Experimental Neurology 211 (2008) 489–493

Contents lists available at ScienceDirect

Experimental Neurology

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

Treadmill training promotes axon regeneration in injured peripheral nerves

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ARTICLE INFO

Article history:
Received 8 October 2007
Revised 25 January 2008
Accepted 16 February 2008
Available online 5 March 2008

Keywords:
Exercise
Recovery
Yellow fluorescent protein
Mouse
Nerve graft

ABSTRACT

Physical activity after spinal cord injury promotes improvements in motor function, but its effects following peripheral nerve injury are less clear. Although axons in peripheral nerves are known to regenerate better than those in the CNS, methods of accelerating regeneration are needed due to the slow overall rate of growth. Therefore we studied the effect of two weeks of treadmill locomotion on the growth of regenerating axons in peripheral nerves following injury. The common fibular nerves of *thy-1-YFP-H* mice, in which a subset of axons in peripheral nerves express yellow fluorescent protein (YFP), were cut and repaired with allografts from non-fluorescent littermates, and then harvested two weeks later. Mice were divided into groups of low-intensity continuous training (CT, 60 min), low-intensity interval training (IT; one group, 10 reps, 20 min total), and high-intensity IT (three groups, 2, 4, and 10 reps). One repetition consisted of 2 min of running and 5 min of rest. Sixty minutes of CT resulted in the highest exercise volume, whereas 2 reps of IT resulted in the lowest volume of exercise. The lengths of regenerating YFP^+ axons were measured in images of longitudinal optical sections of nerves. Axon profiles were significantly longer than control in all exercise groups except the low-intensity IT group. In the CT group and the high-intensity IT groups that trained with 4 or 10 repetitions axons were more than twice as long as unexercised controls. The number of intervals did not impact axon elongation. Axon sprouting was enhanced in IT groups but not the CT group. Thus exercise, even in very small quantities, increases axon elongation in injured peripheral nerves whereas continuous exercise resulting in higher volume (total steps) may have no net impact on axon sprouting.

Introduction

Exercise has been shown to improve motor function following spinal cord injury both in animal models and in clinical studies (Edgerton et al., 1997, Hutchinson et al., 2004, Skinner et al., 1996). The effect of exercise on recovery from peripheral nerve injury has received less attention. Damaged axons in peripheral nerves are capable of significant regeneration but functional recovery in human patients with peripheral nerve injuries is poor (Brushart, 1998). The reason most often cited for poor recovery is that axon regeneration is slow (Fawcett and Keynes, 1990). Seven days of running-wheel training prior to peripheral nerve injury in rats has been found to increase afferent axonal outgrowth as measured from cultured DRG neurons (Molteni et al., 2004). The extent of enhancement was related to the extent of running-wheel usage. Pre-injury exercise may "prime" adult dorsal root ganglion neurons for increased axon regeneration. In another recent study, treadmill training was found to enhance sensory functional recovery, as measured from nerve recordings (Marqueste et al., 2004). However, there is little direct evidence of an effect of post-injury exercise on axon regeneration in injured peripheral nerves.

The data from studies of electrical stimulation, neurotrophins, and peripheral nerve injury are encouraging. If the proximal stump of a transected nerve is stimulated for as little as 1 h at the time of surgical repair, axon regeneration is enhanced (Al-Majed et al., 2000a,b; English et al., 2006, 2007). This enhancement is associated with an increase in BDNF and trkB in the regenerating neurons (Al-Majed et al., 2000a,b) and is dependent on neuronal neurotrophins (English et al., 2006, 2007). Recent experiments have also shown that exercise affects an increase in neurotrophins (e.g., BDNF) (Berchtold et al., 2005, Gomez-Pinilla et al., 2002) and that neurotrophins are required for even control levels of axon regeneration after peripheral nerve injury (English et al., 2005). Therefore, there is sufficient background to hypothesize that exercise should also enhance axon regeneration after peripheral nerve injury.

Treadmill training is readily applied and can be used by both laboratory animals and human subjects. In clinical studies of exercise with human subjects, continuous treadmill locomotion at a moderate to brisk pace is commonly used. When allowed to exercise voluntarily, laboratory mice utilize a markedly different pattern. This pattern is characterized by repeated short-duration runs (2 min) at speeds that are close to maximum, with rest periods interspersed between runs (De Bono et al., 2006), a form of interval training. In this study we provide direct evidence that treadmill training enhances the growth of regenerating axons in injured peripheral nerves. We also show that there are differences in the effects of these two forms of treadmill training on axon regeneration in the first two weeks following injury.

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doi:10.1016/j.expneurol.2008.02.013

A preliminary report of some of these findings has been made (English et al., 2006, 2007).

Methods

Animals and surgical procedures

All experimental procedures conformed to the Guidelines for the Use of Animals in Research of the Society for Neuroscience and were approved by the Institutional Animal Care and Use Committee of Emory University. All experiments were conducted using *thy-1-YFP-H* mice (Feng et al., 2000) on a C57BL/6J background. The *thy-1-YFP-H* mice are maintained as heterozygotes (YFP^+), so that half of all litters born to YFP^+ mice will not contain the transgene. In these transgenic mice, yellow fluorescent protein is present in about 10% of all axons in peripheral nerves, enabling visualization of individual axons using fluorescence microscopy. Mice positive for the transgene (YFP^+) were anesthetized with pentobarbital (90 mg/kg; IP) and the common fibular (CF) nerve was exposed and cut with sharp scissors. The cut nerve was then repaired with a short (3 mm) segment of the CF nerve obtained from a WT littermate of the host mouse that was negative for the transgene. This nerve allograft and the cut ends of the CF nerve in the host mouse were carefully arranged on a short length of Gore-Tex tubing that had been cut in half longitudinally. The cut ends of the nerves were aligned as much as possible with those of the graft, placing the proximal stump of the host nerve with the proximal end of the graft from the donor, and similarly apposing the distal end of the graft with the original distal stump of the cut nerve. It was then secured with a 1:1 mixture of fibrin (3 μL, E.C. 2325986) and fibronectin (3 μL, E.C. 2891492), paired with an equal amount of thrombin (6 μL, E.C. 3.4.21.5) (English et al., 2005). All of these reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). The WT graft used to repair the cut nerve serves as a dark background against which regenerating YFP^+ axons can be visualized.

Exercise

Treadmill training began on the third post operative day. In the CT group ($n=5$), training consisted of 1 h of continuous locomotion at a treadmill speed of 10 m/min, with no incline, applied five days per week for two weeks. All mice had been acclimated to the treadmill for 5 to 10 min twice prior to surgery and none had been on the treadmill for at least 3 days before nerve transection. Mice ran on the treadmill at this speed with little or no persuasion, even on the third post operative day. When mice exercise voluntarily, they do not run continuously. They run at near-maximal speeds for short periods separated by rest periods (De Bono et al., 2006). Therefore, mice in the IT group were run at a faster speed (20 m/min) for 2 min at a time, separated by 5-min rest periods. Training for IT groups consisted of two ($n=4$ mice), four ($n=3$), or ten repetitions ($n=4$) per day. Although the intensity of exercise (steps per unit time) was higher in the high-intensity IT groups, the volume of exercise (total step number) was smaller for these animals due to less total time spent running on the belt. A treadmill speed of 10 m/min results in a step cycle frequency of 147 steps per minute (unpublished findings). Running continuously at this speed for 60 min results in a total volume of 8620 steps. A treadmill speed of 20 m/min results in a step cycle frequency of 295 steps per minute (unpublished findings). Even 10 repetitions at 20 m/min results in a total volume of only 5900 steps. Two repetitions at 20 m/min results in only approximately 1180 steps. Air jets were used judiciously as encouragement for IT mice to keep up with the belt. Mice in the control group remained caged for two weeks after nerve transection and did not receive treadmill training. Results from an additional group of mice that were exposed to 10 repetitions at 10 m/min ($n=3$) in preparing for the study are also reported because these results contrast those from both 10 m/min CT and 20 m/min IT.

Mice subjected to common fibular nerve transection were able to run at 10 m/min for 1 h continuously and at 20 m/min intermittently beginning three days after surgery, despite loss of the ability to dorsiflex the ankle. Exercise capacity in mice varies with strain (Billat et al., 2005, Lerman et al., 2002), with C57BL/6J mice ranking poorly in aerobic performance amongst other strains (Lerman et al., 2002, Lightfoot et al., 2001). The intensity used in this study during high-intensity IT was close to that associated with maximal oxygen consumption for healthy C57BL/6J mice (i.e., 25 m/min, (Schefer and Talan, 1996)). Lerman et al. found that maximal treadmill speed for C57BL/6J mice was only 22.2 m/min (Lerman et al., 2002). Mice evaluated in this study were able to maintain apparently close to maximal running speed for two-min blocks of time and for multiple repetitions despite a significant physical limitation and with little encouragement. Although kinematic data were not collected, it was apparent that locomotor movements were compromised in these mice. Following transection of the common fibular nerve, function of ankle dorsiflexors muscles is lost.

Tissue harvesting and microscopy

At the end of the prescribed exercise period, mice were euthanized with an overdose of pentobarbital (150 mg/kg) and perfused through the heart with saline, followed by periodate-lysate-paraformaldehyde fixative (McLean and Nakane, 1974). The entire sciatic nerve was removed, placed on a microscope slide, and cover slipped using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Edges of the cover slip were sealed with nail polish. Mounted nerves were viewed with a laser scanning confocal microscope (Zeiss LSM510). Horizontal optical sections 10 μm thick were obtained through the full extent of the graft. Stacks of images of adjacent microscope fields were stitched together using Adobe Photoshop. The result was a stack of stitched images containing the entire repaired CF nerve. All visible YFP^+ axon profiles in these stacks of optical sections were measured from their distal tips to the surgical repair site using Image Pro Plus (Media Cybernetics, Silver Spring, MD USA).

Data analysis

Cumulative histograms of the distributions of axon profile lengths in these nerves were constructed with a bin size of 100 μm. Averages of these histograms were computed for each group. Statistical groups consisted of four (2-rep group), three (4-rep group), and four (4-rep group) nerve grafts in which axons were measured. The distribution of axon profile lengths measured in nerve allografts is not statistically normal (English et al., 2005, Groves et al., 2005), a requirement for the use of parametric tests such as t-tests or analysis of variance (ANOVA). Thus, statistical significance of differences between treatment groups in these experiments was evaluated using two different approaches. First, a nonparametric statistical test for independent samples, the Mann–Whitney *U* test, was used to determine whether the distributions of axon profile lengths measured in different treatment groups are from the same population. This method makes no assumption about the nature of these distributions. Second, the median axon profile length was determined in each nerve studied in each mouse. These values were found to be distributed normally, but the variances of different samples were not homogeneous (Levene's test, $p>0.05$), also a requirement for use of parametric statistics. Therefore, they were log-transformed before evaluating the significance of differences between the experimental groups using ANOVA and post hoc (Fisher Least Significant Differences (LSD)) testing. The numbers of axon profiles proximal and distal to the surgical repair site were counted in each nerve graph studied. The ratio of those counts, the number of distal profiles per proximal profile, was calculated as a sprouting index. This index is used as a global measure of the amount of regenerative sprouting that had occurred in the 2-week survival