This is a non-final version of an article published in final form in Eur J Neurosci. 2017 Dec;46(12):2885-2892. doi: 10.1111/ejn.13739. Epub 2017 Nov 6.PMID: 29044770

Effects of forced, passive and voluntary exercise on spinal motoneurons changes after peripheral nerve injury

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Key words: motoneurons, exercise, peripheral nerve injury, synaptic composition, perineuronal nets

Pages: 14

Words: 4063

Number of figures: 2 (2 in color)

Number of tables: 2

ABSTRACT

After peripheral nerve injury, there are important changes at the spinal level that can lead to disorganization of the central circuitry and thus, compromise functional recovery even if axons are able to successfully regenerate and reinnervate their target organs.

Physical rehabilitation is a promising strategy to modulate these plastic changes and thus, to improve functional recovery after damage of the nervous system. Forced exercise in a treadmill is able to partially reverse the synaptic stripping and the loss of perineuronal nets that motoneurons suffer after peripheral nerve injury in animal models.

The aim of this study is to investigate if passive exercise, by means of cycling in a a motorized bicycle, or voluntary free running in a wheel are able to mimic the effects induced by forced exercise on the changes that axotomized motoneurons suffer after peripheral nerve injury. Partial preservation of synapses and perineuronal nets was observed only in axotomized motoneurons from animals subjected to high intensity cycling and the ones that freely ran long distances, but not when low intensity exercise protocols were applied. Therefore, the intensity but not the type of exercise used is the key element to prevent synaptic stripping and loss of perineuronal nets in motoneurons after axotomy.

INTRODUCTION

Peripheral nerves are frequently injured, and despite the ability of axons to regenerate, their functional outcome is commonly poor, usually attributed to the non-specific regeneration of damaged axons. However, other factors can also interfere with functional recovery, among them the changes that occur in neural circuits connectivity at the spinal level. After axotomy, spinal motoneurons suffer massive synaptic stripping that disrupts the spinal circuitry, with an important loss of proprioceptive synapses that never return to basal levels. This leads to dysfunction of the stretch reflex -the monosynaptic circuit between a proprioceptive neuron and the motoneurons of the homologous muscle- after nerve injuries, even if regeneration and reinnervation of peripheral target organs is successful (Alvarez et al., 2010; Cope et al., 1994; Rotterman et al., 2014). Therefore, strategies aimed to modulate the spinal changes induced by peripheral nerve injury, reducing synaptic stripping and preventing disorganization of the spinal circuitry, could benefit functional recovery.

Activity-dependent therapies can be a good strategy to modulate the spinal changes induced by a peripheral nerve injury. In a recent study, we found that forced exercise in a treadmill was able to partially prevent the loss of glutamatergic synapses on motoneurons two weeks after nerve injury, and also to reduce the disorganization of perineuronal nets (PNNs), which are responsible for synaptic stabilization (Arbat-Plana et al., 2015). Other studies had already shown that forced treadmill running following peripheral nerve injury was effective in promoting axonal regeneration (Cobianchi et al., 2013; Gordon and English, 2015; Sabatier et al., 2008). However, for translating these basic research findings to the clinical use, it will be important to evaluate other types of exercise protocols that may be easier to apply in human patients, such as passive or voluntary exercise. For example, motorized cycling is used in clinics to provide passive exercise. In experimental models, passive cycling of the denervated hindpaw at early stages preserves the structure of the end-plates, enhances axonal regeneration and muscle reinnervation (Patcher, 1989; Udina et al., 2011b). In contrast to forced treadmill running, which requires subjects to perform active movements using varying degrees of supraspinal and/or segmental spinal control, passive cycling in a motorized bicycle is performed in anesthetized rats and, thus, it does not need supraspinal

control. In the case of voluntary exercise, animals are allowed to freely run in a wheel and, in contrast to forced exercise, it is non-stressful.

Therefore, the goal of this study was to evaluate if passive or voluntary exercise training can mimic the effects of forced treadmill exercise on the spinal changes observed after nerve injury. We examined the effects of these three protocols of exercise on synaptic stripping and PNN on axotomized motoneurons of the lumbar spinal cord. Since proprioceptive synapses are one of the most affected by synaptic stripping after axotomy, in this study we have used VGlut1 to label these synapses. In contrast to VGlut2, that mainly labels synapses arising from spinal origin, VGlut1 specifically labels proprioceptive afferents from muscle spindles (Alvarez et al., 2004).

It is important to take into account that the synaptic content of motoneurons is quite plastic and indeed there is competition between proprioceptive and corticospinal inputs. A recent study (Jiang et al., 2016) has shown that after dorsal rhyzotomy, the loss of proprioceptive inputs by motoneurons leads to an increase in the number of C-boutons. These C-boutons are the presynaptic terminals of ChAT interneurons (Nagy et al., 1993), and participate in regulating motoneuron excitability (Hellström et al., 2003). In contrast to dorsal rhyzotomy, electrical stimulation of the dorsal root, potentiated proprioceptive inputs on motoneurons and reduced corticospinal connections on cholinergic interneurons and C-boutons on motoneurons. Therefore, we also evaluated if exercise preserves proprioceptive contacts in motoneurons at the expenses of reducing C-boutons or, contrarily to dorsal root stimulation, an activity dependent therapy that activates both peripheral sensory inputs and cortical inputs does not negatively modulate the corticospinal influence on motoneurons.

MATERIAL AND METHODS

Experimental animals

Thirty-two adult female Sprague-Dawley rats (8 weeks old; 250–300 g) from the animal facility of the Universitat Autònoma de Barcelona 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 directive on Animal Care 2010/63/EU. During 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%). Each experimental group (see table 1) had 4 animals.

Retrograde labeling

To identify the motoneuron pools from tibialis anterior (TA) and gastrocnemius medialis (GM) muscles, retrograde tracing was applied one week before intervention. Bilaterally, two retrotracers, Fluorogold (5%; FG, Fluorochrome) and True Blue Chloride (10%; TB, Setareh Biotech, Eugene, OR, USA), were used to identify both motoneuron pools. A small incision to the skin was made to expose the muscle, and then two injections (2.5 µl/injection) were distributed within the muscle with a glass pipette using a Picospritzer (Arbat-Plana et al., 2015; Davalos et al., 2005; Geremia et al., 2007). The tip of the pipette was left for one minute inside the muscle to avoid reflux. The area of application was rinsed with saline to clean any remnants of the tracer and the skin wound was sutured.

Surgical procedure

Under anesthesia, the sciatic nerve was exposed at the mid-thigh and sharply transected. The proximal and distal nerve stumps were rejoined with two epineural 10-0 sutures. The wound was closed and disinfected with povidone iodine, and rats were allowed to recover in a warm environment. Animals were periodically checked after the surgery following a standardized protocol of supervision to detect possible signs of pain or discomfort (physical aspect, response to manipulation, signs of autotomy) that guides the application of corrective measures (closer monitorization, administration of analgesia). None of the animals of the study presented signs that demanded intervention during all the follow-up.

Treadmill running

Prior to surgery, rats subjected to training exercise were placed on a motor-driven rodent treadmill (Treadmill LE 8706, LETICA, Spain) for 60 min twice a week to get them used to the device. During these training sessions, shock grid intensity was set at 0.4 mA to provide a mild negative stimulus to encourage running. The training protocol started 3 days after surgery and consisted of one session of treadmill running 5 days/week until 2 weeks

post-injury. Treadmill running exercise was applied under two protocols (Table 1). In a low intensity group rats walked at a constant treadmill speed of 10 cm/s during two periods of 30 min with 10 minute resting period between, whereas in a high intensity group running started at 10 cm/s and 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).

Bicycle protocol

The motorized bicycle for rats consists of a motor driving two pedals that can rotate at variable rates, 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 (4% induction, 1.5% maintenance) during the sessions. Three days after the injury, animals were subjected 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 subjected to a high-intensity bicycle program (HBP), in which the cycling rate was progressively increased for 60 min, from 45 rpm in steps of 7 rpm every 10 min, until a maximum speed of 75 rpm was reached.

Free-running wheel protocol

Rats in the voluntary exercise group were housed in pairs in cages with stainless-steel running wheels (LE904, Panlab, Spain) and were allowed free access to the wheel 24 h per day. The running distance was monitored daily. All wheels were filmed daily and analyzed using Smart Video Tracking (Panlab) in order to categorize the rats according to running distances. Since there were large individual variations in the time and distance run in the wheels, we divided the animals into: low (LW), medium (HW) or high (HW) distance runners. Animals in LW run 4-5 km/day, in MW 5-6 km/day and in HW up to 10-12 km/day.

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 24h, cryoprotected in 30% sucrose, and stored at 4ºC until use. Samples were embedded in TissueTek, serially cut (20µm thickness) in the transverse plane with a cryostat, and collected onto gelatin-coated glass slides. All sections were blocked with 2% normal bovine serum for 1h, followed by overnight incubation at 4ºC with combinations of primary antibodies (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. Then, series of sections were Nissl-stained (Invitrogen, 1:200) to prevent retrotracer confocal fade. Finally, sections were mounted on slides and coverslipped with Fluoromount-G (SouthernBiotech). Motoneurons labeled with both retrotracer and Nissl were localized and images captured with a scanning confocal microscope (LSM 700 Axio Observer, Carl Zeiss 40x/1,3 Oil DIC M27).

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). First, motoneurons were automatically selected, and a constant threshold was used to segment and obtain an estimated average density for each labeling (Syn, VGlut1, VGat, PNN, Iba1, GFAP). Immunoreactivity was evaluated in a perimeter of 5µm surrounding the neuron soma. This 5µm-thick perimeter covers the synaptic area surrounding the neuron and limits the overlapping with synapses of neighboring motoneurons. Indeed, the maximum depletion in synapses after axotomy has been described close to the soma (Alvarez et al., 2011). Regarding VChat staining, the number of C- boutons surrounding MNs was evaluated by double manual counting. For each animal, 10 to 15 motoneurons of each pool and each side were analyzed.

Statistical analysis

The results were statistically analyzed with SPSS 20.0 (SPSS Inc., Chicago, IL, USA). For quantitative variables, normality was assessed with the Shapiro-Wilk test (Royston, 1993). For normal variables, one-way analysis of variance (ANOVA) analysis with Tukey's post hoc Honest Significant Difference (HSD) test was used to test the significance of the

difference between the lesion side and the contralateral side. For non-normal variables Kruskall-Wallis test was performed. 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 different trained groups compared to the untrained (UT) group. A value of $P < 0.05$ was considered significant.

RESULTS

Axotomized motoneurons suffered massive synaptic stripping of their central dendritic arbor and reduction of PNN around their soma, as previously reported (Arbat-Plana et al., 2015). These alterations were partially reverted in injured animals subjected to a high intensity treadmill running exercise for two weeks after the injury, whereas a protocol of lower intensity had milder effects. In this study, we have evaluated if passive exercise, by means of a motorized cycling apparatus adapted to rats, or voluntary exercise, by means of a wheel where animals could freely run, are able to mimic the effects of forced treadmill running on these changes at the spinal cord. To closely mimic forced exercise, two protocols of motorized cycling were used, low (LBP) and high (HBP) intensity ones. In the group of free running in a wheel, animals were subdivided in three different groups depending on the cumulative distance they ran during the follow up: short (SW), medium (MW) or long (HW).

No significant differences were found at the contralateral intact side between groups in the different parameters related with the synaptic content analyzed.. Therefore, changes in immunofluorescence for synaptic markers were normalized to the ratio of ipsilateral vs contralateral values to minimize image-to-image variability. However, we observed than PNN surrounding intact motoneurons (contralateral side) was higher in some of exercised groups; consequently, these values are express in absolute values.

In the 5µm perimeter surrounding the soma of contralateral non injured motoneurons of untrained animals, the average density of Synaptophysin ranged from 74 to 80 μm^2 in TA and from 87 to 91 μm^2 in GM motoneurons. VGat density ranged from 36 to 42 μm^2 in both TA and GM motoneurons. VGlut1 ranged from 65 to 69 μm^2 in TA motoneurons and from 97 to 103 μm^2 in GM ones. Regarding C-boutons, non-injured motoneurons of TA contented from 0.6 to 1 C-bouton and GM motneurons from 0.9 to 1,5. Density of PNN, evaluated by WFA immunostaining, ranged from 48 to 52 μm^2 in TA motoneurons and from 50 to 56 μm^2 in GM motoneurons. Microglia reactivity, immunostained with iba1, ranged from 78 to 92 μm^2 in

TA motoneurons and from 75 to 87 μm^2 in GM motoneurons, whereas astroglia reactivity, immunostained with GFAP, was minimal (4 to 6 μm^2 and 2 to 4 μm^2 in TA and GM motoneurons respectively).

Synaptic changes of injured motoneurons after different protocols of exercise

Similar to previous results, high intensity treadmill running protocol (HTRP) partially prevented the decrease in Synaptophysin immunoreactivity surrounding axotomized motoneurons 2 weeks post-injury ($p<0.001$). Synaptophysin loss was also partially prevented with HBP in both TA and GM motoneurons ($p<0.001$), but only in TA in HW ($p<0.001$). In contrast, animals subjected to low intensity protocols (LTRP, LBP) or that freely run medium (MW) and low (LW) distances showed similar Synaptophysin loss on injured motoneurons than untrained (UT) control rats (Fig. 1A, 1E).

The decrease of VGlut1 observed after axotomy was also partially reduced in both GA and TA motoneuron pools in animals of HTRP, HBP and HW groups ($p<0.001$), whereas the less intense protocols showed values similar to injured animals not subjected to exercise (Fig. 1B, 1E).

Regarding inhibitory synapses, the increase of VGat observed after injury was partially reduced by HTRP and also by LTRP in TA and GM motoneurons ($p<0.001$). High intensity cycling protocol and freely run long distance exercises were able to partially reduce the increased content of inhibitory VGat synapses on injured motoneurons ($p<0.001$). In contrast, the low intensity passive bicycling and medium and low distance running had no effects on these synapses, showing similar values to those found in UT animals (Fig. 1C,1E).

The counts of synaptic contacts expressing VChat showed a significant decrease of C-boutons surrounding motoneurons. Rats subjected to HTRP, HBP and HW had a similar partial recovery of this loss in both TA and GM motoneurons ($p<0.001$). In contrast, low intensity trained animals did not show significant differences compared to UT rats (Fig.1D,1E).

Perineuronal nets of injured motoneurons after different protocols of exercise

When analyzing the amount of PNN immunoreactivity, we observed that HTRP reduced the decrease that these nets shown 2 weeks after axotomy, similar to the findings described in our previous work. HBP was also able to reduce the decrease of PNN observed after axotomy. Similarly, to what we observed in the other parameters, only when animals were running long distances in the wheel, PNN loss was partially prevented (Fig. 2A, 3D). LTRP also reduced the decrease of PNN observed after sciatic nerve injury, although the difference was not significant.

Glial reactivity after different protocols of exercise

In untrained rats, there was an important increase of microglia reactivity around axotomized motonerons at two weeks, whereas astrocyte reactivity was not significant at this time point. In contrast, if animals were subjected to HTRP, microglia reactivity was drastically reduced around injured motoneurons, and an important astrocyte reaction was observed. The ability to reduce microglia reactivity was not observed with the other type of exercise protocols, including LTRP, high intensity cycling protocol or long distance running in the wheel, and levels of microglia around motoneurons were similar to the ones observed in UT animals. In contrast, the low intensity cycling protocol and freely running in a wheel low distances induced a marked increase in Iba1 reactivity around the injured motoneurons ($p>0.01$ and $p>0.001$ vs UT group respectively; Fig. 2B, D*)*. Regarding astrocyte reactivity, all the protocols of exercise, despite their intensity, induced a marked increase of GFAP immunoreactivity around axotomized motoneurons compared to the non-trained group (Fig. 2C,D).

DISCUSSION

In this study, we have evaluated the effect of different types of exercise on the changes that motoneurons suffer at the spinal level after peripheral nerve injury. Specifically, we have compared forced running in a treadmill, passive cycling in a motorized bicycle or free running in a wheel. 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.

In a previous study, we had already observed that forced running in a treadmill partially prevented synaptic stripping and reduction of PNN of axotomized motoneurons (Arbat-Plana et al., 2015). Although low intensity forced exercise in the form of treadmill running induced a slight preservation of synapses and PNN, the most important changes were observed after a high intensity protocol. Similarly, the present results show that despite the type of exercise, voluntary or passive, only significant effects on synaptic stripping and PNN preservation occur in the rats subjected to a more intense cycling protocol or the ones that voluntarily run longer distances. Thus, 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 loss. In the literature there is controversy about the effects of exercise on functional recovery after peripheral nerve injury, with some studies demonstrating positive effects on motor recovery (Asensio-Pinilla et al., 2009; Sabatier et al., 2008; Udina et al., 2011b) and other pointing adverse effects (Herbison et al., 1974; Soucy et al., 1996). These differences are probably related with the type, duration and intensity of the exercise tested, and also at which point of the regenerative process the treatment is applied (for review, see Udina et al., 2011a). .

Motoneurons have a rich synaptic arbor, with excitatory and inhibitory inputs. By using different antibodies against vesicular transporters, specific synapses can be labeled and measured. Proprioceptive sensory afferents from muscle spindles can be specifically labeled by VGlut1 antibodies (Alvarez et al., 2004), that allow to assess the stretch arc reflex, in which sensory neurons from the muscle spindle directly synapse with motoneurons that innervate the same muscle. VGat transporter labels both GABAergic nerve endings as well as a subpopulation of glycinergic nerve endings (Chaudhry et al., 1998), whereas VChAT is a good marker for the subpopulation of cholinergic interneurons that directly synapse with motoneurons by means of C-boutons. These cholinergic interneurons, among other sources, receive inputs form the corticospinal tract, being an indirect marker of their influence on spinal motoneurons (Jiang et al., 2016). Rat spinal motoneurons have similar amounts of GABAergic, glycinergic and C-boutons, and about half the number of VGlut1 contacts, but VGlut1 contacts are almost double in size (Kitzman, 2007, 2006). Therefore, when analyzing the different markers of synapses around motoneurons by immunofluorescence, higher intensities for Vglut1 could be observed, as we found in our study. Axotomy leads to massive loss of VGlut1 synapses, and an important increase of inhibitory synapses (Arbat-Plana et al., 2015). Forced exercise in a treadmill partially prevented the loss of proprioceptive afferents, mainly modulating the loss of VGlut1 synapses due to the lack of activity of sensory

afferents, and not the loss induced by the axotomy itself (Arbat-Plana et al., 2015). The partial preservation of VGlut1 synaptic contacts by different types of exercise would reduce the disorganization of the spinal circuitry and thus, could contribute to a better functional outcome after nerve injury. Indeed, the marked loss of VGlut1 synapses in axotomized motoneurons is not fully recovered even if there is a successful reinnervation of both the muscle and the muscle spindle by motoneurons and proprioceptive neurons respectively. This limited recovery of the central connectivity has been related with the poor functionality of the stretch reflex after peripheral nerve injury, impairing functional recovery (Bullinger et al., 2011; Rotterman et al., 2014). However, there is a delicate equilibrium of synapses in motoneurons, and different projections can compete against each other. A recent study showed that electrical stimulation of sensory afferents increased VGlut1 contacts in motoneurons, but in parallel to a decrease in the density of corticospinal tract projections. This decrease was mainly due to a reduction in the amount of C-boutons that motoneurons receive from cholinergic interneurons that are directly modulated by the corticospinal tract (Jiang et al., 2016). Therefore, an increased amount of activity in the periphery can potentiate the spinal circuitry but at the expense of weakening the descending influences of the motor cortex. Thus, in this study we also evaluated the effects of exercise on the number of C-boutons on the axotomized motoneurons. After injury, as expected due to the massive synaptic stripping, the number of C-boutons on motoneurons was also drastically reduced. The different protocols of exercise at high intensity, however, significantly increased the number of C-boutons in motoneurons compared to non-treated ones, suggesting that exercise does not potentiate the spinal circuitry at the expense of the corticospinal drive. In fact, the increased activity induced by exercise is more physiological that that induced by electrical stimulation of sensory afferents, since it activates both non-injured afferents but also descending projections. When sensory inputs of the hind limb are interrupted by dorsal rhyzotomy, forced exercise is not able to prevent PNN loss, thus suggesting that the effects of exercise on PNN depend on increased input activity mediated by sensory afferents (Arbat-Plana et al., 2015). On the other hand, destruction of the locus coeruleus and thus, disruption of descending noradrenergic projections also impairs the ability of forced exercise to prevent loss of synapses and PNN in injured motoneurons (unpublished data). Interestingly, even the protocol of passive cycling exercise, with the animal anesthetized, was able to prevent to some extent the loss of C-boutons in injured motoneurons.

Increased activity also modulated glial activation surrounding axotomized motoneurons. As previously described, this reactivity was reduced in animals forced to run

on the treadmill (Cobianchi et al 2013; Arbat-Plana et al., 2015), but it did not occur when animals voluntarily run or passively cycled. Moreover, we observed a higher activation of microglia in animals that run/cycle lower distances compared to non-trained ones. These differences can be due to the amount of stress induced by the different protocols of exercise. Both passive and voluntary exercise probably generate less stress than forced running in a treadmill at an increasing speed. In fact, it is well known that the noradrenergic system is activated by acute stress, and noradrenaline has an anti-inflammatory effect (Heneka et al., 2010). Since noradrenaline has also 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. In contrast, in situations that induce lower levels of stress, like mild running in a wheel or passive cycling at low intensity, microglia reactivity was not attenuated. Perhaps the increased microglia reactivity is counteracting the potential positive effects of exercise on the preservation of synapses and PNN, and thus, stress-related changes might also influence the fate of synapses and PNN. In contrast, the increased astrocyte reactivity induced by all type of exercise seems an unspecific finding, not related with the effects of exercise on motoneurons.

In conclusion, different protocols of exercise, when applied at high intensity, are able to partially revert some of the central changes observed around axotomized motoneurons. Thus, further studies correlating the amount of synaptic and PNN preservation in injured motoneurons with functional recovery are needed in order to fully understand the effects of physical exercise on the injured nervous system.

ACKNOWLEDGMENTS

This study was supported by project grant PI11/00464, TERCEL (RD12/0019/0011) and CIBERNED (CB06/05/1105) funds from the Instituto de Salud Carlos III of Spain, the European Community's Seventh Framework Programme (FP7-HEALTH-2011) under grant agreement nº 602547 (EPIONE), and FEDER funds. We are grateful for the technical help of Jessica Jaramillo and Mónica Espejo.

CONFLICT OF INTEREST

Authors state no competing interests.

CONTRIBUTION OF AUTHORS

The three authors contributed to the design of the work and interpretation of data, participated in the drafting of the paper and approved the final version. AA and EU performed the experiments and AA aquired and analysed the data.

References

Alvarez, F.J., Bullinger, K.L., Titus, H.E., Nardelli, P., Cope, T.C., 2010. Permanent reorganization of Ia afferent synapses on motoneurons after peripheral nerve injuries. Ann. N. Y. Acad. Sci. 1198, 231–41.

Alvarez, F.J., Villalba, R.M., Zerda, R., Schneider, S.P., 2004. Vesicular glutamate transporters in the spinal cord, with special reference to sensory primary afferent synapses. J. Comp. Neurol. 472, 257–280.

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

Asensio-Pinilla, E., Udina, E., Jaramillo, J., Navarro, X., 2009. Electrical stimulation combined with exercise increase axonal regeneration after peripheral nerve injury. Exp. Neurol. 219, 258–65.

Bullinger, K.L., Nardelli, P., Pinter, M.J., Alvarez, F.J., Cope, T.C., 2011. Permanent central synaptic disconnection of proprioceptors after nerve injury and regeneration. II. Loss of functional connectivity with motoneurons. J. Neurophysiol. 106, 2471–2485.

Chaudhry, F.A., Reimer, R.J., Bellocchio, E.E., Danbolt, N.C., Osen, K.K., Edwards, R.H., Storm-Mathisen, J., 1998. The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. J. Neurosci. 18, 9733–9750.

Cobianchi, S., Casals-Diaz, L., Jaramillo, J., Navarro, X., 2013. Differential effects of activity dependent treatments on axonal regeneration and neuropathic pain after peripheral nerve injury. Exp. Neurol. 240, 157–67.

Cope, T.C., Bonasera, S.J., Nichols, T.R., 1994. Reinnervated muscles fail to produce stretch reflexes. J. Neurophysiol. 71, 817–820.

Davalos, D., Grutzendler, J., Yang, G., Kim, J. V, Zuo, Y., Jung, S., Littman, D.R., Dustin,

M.L., Gan, W.-B., 2005. ATP mediates rapid microglial response to local brain injury in vivo. Nat. Neurosci. 8, 752–8.

English, A.W., Wilhelm, J.C., Sabatier, M.J., 2011. Enhancing recovery from peripheral nerve injury using treadmill training. Ann. Anat. 193, 354–61.

Geremia, N.M., Gordon, T., Brushart, T.M., Al-Majed, A.A., Verge, V.M.K., 2007. Electrical stimulation promotes sensory neuron regeneration and growth-associated gene expression. Exp. Neurol. 205, 347–359.

Gordon, T., English, A.W., 2015. Strategies to promote peripheral nerve regeneration: electrical stimulation and/or exercise. Eur. J. Neurosci. 43, 336-50

Hellström, J., Oliveira, A.L.R., Meister, B., Cullheim, S., 2003. Large cholinergic nerve terminals on subsets of motoneurons and their relation to muscarinic receptor type 2. J. Comp. Neurol. 460, 476–486.

Heneka, M.T., Nadrigny, F., Regen, T., Martinez-Hernandez, A., Dumitrescu-Ozimek, L., Terwel, D., Jardanhazi-Kurutz, D., Walter, J., Kirchhoff, F., Hanisch, U.-K., Kummer, M.P., 2010. Locus ceruleus controls Alzheimer's disease pathology by modulating microglial functions through norepinephrine. Proc. Natl. Acad. Sci. U. S. A. 107, 6058–6063.

Herbison, G., Jaweed, M., Ditunno, J., 1974. Effect of swimming on reinnervation of rat skeletal muscle. J. Neurol. Neurosurg. Psychiatryhiatry 1257–1251.

Jiang, Y.-Q., Zaaimi, B., Martin, J.H., 2016. Competition with Primary Sensory Afferents Drives Remodeling of Corticospinal Axons in Mature Spinal Motor Circuits. J. Neurosci. 36, 193–203.

Kitzman, P., 2006. Changes in vesicular glutamate transporter 2, vesicular GABA transporter and vesicular acetylcholine transporter labeling of sacrocaudal motoneurons in the spastic rat. Exp Neurol. 197, 407–419.

Kitzman, P., 2007. VGLUT1 and GLYT2 labeling of sacrocaudal motoneurons in the spinal cord injured spastic rat. Exp Neurol. 204, 195–204.

Nagy, J., Yamamoto, T., Jordan, L., 1993. Evidence for the cholinergic nature of C-terminals associated with subsurfance cisterns in alpha-motoneurons of rat. Synapse 15, 17–32.

Patcher, B.R., Everestein, A., 1989. Passive exercise and reinnervation of the rat denervated

extensor digitorum longus muscle after nerve crush. Am J Phys Med Rehabil., 179-182.

Rotterman, T.M., Nardelli, P., Cope, T.C., Alvarez, F.J., 2014. Normal distribution of VGlut1 synapses on spinal motoneuron dendrites and their reorganization after nerve injury. J. Neurosci. 34, 3475–92.

Sabatier, M.J., Redmon, N., Schwartz, G., English, A.W., 2008. Treadmill training promotes axon regeneration in injured peripheral nerves. Exp. Neurol. 211, 489–93.

Sanders, V.M., Straub, R.H., 2002. Norepinephrine, the β-adrenergic receptor, and immunity. Brain. Behav. Immun. 16, 290–332.

Soucy, M., Seburn, L., Gardiner, P., 1996. Is increased voluntary motor activity beneficial or detrimental during the period of motor nerve regeneration/reinnervation? Can J Appl Physiol 218–24.

Udina, E., Cobianchi, S., Allodi, I., Navarro, X., 2011a. Effects of activity-dependent strategies on regeneration and plasticity after peripheral nerve injuries. Ann. Anat. - Anat. Anzeiger 193, 347–353. doi:10.1016/j.aanat.2011.02.012

Udina, E., Puigdemasa, A., Navarro, X., 2011b. Passive and active exercise improve regeneration and muscle reinnervation after peripheral nerve injury in the rat. Muscle Nerve 43, 500–9.

TABLE AND FIGURE LEGENDS

Table 1. Protocols of physical activity applied in the experimental groups. Animals were subjected for two weeks to forced, voluntary or passive exercise at two different levels of intensity. Animals forced to run in a treadmill were submitted to low (LTRP) or high (HTRP) intensity protocols. Similarly, animals that passively cycle in a motorized bicycle were subdivided in low (LBP) and high (HBP) intensity cycling. Animals that freely ran in a wheel were subdivided in three groups: short, (SD), medium (MD) or long (LD), depending on the distance run at the end of the experiments.

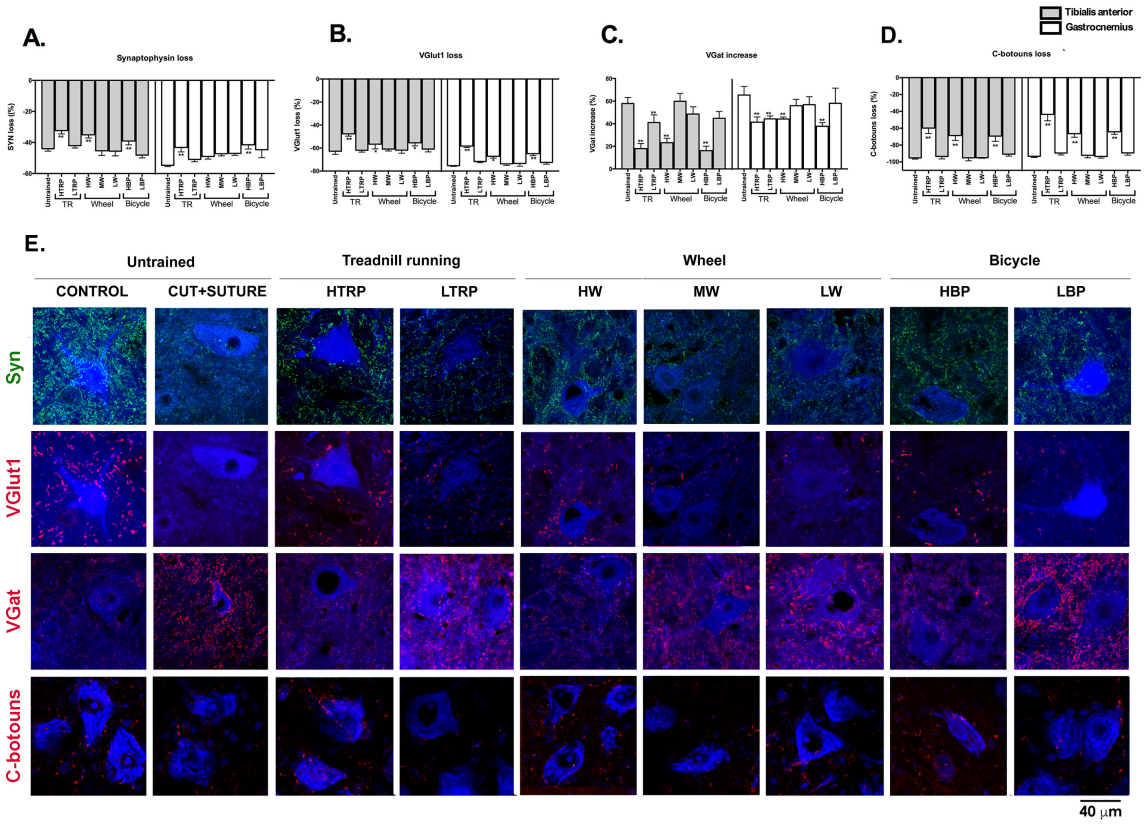
Type of training	Protocol	Group
Untrained	No training (n=4)	UT
Treadmill	Low intensity (n=4)	HTRP
	High intensity (n=4)	LTRP
Running Wheel	Short distance (n=4)	SW
	Medium distance (n=4)	MW
	Large distance (n=4)	LW
Motorized Bicycle	Low intensity (n=4)	LBP
	High intensity (n=4)	HBP

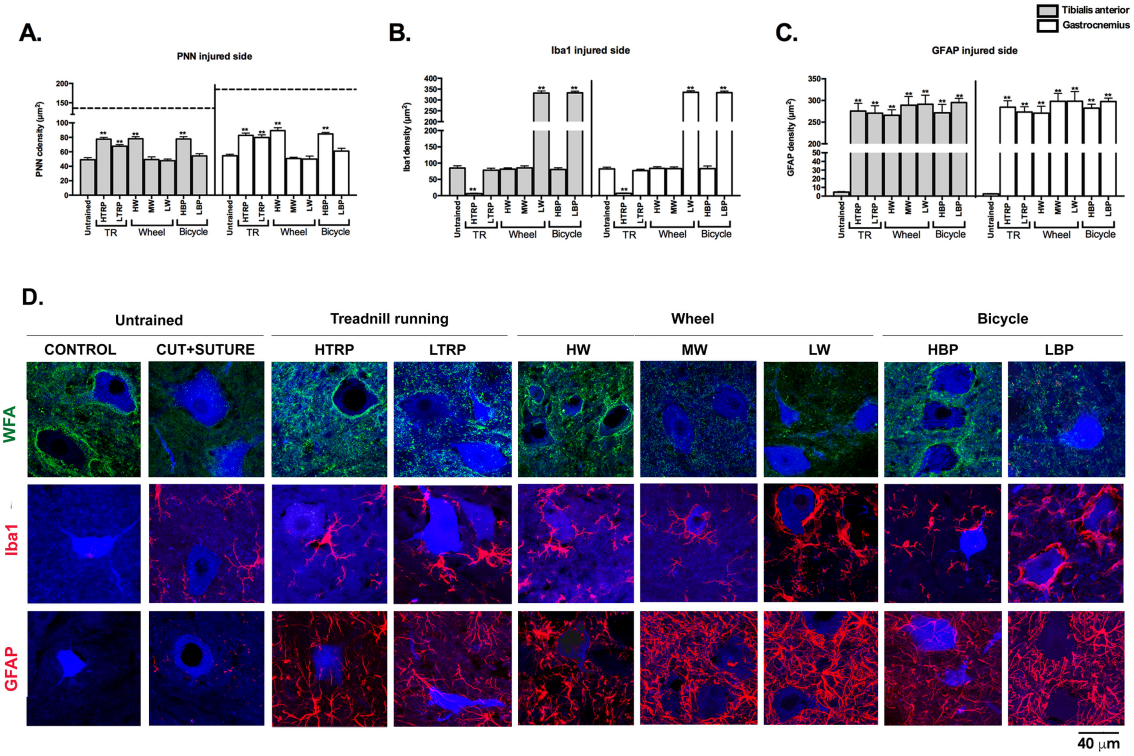
Table 2. Antibodies used in this study.

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	VGat	Strep-Tag fusion protein of rat VGat (aa 2 -115)	Guinea Pig	1:200	Synaptic systems
C-boutons	VChAT	Rat Vesicular Acetylcholine Transporter conjugated to Blue carrier protein (aa 475-500)	Sheep	1:500	Abcam
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

Fig 1. Quantitative analyses of synaptic stripping, excitatory and inhibitory synapses and C-boutons after axotomy in animals subjected to different exercise protocols. Evaluation of Synaptophysin (A), VGlut1(B), Vgat (C) and VChAT (D) immunostaining in TA (gray bars) and GM (white bars) after different training protocols or non-trained animals two weeks after cut and suture of the right sciatic nerve. Values are expressed as percentage of loss/gain versus contralateral non-injured side. Data are expressed as mean+SEM, *p<0.05, **p<0.01 vs axotomized. (E) Representative images from confocal images of spinal cord regions containing black-labeled motoneurons (blue) of TA or GM muscles after sciatic nerve section repaired with direct suture in the different experimental groups. Immunostainings were performed to evaluate amount of synaptic content on motoneurons (Synaptophysin, green), and glutamatergic (VGlut1), GABAergic (VGat) and cholinergic (VChAT) contacts (red).

Fig 2. Evaluation of excitatory and inhibitory synapses, PNN astroglia and microglia reactivity surrounding axotomized motoneurons in non-trained animals and animals subjected to different exercise protocol. Evaluation of PNN (A), Iba1 (B) and GFAP (C) immunostaining in TA (gray bars) and GM (white bars) after different training protocols or non-trained animals two weeks after cut and suture of the right sciatic nerve. Horizontal dotted lines indicate the average of motoneuron labeling in non-injured side. Data are expressed as mean+SEM, *p<0.05, **p<0.01 vs axotomized. (E) Representative images from confocal images of spinal cord regions containing black-labeled motoneurons (blue) of TA or GM muscles after sciatic nerve section repaired with direct suture in the different experimental groups. Immunostainings were performed to evaluate amount of WFA (green), whereas Iba1 (for microglia) and GFAP (for astrocytes) were used to evaluate glial reactivity (red). ¡


A.
Synaptophysin loss
SYN loss (%)
B.
VGlut1 loss
VGlut1 loss (%)
C.
VGat increase
VGat increase (%)
D.
C-botouns loss
C-botouns loss (%)
Tibialis anterior
Gastrocnemius
Untrained
HTRP
LTRP
HW
MW
LW
HBP
LBP
TR
Wheel
Bicycle
E.
Untrained
Treadnill running
Wheel
Bicycle
CONTROL
CUT+SUTURE
HTRP
LTRP
HW
MW
LW
HBP
LBP
Syn
VGlut1
VGat
C-botouns
40 μm


A.
PNN injured side
PNN cdensity (μm²)
B.
Iba1 injured side
Iba1 density (μm²)
C.
GFAP injured side
GFAP density (μm²)
Tibialis anterior
Gastrocnemius
Untrained
HTRP
LTRP
HW
MW
LW
HBP
LBP
TR
Wheel
Bicycle
D.
Untrained
Treadnill running
Wheel
Bicycle
CONTROL
CUT+SUTURE
HTRP
LTRP
HW
MW
LW
HBP
LBP
WFA
Iba1
GFAP
40 μm