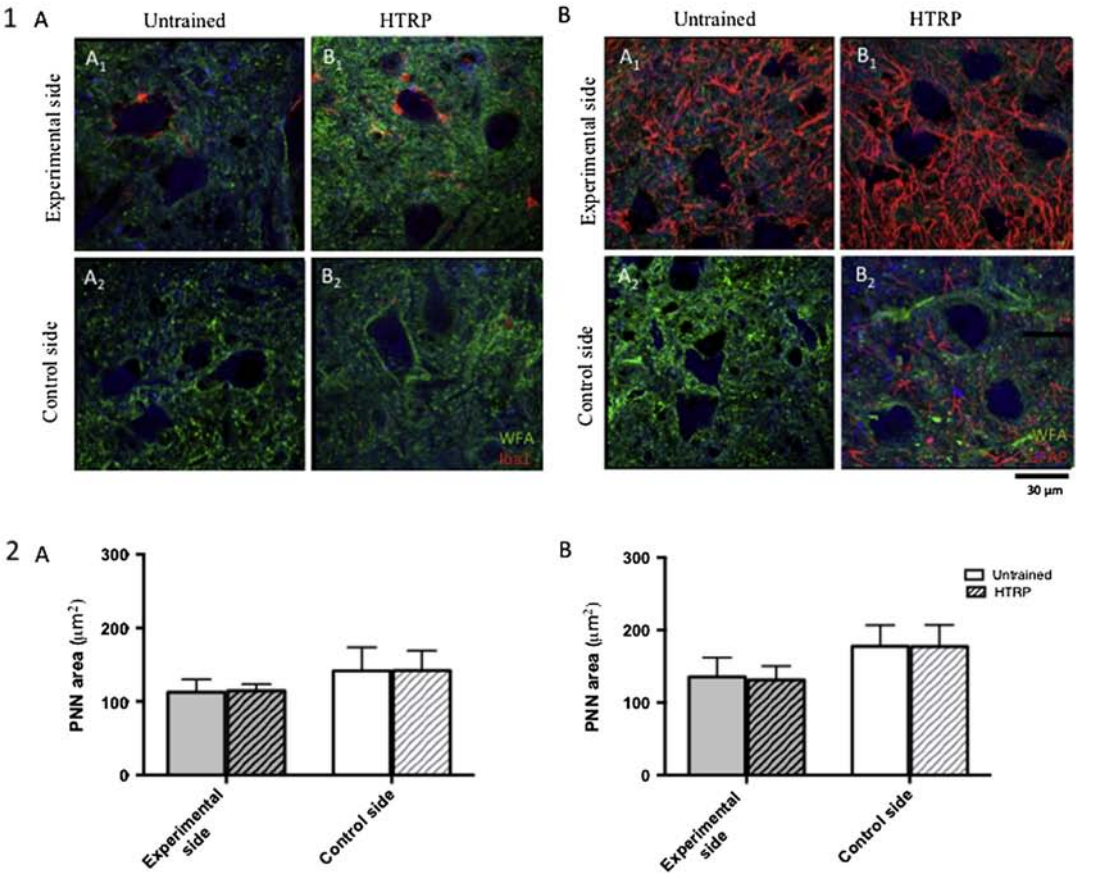

Fig. 8. Glia and PNNs surrounding intact motoneurons that lost the segmentary sensory inputs of the homolateral hindlimb. 1. Confocal images of spinal cord regions stained for PNNs (*W. floribunda*, green) and microglia (Iba1, red, A) or astroglia (GFAP, red, B) 2 weeks post-injury in untrained animals (A) and animals submitted to HTRP. 2. Quantitative analysis of motoneuron PNN of animals submitted to dorsal rhizotomy (L3 to L6) to disrupt the homolateral sensory inputs of the limb. TA (2A) and GM (2B) motoneurons of untrained animals (solid bars) and 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 reverse the loss. In the control non-injured side of trained animals there was reduction in microglia reactivity, a mild increase in astroglia, and PNN were similar to controls (data are expressed as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the stretch reflex circuit. The group of Cope (Alvarez et al., 2010) proposed that Vglut1 synapses never recover after injury and that 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 recovered baseline levels after axotomy. In parallel to the Vglut1 decrease, we observed a marked increase in 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. Up-regulation of gephyrin may be a protective action to compensate for the increased excitability observed in motoneurons after axonal injury.

The synaptic stripping has 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 reverse synaptic stripping. These neurotrophins differentially affected excitatory and inhibitory synapses (Davis-López de Carrizosa et al., 2009). Therefore, we wanted to test whether addition of these neurotrophins to the proximal stump of the nerve could reverse this stripping, by mimicking the trophic support normally received from the muscle. However, we found only marginal preservation of Vglut1 synapses after BDNF administration. Direct application of these factors into the spinal cord could be a more effective method to reverse synaptic stripping (Davis-López de Carrizosa et al., 2009; Novikova et al., 2000), but application at the injury site is a better clinical strategy, since it can be performed in conjunction with surgical repair and it also enhances axonal regeneration. Continuous delivery of these factors, either by slow-release microparticles or by viral vector-mediated transfer of neurotrophins (Blits et al., 2003; Ruitenberg et al., 2002) may be a more effective strategy to guarantee trophic support for longer periods of time. Furthermore, other trophic factors may be important in spinal motoneurons to maintain the synaptic contacts. However, finding the correct combination of factors, with the appropriate dose and timing-pattern, may be too complex to perform. In fact, when the mixture of both BDNF and NT3 were applied, no effect was observed. TrkB mediates the effects of BDNF but also NT4 and NT3 (Klein et al., 1991; Yamamoto et al., 1996). Thus, perhaps NT3 is interfering with BDNF action. Therefore, strategies like

physical exercise, whose effects in the nervous system have been linked with its capacity to up-regulate trophic factors (F. Gomez-Pinilla et al., 2012; Vaynman et al., 2003; Ying et al., 2003) may be a better clinical strategy to modulate the trophic support of axotomized motoneurons.

The effects of exercise on motor function after peripheral nerve injury are a bit controversial, with some studies concluding that exercise has beneficial effects (Asensio-Pinilla et al., 2009; Marqueste et al., 2004; Van Meeteren et al., 1997; Sabatier et al., 2008; Udina et al., 2011a) and others indicating it is detrimental (Gutmann and Jokoubek, 1963; Herbison et al., 1980; Herbison et al., 1974; Van Meeteren et al., 1997; Soucy et al., 1996). Moreover, activity-dependent therapies can also modulate the plastic changes observed after peripheral nerve injury, as reported for the modulation of the H reflex (Asensio-Pinilla et al., 2009; Udina et al., 2011a; Vivó et al., 2008) and of pain responses (Cobianchi et al., 2010, 2013). Since synaptic stripping might be a source of poor functional recovery following peripheral nerve injury, these combined effects of treadmill training have the potential for improved functional recovery after injury. However, the mechanism through which exercise facilitates functional recovery after injuries of the nervous system is not clear. It was proposed that it modulates plasticity by increasing BDNF (Vaynman and Gomez-Pinilla, 2005), but changes in other trophic factors may also play a role (Cobianchi et al., 2013).

Increased plasticity of the nervous system has been related with PNN reduction. Enzymatic degradation of PNN reopens the critical period for experience-dependent plasticity in the visual cortex (Pizzorusso et al., 2002, 2006) and enhances 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 after peripheral nerve injury and when activity dependent therapies are applied. In a spinal cord injury model, it has been described that rehabilitation increased motoneuron PNN (Wang et al., 2011). In our study we characterized the fate of motoneuron PNN after sciatic nerve injury, and how exercise might affect these structures that are critical for the stability of the neural circuits.

Accompanying the synaptic stripping induced by axotomy, we observed disorganization in the PNN surrounding injured motoneurons. A previous paper pointed out that motoneurons do not lose PNN after nerve injury (Kalb and Hockfield, 1988), although they did not perform a quantitative analysis of these nets. In fact, we were still able to detect PNN staining surrounding axotomized motoneurons, although there was a clear reduction of immunoreactivity, with a minimum point at two weeks. It seems logical that these nets, which contribute to stabilize synapses, are affected by the process of synaptic stripping. It is interesting to note that in other neurons of the central nervous system, such as those of the visual system (Guimarães et al., 1990; Lander et al., 1997; Kind et al., 1995; Sur et al., 1988), the somatosensory system (McRae et al., 2007) and the cerebellum (Carulli et al., 2013), PNN formation and maintenance are influenced by the activity of sensory afferents, with increased activity reducing PNN. After a peripheral nerve injury, there is loss of normal sensory inputs from Ia afferents that are also injured and disconnected from their target organ, the muscle spindle. Accompanying this sensory loss, motoneurons are also injured and switch to a pro-regenerative state that includes changes in their dendritic arbor. Therefore, to further understand the mechanism that may explain the reduction of PNN after injury, we also evaluated their fate when motoneurons were not axotomized but had lost sensory inputs from the limb. Interestingly, after transection of the dorsal roots innervating the lower limbs (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.

Moreover, when animals that suffered interruption of the sensory inputs of the hind limb by multiple rhizotomies were also trained on the treadmill, PNN loss was not reversed, suggesting that the effects of exercise on motoneuron PNN depend on increased input activity mediated by sensory afferents (Molteni et al., 2004). We performed a wide dorsal rhizotomy to suppress most of the sensory inputs of the hind limb, and not only the sciatic afferents. Even during the denervation period, exercise can stimulate afferents from proximal non-denervated muscles (Koerber et al., 2006).

Increased activity in the form of treadmill exercise also modulated 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 with this, we observed a significant reduction in microglia processes around motoneurons already at one week. The intensity of exercise was related to the amount of microglia reactivity attenuation, since low intensity treadmill had no effect on microglia activity. Although it has been proposed that reduction in microglia reactivity can prevent neuronal loss (Milligan and Watkins, 2009), others suggest that activated microglia play a predominantly protective role (Cullheim and Thams, 2007). In our study, reduction in microglia activation at early time points was associated with 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 by a reduction in astrocyte reactivity. Nevertheless, we observed a marked increase in astrocytes 2 weeks post-injury in the trained animals, a fact that suggests that astrocyte activation is independent of microglia activation. This increased astrocyte reactivity may be related to the preservation of PNN in axotomized motoneurons of trained animals. A previous study showed that the formation of synapses promoted by astrocytes paralleled the emergence of PNNs in embryonic hippocampus (Pyka et al., 2011). In our study, the increased presence of astrocytes surrounding motoneurons 8 weeks post-injury is coincident with the recovery of PNN immunoreactivity to baseline levels.

In conclusion, our results provide a proof of concept that enhancing functional afferent inputs to spinal neurons may help to prevent the central changes that these neurons suffer after axotomy. Further studies analyzing the functional recovery of the central circuits after treadmill running and its relation to motoneuron PNNs changes would help elucidate the complex effects of activity-dependent therapies on the nervous system.

Acknowledgments

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