Electrical stimulation combined with exercise increase axonal regeneration after peripheral nerve injury

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ARTICLE INFO

Article history:
Received 2 February 2009
Revised 24 April 2009
Accepted 22 May 2009
Available online 3 June 2009

Keywords:
Electrical stimulation
Exercise
Nerve regeneration
Reinnervation
Spinal reflexes
Treadmill

ABSTRACT

Although injured peripheral axons are able to regenerate, functional recovery is usually poor after nerve transection. In this study we aim to elucidate the role of neuronal activity, induced by nerve electrical stimulation and by exercise, in promoting axonal regeneration and modulating plasticity in the spinal cord after nerve injury. Four groups of adult rats were subjected to sciatic nerve transection and suture repair. Two groups received electrical stimulation (3 V, 0.1 ms at 20 Hz) for 1 h, immediately after injury (ESa) or during 4 weeks (1 h daily; Esc). A third group (ES+TR) received 1 h electrical stimulation and was submitted to treadmill running during 4 weeks (5 m/min, 2 h daily). A fourth group performed only exercise (TR), whereas an untreated group served as control (C). Nerve conduction, H reflex and algesimetry tests were performed at 1, 3, 5, 7 and 9 weeks after surgery, to assess muscle reinnervation and changes in excitability of spinal cord circuitry. Histological analysis was made at the end of the follow-up. Groups that received acute ES and/or were forced to exercise in the treadmill showed higher levels of muscle reinnervation and increased numbers of regenerated myelinated axons when compared to control animals or animals that received chronic ES. Combining ESa with treadmill training significantly improved muscle reinnervation during the initial phase. The facilitation of the monosynaptic H reflex in the injured limb was reduced in all treated groups, suggesting that the maintenance of activity helps to prevent the development of hyperreflexia.

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Introduction

After section of a peripheral nerve there is a complete loss of sensory and motor functions in the denervated limb. Despite the capacity of peripheral axons to regenerate and the refined microsurgery repair techniques used nowadays, the degree of functional recovery is still poor after nerve transection. Distance from the injury to the original target, slow regeneration of axons across the injury site, progressive decline in the regenerative capacity of axotomized neurons, failure of chronically denervated Schwann cells to support axonal regeneration and muscle atrophy are considered factors limiting recovery (Irintchev et al., 1990; Brushart et al., 2002; Gordon et al., 2003; Fenrich and Gordon, 2004). The accuracy of specific peripheral reinnervation and the restitution of adequate connectivity in spinal circuits and central nervous system integration are also important factors determining an adequate functional recovery. In parallel to distal axonal degeneration, nerve injuries also provoke remodeling of the spinal circuits that contribute to positive symptoms, such as hyperreflexia and hyperalgesia (Navarro et al., 2007). Changes at the spinal cord level may persist long time after reinnervation and impair motor control and sensory processing, contributing to chronic deficits after severe nerve injuries (De Medinaceli, 1988; Gramsbergen et al., 2000; Valero-Cabrè and Navarro, 2001). Thus, it is important to promote regeneration and accelerate reinnervation, but also to modulate the plastic changes at the spinal cord level to improve functional recovery.

Among the different strategies investigated for promoting axonal regeneration, application of electrical stimulation to the injured peripheral nerve is a promising approach to enhance axonal regeneration (Nix and Hopf, 1983; Al-Majed et al., 2000a; Brushart et al., 2005; Ceremia et al., 2007; Vivó et al., 2008) that has already been tested in humans (Gordon et al., 2007). Electrical stimulation of the nerve for 1 h after lesion enhances the early expression of BDNF and pro-regenerative associated genes, and facilitates that sensory (Geremia et al., 2007) and motor (Al-Majed et al., 2000b, 2004) axons grow faster across the suture site after transection of the femoral nerve in rats. Electrical stimulation is an artificial way to induce activity in the axotomized neurons, whereas exercise is a natural way of activity. Locomotor training exercise has been also shown to improve functional recovery after peripheral nerve lesions (Van Meeteren et al., 1997; Molteni et al., 2004; Marqueste et al., 2004; Sabatier et al., 2008) and its effects were related with the increased expression of BDNF and its receptor trkB (Gómez-Pinilla et al., 2001). However, other studies have shown deleterious effects of exercise on axon rege-
neration and functional recovery (Guttmann and Jakoubek, 1963; Herbison et al., 1974b, 1980a,b). The controversy may be due to the different protocols used, since the training pattern and duration seem to affect nerve regeneration (Van Meeteren et al., 1997, 1998; Sabatier et al., 2008). High frequency or high speed training can cause considerable muscle damage (Herbison et al., 1973, 1980a,b) and inhibit collateral and terminal axonal sprouting (Tam et al., 2001), whereas mild exercise, as treadmill training at low speed, can be an adequate protocol to benefit axonal regeneration and muscle reinnervation.

In a recent study we showed that acute electrical stimulation not only promoted functional recovery after sciatic nerve section and suture repair in rats but also modulated the hyperreflexia observed after the lesion (Vivó et al., 2008). Here, using the same model, we studied if chronic daily application of electrical stimulation could improve the effects observed with acute treatment (just 1 h after nerve repair). We also wanted to further elucidate the effects of activity, induced by nerve stimulation or by treadmill exercise, on axonal regeneration and spinal plastic changes. Electrophysiological tests were used to evaluate muscle reinnervation and spinal H reflex responses. Algesimetry tests were used to assess the responses to pain stimulation. The number of myelinated regenerated axons was counted in the distal tibial nerve at the end of follow-up.

Materials and methods

Experimental design

Eight weeks-old female Sprague-Dawley rats (250±30 g) were divided in five groups. Under anesthesia with pentobarbital (40 mg/kg i.p.) the right sciatic nerve was exposed at the mid thigh, transected at 90 mm from the tip of the third toe, and repaired by epineurial suture, maintaining the fascicular alignment of tibial, peroneal and sural branches. Four groups followed different activity treatments. One group received acute electrical stimulation (group ESa, n=17) applied to the repaired nerve immediately after surgery for 1 h, whereas a second group received chronic electrical stimulation (group ESC, n=8) for 1 h daily, 5 days a week, during 4 weeks. Another group was trained by running on a treadmill (group TR, n=10) 2 h, 5 days a week, during 4 weeks, beginning 5 days after surgery. In a fourth group, ESa was combined with the same treadmill training, (group ES+TR, n=10). A fifth group of injured rats was untreated and served as control (C). For acute electrical stimulation, immediately after repair with the wound open, the sciatic nerve was stimulated at the proximal stump with pulses of 0.1 ms duration and suprathreshold intensity (3 V) delivered at 20 Hz (Grass S44, Quincy MA). The cathode was a thin wire bare at the tip and gently twisted around the sciatic nerve near the sciatic notch, and the anode was a thin needle inserted in the near muscle. This pattern of stimulation has already been shown to promote motor nerve regeneration in rats and not produce harmful effects (Al-Majed et al., 2000a; Vivó et al., 2008). At the end of the procedure, the electrodes were removed and the wound was sutured and disinfected. For chronic electrical stimulation, the same stimulation pattern was applied to the anesthetized rats by means of two thin needles transcutaneously inserted, the cathode at the sciatic notch and the anode the 0.5 cm caudally. Animals were anesthetized with isofluorane, and kept warm and rehydrated during and after the procedure.

Rats of groups TR and ES+TR followed active exercise in a treadmill (Treadmill LE 8706 LETICA, Spain) daily from day 5 postoperation to one month. The animals were forced to walk on the horizontal treadmill at a speed of 5 m/min, during two periods of 30 min with a 10 min resting in between. Treadmill training was started at day 5 postoperation to allow enough time for nerve suture and wound healing, and was given until day 30 when reinnervation of distal targets in the hindpaw was expected to avoid possible detrimental effects on the partially innervated muscles (Tam et al., 2001).

All rats were kept on standard laboratory food and tap water ad libitum with a light–dark cycle of 12 h. In order to avoid autotomy after denervation, the animals were treated with amitriptyline (150 μg/ml in the drinking water). This treatment has been demonstrated that effectively reduces the development of autotomy due to neuropathic pain, and does not affect peripheral nerve function and regeneration (Navarro et al., 1994). The experimental procedures were approved by the Ethical Committee of our institution, and followed the rules of the European Communities Council Directive 86/609/EEC.

Neurophysiological tests

Muscle reinnervation was assessed by nerve conduction tests performed at 7, 21, 31, 49 and 60 days postoperation (dpo). Under pentobarbital anesthesia, the sciatric nerve was stimulated with single electrical pulses (0.1 ms duration, incremental intensity until the supramaximal level) delivered by monopolar needles percutaneously placed at the sciatic notch proximal to the lesion, and the compound muscle action potentials of tibialis anterior (TA) and plantar (PL) muscles were recorded by means of monopolar needles (28G) inserted on the muscle belly and displayed in an oscilloscope (Sapphire 4M, Vickers) (Valero-Cabrè and Navarro 2001, 2002). The latency to the onset of the responses was measured in milliseconds. The maximal amplitude of the M wave was measured in millivolts, and expressed as the percentage with respect to values obtained from the contralateral intact hindlimb for each rat, in order to evaluate muscle reinnervation. For spinal reflex testing, the maximal baseline to peak amplitude of the H wave was measured, using the recording where the H wave achieved the highest amplitude that usually corresponded to near the supramaximal stimulation for the M wave. To assess reflex facilitation, i.e. the proportion of all reinnervated muscle fibers that are activated by the H reflex, the maximal amplitudes of the M and H waves were used to calculate the H/M amplitude ratio for each rat at each time tested (Valero-Cabrè and Navarro 2001).

Thermal and mechanical algesimetry tests were performed prior to electrophysiological tests. Thermal nociception was evaluated by a heat-radiation method using the plantar test (Ugo Basile, Comerio, Italy). Rats were placed into an elevated plastic box with a glass floor and allowed to acclimatize to the environment before testing. The beam of a projection lamp was focused from below the glass floor onto the plantar surface of the hindpaw. The time to withdrawal of the heated paw (latency withdrawal) was measured through a time-meter coupled with infrared detectors. The maximal time of stimulation was limited to 20 s to avoid skin damage. A mean of three trials separated by 15 min resting periods was obtained each time. The values were normalized as the percentage of withdrawal latency of the operated versus the contralateral intact hindpaw each testing day.

Mechanical test was performed by using a 0.8 mm diameter spring wire connected to an Electronic Von Frey algesimeter (Bioseb, Chaville, France). In this case, rats were placed into individual transparent plastic cubicles with a wire mesh at the bottom, where they received mechanical stimulus onto the middle of the hindpaw plantar surface. Paw withdrawal pressure was recorded three times in both hindpaws of each animal, and the mean of these values was used for calculating the percentage of the injured versus the contralateral intact hindpaw.

Histological evaluation of nerve regeneration

At 60 days follow-up rats of each group were anesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The regenerated nerve was dissected and a segment of the tibial nerve at the ankle was harvested. The nerve segments were...
fixed in glutaraldehyde–paraformaldehyde (3%–3%) in 0.1 M cacodylate buffer (pH 7.4, 4 °C), postfixed in 2% OsO₄, dehydrated through ethanol series, and embedded in Epon. Transverse semithin sections (0.5 μm) of the tibial nerve were stained with toluidine blue and examined under light microscopy. Images were acquired with an Olympus DP50 camera. Measurements of the cross-sectional area of the whole nerve, and counts of the myelinated fibers were performed using NIH Image software. The density of myelinated fibers was obtained from counts in fields chosen by systematic sampling representing at least 50% of the total area of the nerve (Gómez et al., 1996). The total number of regenerated myelinated fibers was calculated by multiplying the myelinated fiber density per the cross-sectional area of the nerve.

Data analysis

All data are presented as the group mean±SEM. The results have been compared between groups by two-way ANOVA with group and dpo as factors, followed by Bonferroni test between all pairs of groups. A value of \( p < 0.05\) was considered to be statistically significant.

Results

Muscle reinnervation

Electrophysiological tests performed at 1 week post-injury evidenced complete denervation of the hindlimb muscles. Reinnervation of the proximal TA muscle was found from 21 dpo, as evidenced by M waves of small amplitude and long latency, and progressed with time as judged by the increasing amplitude of the M waves (Fig. 1A). Group ESa, treated with acute electrical stimulation, achieved levels of reinnervation at 60 dpo of 34±3% with respect to the contralateral intact hindlimb, significantly higher than the 27±2% of the control group. In contrast, group ESc with chronic electrical stimulation showed a similar evolution to that of the control group. Group TR had a reinnervation course overlapping with that of group ESa, but a slightly better final recovery of 39±4%. Group ES+TR, with combined electrical stimulation and treadmill, had the highest M amplitudes during the first 7 weeks, and achieved final levels of reinnervation similar than group TR. Latencies of the M wave in the different groups were considerably longer than normal during the first stages of

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Fig. 1. Recovery of the M wave amplitude in percentage with respect to contralateral control values (A, C), and of the M wave onset latency in absolute values (B, D) for the tibialis anterior muscle (A, B) and the plantar muscle (C, D) in rats untreated (group C) and treated with acute electrical stimulation (ESa), chronic electrical stimulation (ESc), treadmill training (TR) or combination of acute electrical stimulation and treadmill (ES+TR) after section and suture repair of the sciatic nerve. Combination of ESa and TR enhanced muscle reinnervation at early time points, whereas ESa and TR showed significant improvement at the end of follow-up. At early stages of reinnervation, groups ES+TR, ESa and TR had shorter latencies than groups C and ESc, differences being reduced at later times. For the plantar muscle a penalization value of 40 ms was applied to animals without response at 31 dpo to reflect in the mean of the group the animals with more delayed reinnervation. (a) \( p < 0.05 \) group ES+TR vs all other groups; (b) \( p < 0.05 \) group ES+TR vs groups C and ESc; (c) \( p < 0.05 \) groups ES+TR, TR and ESa vs C and ESc; (d) \( p < 0.05 \) groups ESa, ESc and TR vs group C; (e) \( p < 0.05 \) groups ESa and TR vs group ESc; (f) \( p < 0.05 \) groups ESa and TR vs group C; (g) \( p < 0.05 \) group ESa vs group ESc.
reinnervation, and shortened with time. At 21 dpo the M wave latencies were significantly reduced in groups ESa, TR and ES+TR (5.1±0.3, 5.8±0.5 and 5.0±0.4 ms, respectively) when compared to the control group (7.3±0.3 ms) and the ESc group (8.3±0.7 ms) (Fig. 1B). However, these differences tended to minimize along the follow-up, and at 60 days the latencies were similar in all the groups (around 2.5 ms), but still 60% longer than in the contralateral intact hindlimb.

Reinnervation of the PL muscles started from 31 dpo, later than in the TA muscle due to its more distal localization (Fig. 1C). The final level of reinnervation was significantly higher in groups ESa (11±2%), TR (9.4±1%) and ES+TR (10.4±2%) in comparison with groups C (6±1%) and ESc (4±1%). The combination of electrical stimulation and exercise showed better reinnervation at early times than single treatments and controls. At early stages of reinnervation (31dpo) latencies of the plantar M wave (Fig. 1D) were shorter in groups ESa, TR and ES+TR (32.4±2.4, 33.0±4.0 and 26.7±5 ms, respectively) than in control (37.5±1.8 ms) and ESc groups (40±0 ms). These trends, although not significant, could still be observed at 60 dpo, when groups ESa, TR and ES+TR had shorter latencies (6.8±0.4, 6.6±0.5 and 6.0±0.2 ms, respectively) compared with groups C and ESc (7.8±0.9 and 7.8±0.3 ms, respectively). For all the groups, latencies at the end of follow-up were more than 200% of normal values.

**Spinal reflex recovery**

Recording of the H wave in the nerve conduction tests provides a reliable assessment of the functional state of the monosynaptic spinal reflex circuit. In the control injured rats, the H reflex appeared highly facilitated during the early and mid phases of reinnervation, as indicated by the marked increase of the H/M amplitude ratio above normal values. At early times of reinnervation, H waves were identified by its latency and relatively constant appearance at increasing stimulation intensity, and if polyphasic the highest peak measured. The H/M ratio showed a 5-fold increase at 31 dpo in the TA muscle and at 49 dpo in the PL muscle (Fig. 2) in animals of group C, and tended to decrease in parallel with progressive reinnervation, although by 60 dpo the values were still higher than in the contralateral limb. All the treated groups presented significantly lower values than group C at 21 and 31 dpo for the TA muscle H/M ratio, and remained stable during follow-up. At 60 dpo, group ES+TR had a significantly lower ratio compared with group C (18±5% vs 38±3%). For the PL muscle, the H/M ratio averaged 47% in groups C and ESc at 49 dpo while it was around 30% in groups ESa, TR and ES+TR.

**Algesimetry results**

Withdrawal responses to heat stimulation evaluated by plantar algesimetry had a latency of about 12–15 s in the intact hindpaw. In the denervated paws there was no response to stimuli applied on the

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**Fig. 2.** Plots of the H/M amplitude ratio in the tibialis anterior and plantar muscles during follow-up in rats untreated (group C) and treated with acute electrical stimulation (ESa), chronic electrical stimulation (ESc), treadmill training (TR) and combination of acute electrical stimulation and treadmill (ES+TR). All the activity treatments tested decreased the H/M ratio when compared to the control group during the early phase of reinnervation. (a) p<0.05 group C vs all other groups; (b) p<0.05 group C vs group ES+TR; (c) p<0.05 group ES+TR vs all other groups; (d) p<0.05 group ESc and C vs ESa and ES+TR; (e) p<0.05 group C vs ESa, TR and ES+TR.

**Fig. 3.** Plots of withdrawal thresholds to heat (Plantar test) and mechanical (Von Frey test) stimulation in the operated right hindpaw with respect to the intact left hindpaw during follow-up in rats untreated (group C) and treated with acute electrical stimulation (ESa), chronic electrical stimulation (ESc), treadmill training (TR) and combination of both (ES+TR). Treadmill exercise in combination with electrical stimulation enhances the initial recovery of nociceptive responses. (a) p<0.05 group ES+TR vs C; (b) p<0.05 group ES+TR vs ESc; (c) p<0.05 group TR vs C.
sciatic territory of the sole at 7 dpo (Fig. 3). The lack of withdrawal response is attributable to the absence of sensation to the painful stimuli, since proximal muscles in the leg (quadriceps and biceps femoris) are normally innervated and allow withdrawal of the paw when stimulation is applied to an innervated area, such as the margin of the medial side of the hindpaw supplied by the intact saphenous nerve (Vivó et al., 2008; Casals-Díaz et al., 2009). During follow-up, groups treated with electrical stimulation and exercise showed a lower withdrawal threshold to heat stimulation at 30 dpo than group C, suggesting earlier skin reinnervation. The withdrawal latencies at 30 dpo averaged 119±4% of the contralateral intact paw in group ESc and 110±8% in group ES+TR, significantly faster compared to 155±17% in group C (p<0.05). Groups Esa and TR had intermediate values. Withdrawal responses to mechanical pressure in the contralateral paw were found at 15–20 g. In the injured paw there were no withdrawal responses to mechanical stimuli until 21 dpo, when they reappeared at two fold higher values with respect to the intact paw. At 30 dpo the mean withdrawal pressure was significantly lower in groups ES+TR (116±12% of the contralateral paw), Esa (132±26%) and TR (120±13%) than in the control group (176±17%). The withdrawal responses in both nociceptive tests tended to decrease slightly during follow-up, indicating progressive reinnervation of the skin, but without significant differences between groups at later times. No evidences of noticeable hyperalgesic responses (withdrawal thresholds lower than normal) were found in any of the groups.

Fig. 4. Semithin sections of the tibial nerve at the ankle level 2 months after section and suture repair of the sciatic nerve. Representative microphotographs of an intact nerve (A), a nerve from an animal of group C (B), group Esa (C), group ESc (D), group TR (E) and group ES+TR (F). Nerves show the typical aspect of a regenerative nerve, with myelinated fibers of smaller size than in intact nerves, which tended to cluster in minifascicles. Bar = 10 μm.

Fig. 5. Histogram graph of the number of regenerated myelinated fibers (MF) found in the tibial nerve at the ankle in intact rats, and in sciatic nerve injured rats without treatment (group C), treated with acute electrical stimulation (group Esa), chronic electrical stimulation (group ESc), treadmill training (group TR) and the combination of acute electrical stimulation and treadmill (group ES+TR). The higher number of myelinated fibers in the regenerating nerves compared to an intact nerve suggests an increased capacity of sprouting after Esa and TR. Both Esa and TR increased the number of regenerated fibers, but only the group with combined Esa and TR showed a significant difference versus the control group. *p<0.05 ES+TR vs C.
although the changes in mean thresholds at longer survival times (i.e. 48 and 59 days) in individual groups may reflect states of transient hyperalgesia in some animals, which has been reported during reinnervation after crush nerve injuries (Vogelaar et al., 2004; Casals-Díaz et al., 2009).

**Morphological evaluation of regenerated nerves**

Regenerated tibial nerves taken just proximal to the ankle at the end of follow-up were analyzed under light microscopy. Transverse nerve sections showed the typical appearance of regenerating nerves (Figs. 4B–E), characterized by the presence of myelinated fibers of small and medium size, clustered in small fascicles, and an enlarged area of connective matrix. Nerves of groups ESa, TR and ES+TR showed a higher density of myelinated fibers compared to nerves from groups C and ESc (Fig. 4). The estimated number of myelinated regenerated fibers (Fig. 5) was about 4555±269 and 4603±943 in groups ESa and TR respectively, whereas the control group had a mean of 2403±196 and group ESc 3132±950. Combination of electrical stimulation and treadmill resulted in a slightly higher number of regenerated fibers (5124±404) than in groups ESa and TR and significantly higher than in group C. The number of regenerated fibers in groups that received ESa and/or TR was higher than in intact nerves (2780±32), indicating sustained regenerative sprouting.

**Discussion**

In this study we compared the effects of different patterns of activity, acute or daily electrical stimulation, treadmill training and combination of acute electrical stimulation with treadmill, on the functional and histological recovery and the modulation of central plasticity after sciatic nerve section and repair in the rat. Application of brief electrical stimulation to the injured nerve enhanced motor reinnervation, and increased the number of regenerated axons distal to the lesion. In contrast, when electrical stimulation was applied daily after the lesion for 4 weeks, regeneration was not improved compared to a control (non-stimulated) group. This suggests that chronic electrical stimulation could be detrimental for axonal regeneration. Interestingly, both protocols of electrical stimulation reduced the hyperreflexia observed after peripheral nerve lesion, as demonstrated by the reduced H/M ratio in the TA muscle compared to the control group, although chronic stimulation did not produce such a modulatory effect in the distal plantar muscles. With four weeks of a mild regimen of treadmill training, functional recovery was similar to the acute electrical stimulation group, and there was also a reduction of the H/M ratio. Combining ESa with treadmill training significantly enhanced the onset of muscle reinnervation, although motor recovery at the end of follow-up was similar to that observed when each treatment was applied individually.

**Electrical stimulation to promote nerve regeneration**

In agreement with a previous work from our laboratory (Vivó et al., 2008), we found here that acute application of electrical stimulation on the injured rat sciatic nerve promotes motor reinnervation and slightly sensory recovery. This beneficial effect was lost when the same stimulation protocol was applied daily for 4 weeks. We decided to apply daily electrical stimulation postoperation to assess if it might improve the effects of a single stimulation session, and enhance the modulation of spinal reflex facilitation. Previous works comparing different periods of stimulation after nerve injury have shown controversial effects in promoting axonal regeneration. Similar effects were found between 2-week or 1-week periods and only 1 h of continuous stimulation at 20 Hz applied by means of an implanted stimulator to the sectioned and repaired femoral nerve in rats, resulting in significant acceleration of motor axons regeneration and preferential motor reinnervation (Al-Majed et al., 2000a). In contrast, the same group reported that stimulation for 1 h almost doubled the number of sensory neurons that regressed into the femoral muscle and cutaneous branches after nerve repair, whereas stimulation for periods of 1, 7 or 14 days was not effective (Geremia et al., 2007). Recently, it has been reported that daily transcutaneous electrical stimulation lead to delayed regeneration after a crush lesion of the sciatic nerve in the mouse, the effects being more pronounced with high- (100 Hz) than with low-frequency (4 Hz) stimulation (Baptista et al., 2008). Chronic electrical stimulation delivered up to 8 h per day to chronically axotomized neurons slightly spared the initial axonal atrophy but was counterproductive over long periods exacerbating the atrophy, possibly by affecting the synthesis and transport of cytoskeletal proteins (Gordon et al., 1991).

On another hand, both acute and chronic electrical stimulation reduced the hyperreflexia (estimated by the H/M ratio) observed in the untreated animals after lesion (Valero-Cabré and Navarro, 2001). This may suggest that electrical stimulation promotes nerve regeneration and modulates spinal plastic changes by different mechanisms of action. The pro-regenerative effects of electrical stimulation have been related with an early increased expression of regeneration-related genes and neurotrophins in the axotomized neurons (Al-Majed et al., 2004; English et al., 2007; Geremia et al., 2007). Thus, electrical stimulation partially mimics the cellular response triggered by axotomy, as it accelerates that the stimulated neurons switch its transmitter state to a regenerative state. However, electrical stimulation of intact nerves does not lead to regenerative changes as strong as axotomy (Mader et al., 2004; Udina et al., 2008). Interestingly, electrical stimulation reverses the hyperreflexia induced by nerve injury. A recent study showed that electrical stimulation (1 h, 20 Hz) applied just after injury but not later modulates post–axotomy hyperreflexia by altering retrograde axonal transport of signals triggered by the axotomy (Bichler et al., 2007). Therefore, it seems that electrical stimulation would modulate spinal reflex changes post-injury by blocking retrograde signal transport contrarily to antidromic excitation of the axon that “primes” the axotomized neuron and leads to accelerated regenerative sprout after lesion. The early neuronal response to injury is triggered by the rapid ion influx at the injury that propagates retrogradely to the soma (Berdan et al., 1993; Mandolesi et al., 2004). Later on, signals are conducted through retrograde transport. Some of these signals are negative (interruption of the normal supply of trophic factors), but others are de novo synthesized or modified proteins from the axon injury site (Ambron and Walters, 1996; Perlson et al., 2004). Therefore, when applying daily electrical stimulation, retrograde transport might be repeatedly impaired and the capacity of the neuron to regenerate reduced.

**Treadmill training to promote nerve regeneration**

When rats were trained in a treadmill for 4 weeks after the injury, muscle reinnervation was also enhanced. This is in agreement with previous studies that showed that mild exercise enhances axonal regeneration. Continuous low-intensity treadmill training for 1 h/day or intermittent high-intensity training increased the length of regenerated axons after autograft repair of the peroneal nerve in mice (Sabatier et al., 2008). Increased activity also promoted motor and sensory functional recovery after nerve crush and section lesions in rats (Van Meeteren et al., 1997; Marqueste et al., 2004). Running exercise during the denervation and reinnervation period has also been shown to have beneficial effects on muscle functional properties (Marqueste et al., 2004). When applied 7 days before the lesion, exercise appears to enhance the condition of the sciatic nerve, with less axon injury and more axon regeneration (Molteni et al., 2004). In contrast, other studies have shown deleterious effects in axon regeneration after intense swimming or treadmill exercise (Herbison et al., 1974a, 1980b; Guttmann and Jakovle, 1963; Van Meeteren et al., 1998). The type, timing and
intensity of training play important roles in the effects of exercise on regeneration and functional recovery after traumatic nerve injuries. On the one hand, the success of rehabilitation protocols is task-dependent, and unspecific training can be even detrimental. On another hand, high frequency or high speed training could cause muscle damage (Herbison et al., 1980a,b; Van Meeteren et al., 1998). With the mild protocol of exercise used in this study (treadmill training at low speed), we found enhanced reinnervation and increased motor nerve conduction velocity (showed here by shorter onset latencies of the M waves) as already reported in other studies (Van Meeteren et al., 1997; Marquete et al., 2004; Sabatier et al., 2008).

Motor recovery and shortening of M wave latencies were similarly enhanced by treadmill training and acute electrical stimulation (see Fig. 1). Both electrical stimulation and exercise share some common mechanisms of action. Indeed, both types of activity have been related to increased expression of neurotrophin BDNF and its receptor trkB (Al-Majed et al., 2000b; Vaynman and Gómez-Pinilla, 2005). The presence of trkB seems crucial to sustain axonal regeneration (Boyd and Gordon, 2001), although the role of BDNF in nerve regeneration is controversial in the literature. Deprivation of endogenous BDNF results in impairment of growth and myelination of regenerating axons (Zhang et al., 2000). A recent study showed that local continuous release of BDNF increased axonal growth over time and induced faster nerve regeneration in rats after sciatic nerve resection and tube repair (Vögelin et al., 2006). In contrast, BDNF was not effective in promoting motor axon regeneration after cut and acute repair of the sciatic nerve (Boyd and Gordon, 2002), but it enhanced axonal regeneration in a dose dependent manner when applied to a model of chronic axotomy, suggesting that the effects of BDNF may depend on the dose and also on the severity of the lesion, being less/not effective when applied at high doses or after mild lesions. On another hand, BDNF has also been highlighted as a key mediator of the capacity of exercise to modulate neural plasticity (Vaynman and Gómez-Pinilla, 2005). Exercise can also influence the injured neurons by stimulating sensory afferents and exerting positive effects in cell circuitry (Molteni et al., 2004). Even during the denervation period, active exercise stimulates muscle afferents of proximal innervated muscles, which may influence the axotomized motoneurons by normally silent spinal synaptic connections (Koerber et al., 2006). In fact, manual sensory stimulation of the injured facial nerve territory improves functional recovery in rats, indicating that activation of the intact sensory afferents could enhance motor regeneration (Angelov et al., 2007). Finally, exercise can facilitate the expression of signals from the muscle to the innervating axons (Pachtar and Eberstein, 1996).

Electrical stimulation combined with treadmill training enhances regeneration

Combination of acute electrical stimulation with exercise improved nerve regeneration, as shown by the higher levels of muscle reinnervation and shorter latencies at early time points when compared with the other groups. Thus, both types of activity were synergistic during the early stage of regeneration. It can be hypothesized that the initial priming effect of electrical stimulation was added to the effects of daily exercise in enhancing axonal elongation and thus the onset and degree of target reinnervation. The pro-regenerative effects of both treatments are neurotrophin-dependent (see above), whereas exercise may also contribute by the patterned activation of afferent inputs. Nevertheless, at the end of follow-up, the degree of motor reinnervation was similar in the three groups ESa, TR and ES+TR. Regenerating motor fibers that reach the muscle are capable of making collateral and terminal branches reinnervating larger motor units than normal to compensate for a reduced number of regenerated axons (Brown et al., 1980; Rafuse and Gordon, 1996). Therefore, at late stages of regeneration electrophy-

siological and muscle tension measurements of the reinnervated muscles may not reflect slight differences in the number of regenerated axons and the rate of regeneration, and thus the differences between groups may be reduced. The histological findings agree with our functional evaluation, since the groups with improved motor recovery (ESa and TR) also had higher numbers of myelinated fibers in the tibial nerve, although only the group with combined treatment (ES+TR) had significantly more regenerated axons than the control group. This increased number of regenerated fibers is consistent with an enhancement of regeneration and a shortening of the staggered regrowth, reported to be induced by electrical stimulation (Al-Majed et al., 2000a; Brushart et al., 2002). Since each injured axon gives several regenerating sprouts in the nerve trunk (Horch and Lisney, 1981; Mackinnon et al., 1991), the marked increase of myelinated axons in these groups (above the number in intact nerves) supports the view that activity enhances growth and sprouting of regenerating axons (Sabatier et al., 2008), and that these effects are maintained for a long distance from the injury.

Conclusion

The results of this study further demonstrate that acute electrical stimulation is able to accelerate axonal regeneration and enhance muscle reinnervation after sciatic nerve injury. There was a synergistic effect when acute stimulation was combined with moderate intensity active exercise only in the early phase of regeneration, suggesting additive mechanisms involved in the two treatments, but no need for maintaining activity on the long term. Our results also show an activity-dependent modulation of spinal hyperreflexia by electrical stimulation and treadmill training. These findings point out to the interest of combined activity-dependent therapies for promoting functional recovery after peripheral nerve injuries.

Acknowledgments

This research was supported by grant CP-FP-INFOS 224012 (TIME project) from the EC, grants PI060201 and PI080598 and CIBERNED funds from the Fondo de Investigación Sanitaria of Spain.

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