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Delayed Treadmill Training and Synaptic Stripping of Axotomized Motoneurons

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Delayed Treadmill Training and Synaptic Stripping of Axotomized Motoneurons

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B.S., Biology, 2010

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An abstract of
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Abstract

Delayed Treadmill Training and Synaptic Stripping of Axotomized Motoneurons
By Natalee Kay Wilson

When a peripheral nerve is injured, sensory and motor axons in the periphery are disconnected from the central nervous system (CNS). In addition, synaptic inputs withdraw from the somata and proximal dendrites of the injured motoneuron, a process known as synaptic stripping. After moderate daily treadmill training, the expected loss of synaptic inputs onto axotomized motoneurons due to synaptic stripping is not observed. Whether this effect of treadmill training is due to the prevention of the withdrawal of synaptic inputs or a restoration of synapses is not clear. In this study, the onset of treadmill training was delayed until one week following peripheral nerve transection. At that time, substantial withdrawal of synapses from the axotomized motoneurons had occurred. The ability of treadmill training to restore synapses onto these motoneurons was evaluated. Synaptic coverage was assayed by measuring the proportion of somata and proximal dendrites of retrogradely labeled motoneurons covered by terminals immunopositive for markers of excitatory VGLUT1 and inhibitory GAD67 synapses. The coverage of axotomized motoneurons by terminals containing VGLUT1 in delayed trained mice was similar to that found in intact controls, suggesting a restoration of VGLUT1+ boutons onto the somata and proximal dendrites of the motoneuron. The expected loss of GAD67+ boutons that was observed in axotomized motoneurons in untrained mice was not found in trained mice, strongly suggesting that the delayed treadmill training had a restorative effect. Thus, if treadmill training is delayed by one week after nerve transection, the anticipated loss of synaptic coverage on the axotomized motoneurons does not occur. This is evidence for treadmill training having a restorative effect on the stripped synapses.

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Introduction

Injured peripheral nerves are capable of regeneration and reinnervation of their targets. Despite this capacity, patients with peripheral nerve injuries have poor functional recovery. Functional recovery never reaches a level of functional recovery comparable to before injury (Frostick et al., 1998). Incorrect reinnervation of the targets of regenerating axons, slow axon regeneration times (regenerating axons in peripheral nerves grow at a rate of 1mm per day) leading to degeneration of the distal regions of the injured nerves, and changes in the circuitry in the central nervous system all may contribute to this poor functional recovery. Traumatic injuries to peripheral nerves result in changes in the circuitry of the central nervous system (CNS), including the spinal cord and regions of the brain (Navarro et al., 2007). The location of motoneurons and spinal circuitry of various types of excitatory and inhibitory synapses are shown in Figure 1. One such change within the CNS resulting from peripheral nerve injury is known as synaptic stripping (Kreutzberg et al, 1968; Oliveira et al., 2008). Synaptic stripping is the withdrawal of synaptic inputs from the somata and proximal dendrites of motoneurons following axotomy. Unlike the peripheral nervous system where axons can regenerate, axotomy-induced synaptic stripping persists for long periods (Alvarez et al, 2010; Chen et al, 2010; Hughes et al, 2005). Synaptic stripping thus represents a potential factor relating to the poor functional recovery that is characteristic of patients with peripheral nerve injuries.

Synaptic stripping had been shown to occur in various animal models including mice, rats, rabbits (Hamberger et al., 1970), and cats (Lindå et al, 1992). The exact mechanism of synaptic stripping is not known. Synaptic stripping has been suggested to occur as the result of a Major Histocompatability Complex I (MHCI) immune response (Oliveira and Culheim et al., 2008) and is accompanied by a robust glial response, both microglial (Kreutzberg et al., 1968) and astrocytes (Graeber et al., 1986). How these

cellular effects might contribute to the withdrawal of synapses from the axotomized motoneurons is not clear but this association is strong. Transection of both sensory and motor axons may contribute to synaptic stripping. Signals from damaged sensory inputs in the muscle may lead to withdrawal of primary afferent terminals (Mendell et al., 2001) and thus contribute to synaptic stripping. Stripping of synaptic terminals from brainstem motoneurons occurs after transection of the facial nerve, which does not contain sensory axons, has been interpreted as evidence that the withdrawal of terminals is the result of a loss of retrograde neurotrophic molecules from the axotomized motoneurons (Titmus et al., 1990).

This latter mechanism of synaptic stripping involves neurotrophins that are thought to be released from the somata and proximal dendrites of motoneurons and contribute to the stabilization of synaptic terminals (Davis-Lopez de Carrizosa et al., 2009). The sources of these retrograde signaling molecules may be intrinsic (motoneuron derived) and/or extrinsic (target derived) to the motoneuron. Neurotrophins are a family of four proteins: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Among many functions attributed to neurotrophins, they are thought to mediate plasticity within the CNS (Mendell et al., 2001). Synaptic plasticity following peripheral nerve injury may be dependent on neurotrophins. Axotomy is thought to result in a lack of neurotrophic support to the motoneuron resulting in the signal for withdrawal of synaptic terminals (Titmus et al., 1990). Neurotrophins may also promote synaptic survival. Muscle derived trophic molecules provide the synapses with the neurotrophic support (de la Cruz et al., 1996). After axotomy, these retrograde factors are no longer available to support the synapses and synaptic stripping results.

An example of neurotrophins inducing plasticity within the CNS is BDNF. If intact mice are made null for the gene for BDNF as adults, nearly half of the synaptic terminals

on spinal motoneurons are withdrawn (Krakowiak et al., 2011). When the proximal stump of a cut eye muscle nerve was treated with BDNF, neurotrophin-3, or a mixture of both neurotrophins, the synaptic stripping observed without the treatment was not observed. Treatment with the neurotrophin NGF similarly restored synaptic inputs on axotomized motoneurons (Davis-Lopez de Carrizosa et al., 2010). These findings are consistent with the idea that synaptic inputs onto motoneurons are maintained by retrograde neurotrophic signaling, especially involving BDNF.

Intrinsic factors, changes within the motoneuron itself, may affect the process of synaptic stripping. There is a selective stripping of excitatory boutons after peripheral nerve axotomy. These excitatory boutons have spherical vesicles, which is characteristic of excitatory synaptic terminals (Lindå et al., 2000). Inhibitory terminals, containing flattened or pleiomorphic vesicles (Triller and Korn, 1981), are much less often stripped. There is a selective stripping of excitatory, glutamatergic terminals compared to inhibitory GABAergic and glycinergic terminals after nerve injury (Kreutzburg et al., 1968). The preferential withdrawal of excitatory terminals when the facial nerve is transected is evidence that damage to primary afferent axons in the cut nerves (Mendall et al, 2001) or retrograde signals to axotomized motoneurons represents a potential source of synaptic stripping.

Exercise, such as treadmill training, is a method in which to stimulate the production of endogenous neurotrophins and evaluate their effect on CNS plasticity and synaptic stripping (Gomez-Pinilla et al., 2001). Exercise has been shown to induce CNS plasticity as the result of regulation of neurotrophins. Daily exercise increased the amount of BDNF within the spinal cords of mice (Gomez-Pinilla et al., 2002; Wood et al., 2011). It has been shown that daily treadmill training enhances axon regeneration after peripheral nerve injury (Sabatier et al., 2008). A similar treadmill training exercise regime was used to study the effects of treadmill training on synaptic stripping. After only one week

following transection of peripheral nerve, a significant (45-50%) reduction in the coverage of motoneuron somata by synaptic inputs is found. If mice were trained daily for two weeks, beginning three days after nerve transection, the anticipated decrease in synaptic coverage around the somata and proximal dendrites resulting from synaptic stripping was no longer observed (Krakowiak et al., 2011). Treadmill training of BDNF KO mice increased the amount of synaptic coverage comparable to intact BDNF KO mice, indicating that at least part of the treadmill training effect on the stripping of synapses was BDNF-independent (Krakowiak et al., 2011). BDNF may be required for the maintenance of synaptic inputs, but there are other factors involved as well. Whether the mechanism of this treadmill training effect is a blockage of axotomy-induced stripping or the restoration of recently stripped synaptic inputs is not clear. The goal of this study is to differentiate between these two mechanisms by investigating the effects of delayed treadmill training on synaptic stripping on axotomized motoneurons.

The contrast between these two possible mechanisms is important to understanding what kind of effect exercise has on synaptic withdrawal. Since it has been shown that treadmill training affects the process of synaptic stripping, it is crucial to determine whether the effect is preventative or restorative. Treadmill training might induce a change in the motoneurons to produce or strengthen signals, such as neurotrophins, that might block the detachment of the synapses. This would describe a preventative mechanism whereby the synapses are blocked from withdrawing from the motoneuron. Alternatively, the mechanism might be restorative. Once the synapses have pulled away from the axotomized motoneuron, treadmill training may induce a signal to restore the inputs back onto the motoneuron. In order to investigate the nature of this effect of treadmill training, mice began training one week after peripheral nerve transection. At that time, a significant amount of synaptic withdrawal has occurred (Krakowiak et al., 2011). If the treadmill training induces only a preventative effect, the

delayed onset of exercise would not produce an effect; the synapses would already be withdrawn. A reduction in synaptic coverage around the axotomized motoneuron would remain, and the amount of synaptic coverage of the motoneuron would be similar to that seen in axotomized, untrained mice. Such an outcome also would indicate that treadmill training would have to be more immediate in order to have an effect. If delayed treadmill training induced a restoration of stripped synapses, a reduction in synaptic coverage would no longer be observed at the end of the training period. The coverage around the motoneuron would be similar to intact controls, indicating that synapses were restored onto the axotomized motoneuron.

The experiments in this project are designed to address the hypothesis that if the onset of treadmill training is delayed until one week after nerve injury, when synapses have been withdrawn, it will have no effect on synaptic coverage around the somata and proximal dendrites of the axotomized motoneurons. Mice were treadmill trained beginning one week following transection of the sciatic nerve, when significant synaptic stripping has already occurred, and then the coverage of the somata of motoneurons by different types of synaptic terminals was evaluated one or two weeks later. The extent of coverage of histological profiles of the somata and proximal dendrites of axotomized motoneurons that is covered by synapses was evaluated using immunofluorescence for specific synaptic vesicles. The amount of synaptic coverage in delayed trained mice was compared to the coverage in intact mice and to data from untrained mice receiving similar nerve injuries. Because treadmill training also might change the relative composition different types of synapses onto motoneurons, this analysis included synaptic terminals from different sources. Glutamate decarboxylase (GAD) is the enzyme responsible for the catalyzation of glutamate to γ-aminobutyric acid (GABA). GABA is the major inhibitory neurotransmitter in the central nervous system. There are two isoforms of GAD in mammals, GAD67 and GAD65. In the spinal cord, GAD67 is

found at synaptic boutons formed mainly from spinal interneurons. The presence of GAD67 in synaptic boutons is considered evidence for the presence of inhibitory synaptic inputs. The vesicular glutamate transporters (VGLUTs) are responsible for loading of the excitatory neurotransmitter glutamate into synaptic vesicles. In the spinal cord, one isoform, VGLUT1, is found nearly exclusively in excitatory, glutamatergic synaptic terminals of primary afferent neurons. These are dorsal root ganglion cells. Immunohistofluorescence was used to evaluate the proportions of the synaptic boutons on motoneurons of each of these types in each of the experimental animal groups.

Methods

Animals:

Experiments were conducted using adult female mice (>2 months old) weighing 17g-30g. All mice used in the following experiments were genotypically wild-type on a C57BL/6J background. All experiments were approved by the Institutional Animal Care and Use Committee of Emory University and were in accordance with the Principles of the Use of Animals in Research of the Society for Neuroscience.

Muscle Injections/Retrograde Labeling:

All procedures performed under aseptic conditions. For all mice, spinal motoneuron cell bodies were retrogradely labeled using cholera toxin B tracer conjugated to Alexafluor 594. Three days prior to nerve transection, mice were anesthetized with isoflurane via nose cone administration and muscles were located visually. Injections of 1μl of cholera toxin B-594 solution (1mg/mL) were made into the lateral gastrocnemius, medial gastrocnemius, and soleus muscles using a 35G injection needle. Following muscle injection and recovery from anesthesia, animals were returned to their cages. Injections were made bilaterally.

Surgical Methods:

Mice underwent survival surgery while anesthetized with isoflurane via nose cone administration. All surgical procedures performed under aseptic conditions. The sciatic nerve was located in the right mid thigh and transected unilaterally using sharp scissors. A 1mm section of the nerve was cut and removed to avoid reinnervation, and the proximal segment of the cut nerve was left disconnected from the distal segment (i.e. unrepaired). On the left sides of each animal, the sciatic nerve remained intact for

comparison. Following nerve transection, all wounds were closed and, once they had recovered from anesthesia, animals were returned to their cages.

There were two delayed training groups in this study. One week following nerve transection, one group of mice was exposed to one week of daily treadmill training. A second group of mice was exposed to two weeks of daily treadmill training, once again after one week post-surgical recovery. Control groups studied included mice with intact sciatic nerves that were not subjected to treadmill training and mice with transected sciatic nerves that were also untrained. The groups of mice used in this study are shown in Table 1. Following their last treadmill training session at the end of the week, mice were euthanized.

Treadmill Training:

A treadmill with enclosed, individual lanes was used for this experiment. Mice were given a 5min acclimation period and monitored during each training session. An interval treadmill training paradigm was used. Mice were run at the relatively fast treadmill speed of 20m/min, 4 repetitions at 2min each (with a 5min rest between each repetition), on a level surface, five days/week. Treadmill training for the two experimental groups began one week after surgery. Group 1 mice were subjected to one week of treadmill training. Group 2 mice were subjected to two weeks of treadmill training.

Immunohistochemistry:

Mice were euthanized with an overdose of pentobarbital (150mg/kg, i.p.) and perfused through the heart with saline followed by periodate-lysate-paraformaldehyde fixative (McLean and Nakane, 1974). Spinal cord segments L3-L5 were harvested and cryoprotected in 20% sucrose. Spinal cords were transversely sectioned on a cryostat

at 20μm. Sections were mounted on Superfrost Plus slides. Incubations and washes were performed using 0.1M phosphate-buffered saline (PBS) containing 0.4% Triton X (PBS-T) and 10% normal goat serum (NGS). Tissue was incubated in PBS-T overnight at 4°C and followed by incubation with various antibodies. An antibody to GAD67 (1:1000) was used to identify those boutons containing this enzyme. Biotinylated anti-mouse secondary antibodies (1:200) were used in combination with with streptavidin conjugated to Alexafluor 488 (1:200) to amplify the binding of these anitbodies. An antibody to the vesicular glutamate transporter 1 (VGLUT1) (1:1000) was used to identify glutamatergic synaptic terminals of primary afferent neurons on motoneurons. A goat anti-guinea pig secondary antibody conjugated to Alexafluor 647 (1:200) was used detect VGLUT1 antibody binding. Rabbit anti-cholera toxin B (1:200) followed by goat anti-Rabbit immunoglobulin conjugated to Alexfluor 594 (1:200) was used to amplify retrograde labeling of motoneurons. Slides were then coverslipped with Entellan. Retrogradely labeled motoneurons (red) with synaptic boutons immunoreactive for VGLUT1 (blue) and GAD67 (green) are shown in Figure 2.

Image Analysis:

A Zeiss LSM510 confocal microscope was used to obtain thin (<1 micrometer) optical sections from cryostat sections through the spinal cord. Images containing twenty retrogradely labeled motoneurons were obtained at high magnification (63x) from each side of the spinal cord of each animal studied. In each animal, the side of the spinal cord contralateral to the side in which the sciatic nerve had been transected was considered an unoperated control. Motoneuron cell bodies were identified by the presence of the fluorescent retrograde label (CTB), as amplified by antibodies conjugated to Alexafluor 594. Only motoneuron profiles containing a distinct nuclear shadow were studied. Using the computer program FIJI (Fiji Is Just ImageJ), brightness and contrast enhancement

was applied to the images to help delineate the cell surface in images containing motoneuron profiles, but no other image processing was performed. The coverage of the somatic surface of each such motoneuron by GABA-ergic (GAD67+) and glutamatergic (VGLUT1+) positive boutons was studied using FIJI. All measurements were made without knowledge of the experimental condition of the neurons being studied. A free-line tool was used to draw a region of interest around the perimeter of the motoneuron profile. The perimeter of the motoneuron was established using only the (red) channel containing the retrograde label in the motoneuron so that only synaptic boutons attached to the soma and proximal dendrites of the motoneuron were included in measurements of immunoreactivity. A plot profile was then generated indicating the intensity of the fluorescence along the perimeter trace. A threshold amount at which the fluorescence was considered to indicate the presence of a synaptic terminal was then established and the proportion of the cell profile perimeter with immunoreactivity exceeding that threshold was calculated. A single retrogradely labeled motonueuron with immunoreactive GAD67 boutons is shown in Figure 3, where letters correlate with letters on the profile plot to represent boutons. Letters on the profile plot indicate location of synaptic boutons as they intersect with the periphery line (region of interest). The grey bar denotes threshold value. Mean values for the percentage of this synaptic coverage were calculated for GABA-ergic, and glutamatergic boutons for each animal in the experimental groups.

Statistical Analysis:

The contralateral side of the spinal cord of each animal was treated as an independent subject. Data for VGLUT1 and GAD67 for each of the groups studied (Table I) were subjected to a one-way analysis of variance (ANOVA). Because in both cases, the omnibus test was significant (VGLUT1, $F_{4,21}$= 4.643, p=0.0076; GAD67, $F_{4,21}$=6.66, p=0.0013), post-hoc paired testing (Fishers least significant difference (LSD))

was conducted. A value of $p<0.05$ was used for significance of differences between groups.

Results

Effect of delayed treadmill training on synaptic coverage by VGLUT1+ terminals.

Nearly all of the excitatory synaptic inputs made onto motoneurons by primary afferent axons contain VGLUT1 (Hughes et al, 2005). The mean percent coverage (±SEM) by excitatory VGLUT1+ terminals on the soma and proximal dendrites of the retrogradely label motoneurons in delayed trained mice is shown in Figure 4. Data from the sides of the spinal cord in which the sciatic nerve was cut are referred to in this figure as Cut. Data from the contralateral side of the spinal cord, the side of the animal where no nerves were cut, are referred to as Uncut. In mice treated with one week of daily treadmill training beginning one week after sciatic nerve transection, the proportion of the surfaces of profiles of retrogradely labeled motoneurons covered by VGLUT1 positive inputs (mean= 7.48%±1.89%) is not significantly different (LSD, p=0.21) from that found on profiles of motoneurons on the contralateral, intact sides of the animals (mean=10.31%±1.08%). In mice treated with two weeks of daily treadmill training beginning one week post nerve transection, the mean percent of the axotomized motoneuron cell surface covered by VGLUT1+ terminals (mean=7.44%±1.25%) was not significantly different (LSD, p=0.05) from that observed on motoneurons in the contralateral, intact side of the spinal cord (mean=11.57%±1.45%). Since a reduction in synaptic coverage is known to occur after one week post nerve transection due to synaptic stripping (Krakowiak et al., 2010), this lack of significant difference between the two sides of these delayed treadmill trained animals is evidence that a restoration of VGLUT1+ synapses around the soma and proximal dendrites the axotomized motoneuron had occurred with treadmill training.

The individual circles in Figure 4 indicate the average percent coverage observed in individual animal cases. Although a substantial variability was observed between the

animals for both the one week and two week treadmill trained groups, no significant differences were found in any paired comparison. The effect of one week of training is similar to two weeks of training. The two sets of data were thus combined.

The effects of delayed treadmill training on the mean percent coverage (±SEM) by VGLUT1 immunopositive excitiatory synaptic boutons surrounding motoneuron somata and proximal dendrites is shown for trained and untrained mice in Figure 5. Data from the Cut and contralateral Uncut sides are shown in comparison to data from Intact controls. Intact controls were intact on both sides of the animal and were not exposed to training (mean=7.64%±1.78%). No significant differences were found between data from intact mice and from motoneurons on the Uncut sides of either untrained (LSD, p=0.84) or trained (LSD, p=0.06) mice. In addition, there was no significant difference between VGLUT1 synaptic coverage on motoneurons on the Uncut sides of untrained and trained mice (LSD, p=0.10). In contrast, percent VGLUT1 coverage of axotomized motoneurons in untrained mice (mean=2.10%±0.77%) is significantly less than all other groups (LSD, p=0.04). This >70% reduction in coverage by VGLUT1+ synaptic terminals represents the expected effect of synaptic withdrawal following peripheral axotomy. This reduction also represents the presumed baseline of synaptic coverage at onset of delayed treadmill training. Percent VGLUT1 coverage on motoneurons on the Cut sides of trained mice (mean=7.46%±1.02%) is significantly higher than on axotomized motoneurons of untrained mice (LSD, p=0.03) and not significantly different from that found in uncut and intact controls (LSD, p=0.92). These results are strong evidence for treadmill training inducing a restorative effect on VGLUT1 positive terminals.

Effect of delayed treadmill training on synaptic stripping of GAD67+ terminals.

The mean percent coverage (±SEM) by inhibitory GAD67+ terminals on the soma and proximal dendrites of the retrogradely labeled motoneurons in delayed trained mice is shown in Figure 6. Data from the sides of the spinal cord in which the sciatic nerve was cut are referred to in this figure as Cut. For comparison, similar data from intact motoneurons on the contralateral (Uncut) sides of the same mice are shown. After one week of delayed treadmill training, the synaptic coverage by GAD67+ boutons (Cut, mean=22.14%±3.61%; Uncut, mean=18.10%±2.16%) is similar on the two sides of the animals. Although a small increase in coverage by GAD67+ boutons is found on the cut sides of these animals, relative to the coverage on intact motoneurons, this increase is not significant (LSD, p=0.47). The synaptic coverage by GAD67+ terminals on axotomized motoneurons in mice exposed to two weeks delayed treadmill training post nerve transection (mean=18.13%±1.99%) also is not significantly different than that on contralateral intact motoneurons (mean=13.57%±2.94%). A slight increase in coverage by GAD67+ boutons in cut animals is noted, but this increase is not statistically significant (LSD, p=0.12). These observations are consistent with a restoration of GAD67+ synapses around the soma and proximal dendrites of the axotomized motoneurons induced by treadmill training.

The effects of delayed treadmill training on the mean percent coverage (±SEM) of GAD67 immunopositive inhibitory synaptic boutons surrounding motoneuron somata and proximal dendrites is shown for trained and untrained mice with cut and contralateral uncut sides are shown in Figure 7. As noted above, because no significant differences were found between one and two week training periods, data for trained mice were pooled. Data from the different experimental groups are shown in comparison to data from intact controls (mean=12.19%±1.94%).

In untrained mice, a significant reduction in coverage by GAD67+ synaptic terminals was noted, compared to intact controls. This result is an expected effect of withdrawal of GAD67+ synapses from axotomy-induced synaptic stripping. This finding in the untrained group represents the presumed GAD67 synaptic coverage at which the animal started after the week delay before training. Delayed treadmill training resulted in a marked increase in GAD67+ synaptic coverage. The percent coverage on motoneurons on the cut sides of trained mice (mean=19.92%±1.94%) is significantly higher than in untrained mice (mean=1.94%±1.64%) (LSD, $p=0.0003$). This increased coverage by GAD67+ boutons in trained mice is also significantly different from that of all of other groups studied (LSD, $p<0.05$). It is thus evidence that any synaptic remodeling initiated by treadmill training has a greater than restorative effect.

On the sides of the untrained mice in which no nerves were cut, there also was a significant reduction in GAD67+ synaptic coverage, relative to intact controls (LSD, $p=0.04$). Thus unilateral nerve transection has an effect on inhibitory synaptic inputs on both sides of the spinal cord. With training, the percent GAD67+ coverage returned to a level similar to that of intact mice (LSD, $p=0.30$), suggesting that treadmill training also influences inhibitory synaptic connections onto motoneurons on both sides of the spinal cord.

Discussion

When a peripheral nerve is injured, sensory and motor inputs in the periphery are subsequently disconnected from the central nervous system (CNS). Synaptic inputs withdraw from the somata and proximal dendrites of the motoneuron in a process known as synaptic stripping (Kreutzberg et al., 1968; Oliveira et al., 2008). A lack of stabilizing neurotrophic support from the axotomized motoneurons may contribute to this withdrawal process (Titmus et al., 1990). Synaptic stripping has been shown to occur in axotomized motoneurons where only motor neurons are affected. The changes induced by axotomy are thought to occur from the motoneuron and not only from signals from axotomized neurons forming inputs onto the motoneuron (Liebermann et al., 1971). This idea also is supported by the observation of stripping of inhibitory boutons (Lindå et al., 2000) that arise from neurons within the central nervous system. In addition, treatment with neurotrophins such as BDNF and NT-3 has been shown to reverse the effects of axotomy-induced synaptic stripping (Davis-Lopez de Carrizosa et al., 2009; Davis-Lopez de Carrizosa et al., 2010). Treadmill training increases the expression of such neurotrophins as BDNF and NT-3 (Gomez-Pinilla et al., 2002) and has also been shown to block the expected loss of synaptic coverage due to synaptic stripping (Krakowiak et al., 2011). Whether the effect of treadmill training was preventing the withdrawal of synaptic inputs or restoring synapses was the primary goal of this experiment.

The main finding of this study is that if treadmill training is delayed by one week after nerve transection, when a significant amount of stripping has occurred, the anticipated loss of synaptic coverage on the axotomized motoneurons does not occur. This is evidence for treadmill training having a restorative effect. The coverage of axotomized motoneurons by terminals containing VGLUT1+ in delayed trained mice was similar to that found in intact controls. This is evidence for the restoration of VGLUT1+ boutons onto the somata and proximal dendrites of the motoneuron. The expected loss

of GAD67+ boutons that was observed in axotomized motoneurons in untrained mice was not found in trained mice, strongly suggesting that the delayed treadmill training had a restorative effect similar to that seen with VGLUT1+ boutons. The effect of treadmill training had the interesting effect of actually *increasing* the proportion of the somata and proximal dendrites of axotomized motoneurons that was in contact with GAD67+ boutons relative to intact controls.

Although both the effects of axotomy to induce synaptic stripping and of treadmill training to restore synaptic coverage to axotomized motoneurons might seem similar between these two types of synapses, differences observed for the different synaptic types might be used to suggest that the mechanisms involved are not the same. There was no significant effect of axotomy on the coverage by VGLUT1+ terminals on contralateral, intact motoneurons, but there was such an effect for GAD67+ terminals. The axotomy induced effect appears to be rescued by treadmill training. This is significant because these are effects seen on the uncut side of the animal suggesting a role of axotomy affecting more than just the side of transection. Training also resulted in an increased coverage by GAD67+ inhibitory synapses on axotomized motoneurons above intact levels whereas VGLU1+ synaptic coverage only increased to intact levels with training.

While the loss of synaptic coverage following axotomy is no longer observed after treadmill training, this was only observed in this study for VGLUT1+ and GAD67+ boutons. The use of the term restorative refers to the return of the percent synaptic coverage on the axotomized motoneurons from the anticipated reduced amount at the onset of training to levels found in intact mice. This effect is observed for both types of synapses, VGLUT1 and GAD67. Although the effect can be termed restorative, the exact mechanism of the effect is not known. The term restoration does not necessarily imply the restored synaptic coverage observed with treadmill training after nerve

transection is due to due new synapse formation in the CNS. This is the most direct explanation, although no data exists to confirm or refute this postulate. Enlargement of retained synaptic terminals might also be a source of the restorative effect. Similarly, whether the restored synapses are the same terminals that were originally withdrawn following nerve transection or newly formed synapses of the same excitatory/inhibitory character is not clear. Until the sources of the restored synaptic connections can be determine, this too will remain to be determined. While it is likely that other types of excitatory and inhibitory synapses would follow the same mechanism, further study would be required to ascertain the effect of axotomy and treadmill training on each specific type of synapse.

As treadmill training has been shown to enhance axon regeneration after peripheral nerve injury (Sabatier et al., 2008) without an increase in the misdirection of regenerating axons (English et al., 2009), it is of interest with respect to its use as a possible enhancement of functional recovery. The plastic changes within the CNS that are induced by peripheral axotomy (Alvarez et al., 2010) and reduction of afferent inputs are likely an important contributor to the poor functional recovery found following peripheral nerve injuries (Frostick et al., 1998). The evidence for a restorative effect of treadmill training on withdrawn synapses might indicate a possible treatment in which recovery from peripheral nerve injury might be enhanced. Further investigation of possibly synaptic reformations would be necessary to determine the potential for treadmill training in recovery from peripheral nerve injuries.

Number of Mice	Nerve Transection	Treadmill Training	Type of Training	Duration of Treadmill Training
n=4	Yes, unilaterally	Yes	Delayed Interval Training	1 week
n=5	Yes, unilaterally	Yes	Delayed Interval Training	2 weeks
n=2	Yes, unilaterally	No	N/A	N/A
n=2	No	No	N/A	N/A

Table 1. Groups of mice used in this study.

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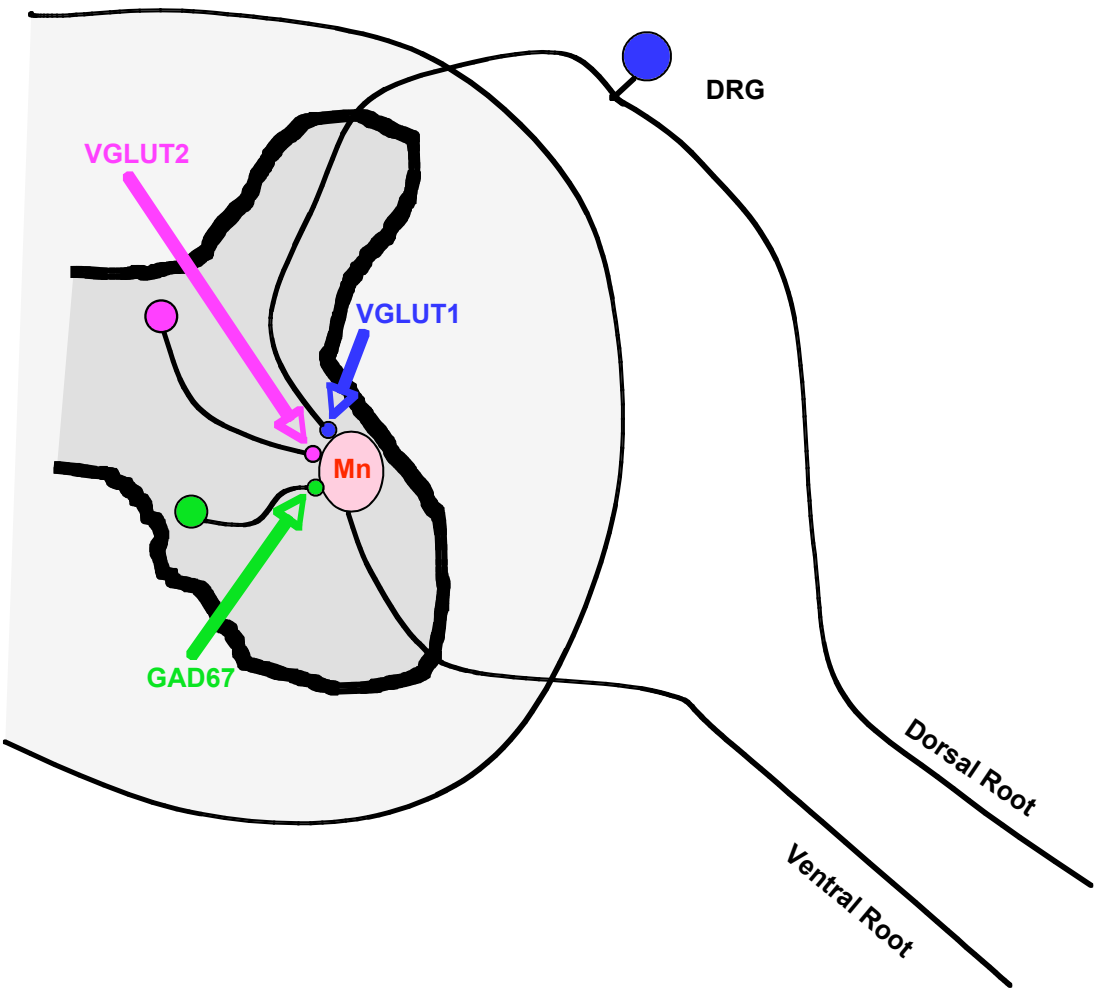

Figure 1. Transverse section of the spinal cord. Figure illustrates location of motoneurons in Lamina IX of the spinal cords and location of various types of synapses.

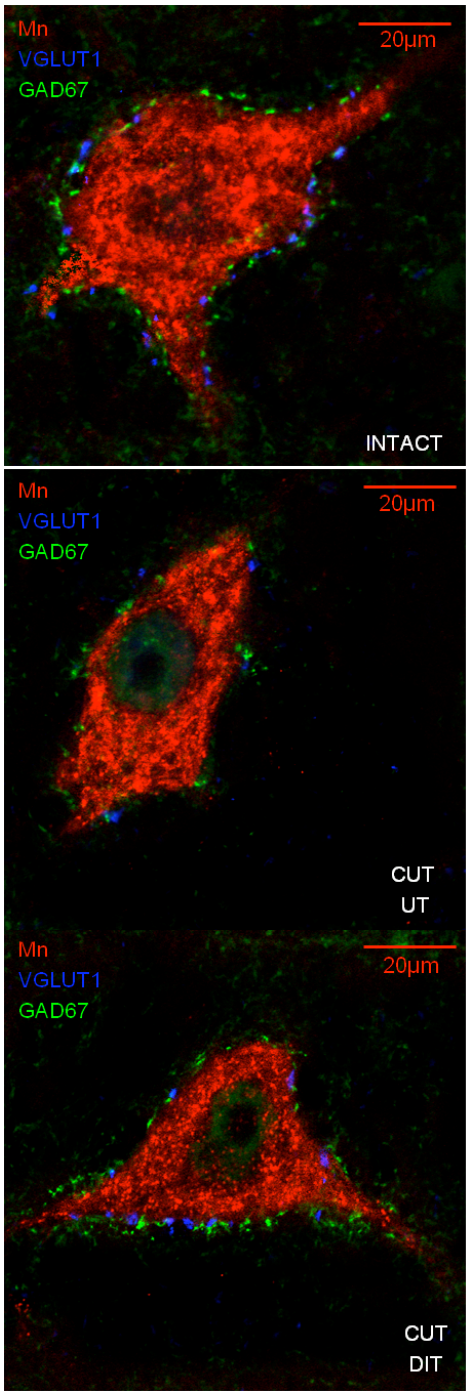

Figure 2. Retrogradely labeled motoneurons (red) with synaptic boutons immunopostive for VGLUT1 (blue) and GAD67 (green). Background has been dimmed (burned) to emphasize boutons around the somata and proximal dendrites of the motoneuron. A. Intact B. CUT (nerve transected) and untrained C. CUT (nerve transected) and delayed treadmill trained

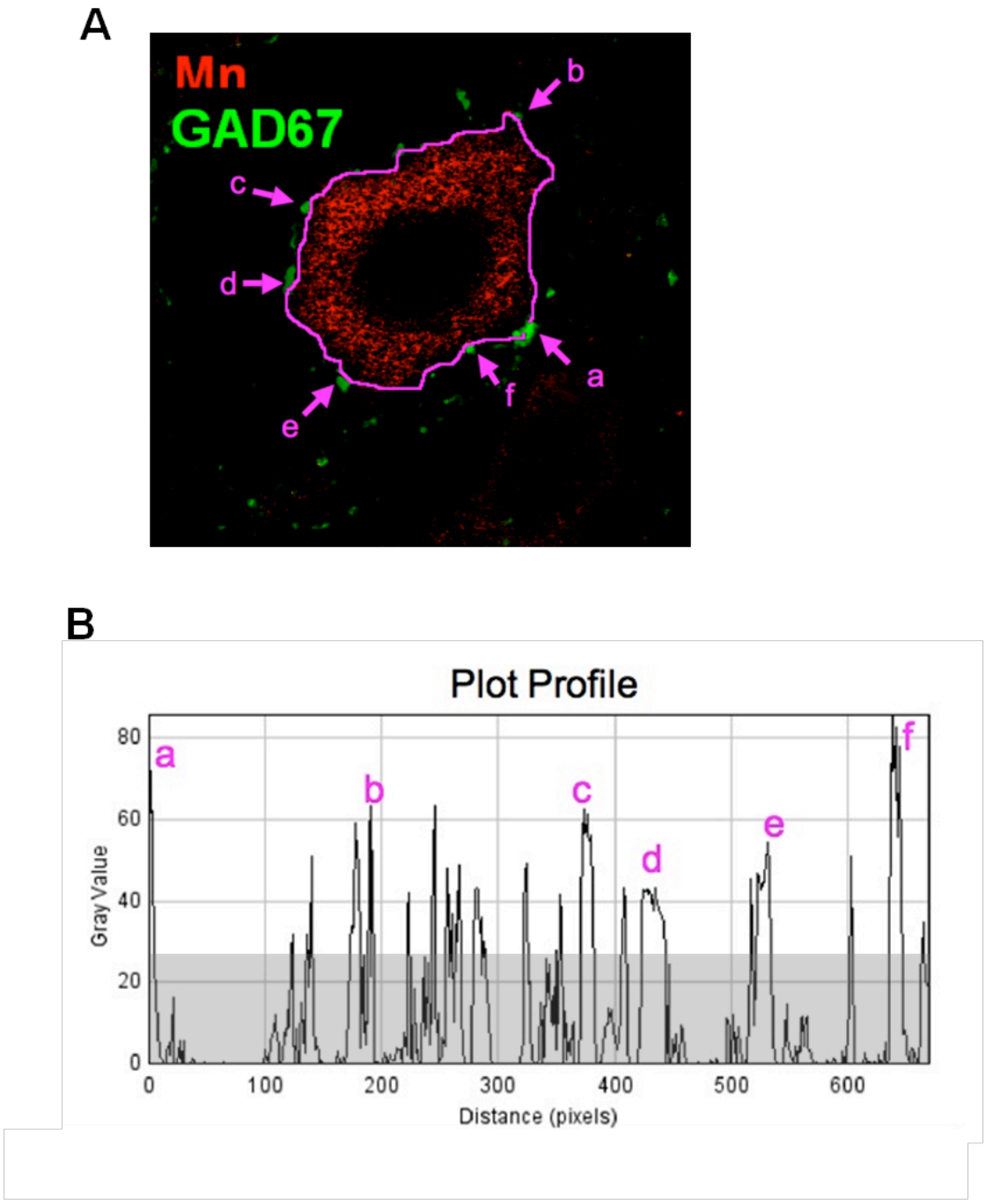

Figure 3. A. Single retrogradely labeled motonueuron with immunopostive GAD67 boutons. Letters correlate with letters on the profile plot to represent boutons. B. Profile plot: Letters indicate location of synaptic boutons as they intersect with the periphery line (region of interest). Grey bar denotes threshold value.

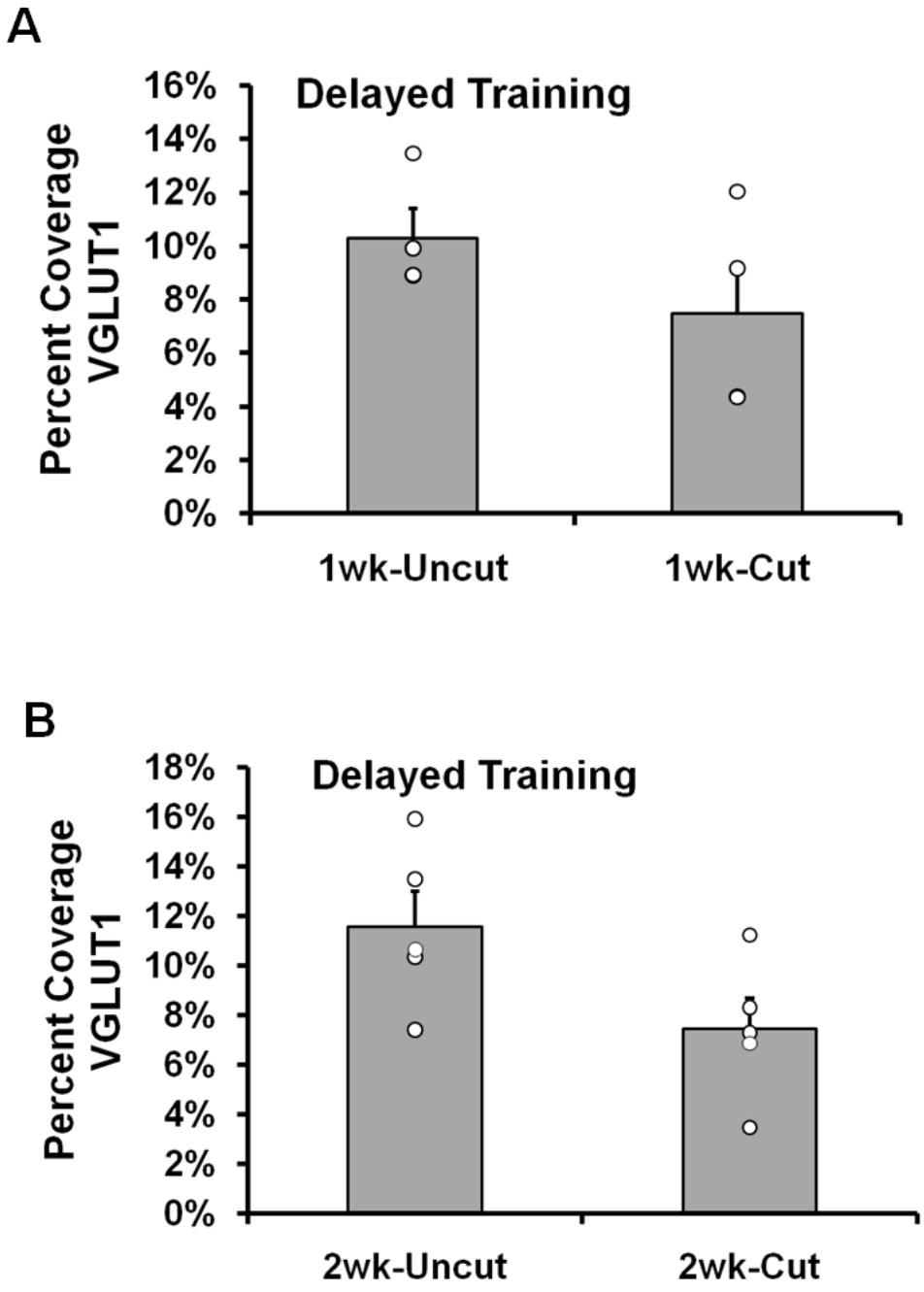

Figure 4. Effect of delayed treadmill training on VGLUT1 immunopositive excitatory synapses. The mean percent synaptic coverage (±SEM) by VGLUT1+ terminals for one week (A) and two weeks (B) of treadmill training. Cut and Uncut refer to the two sides of these animals.

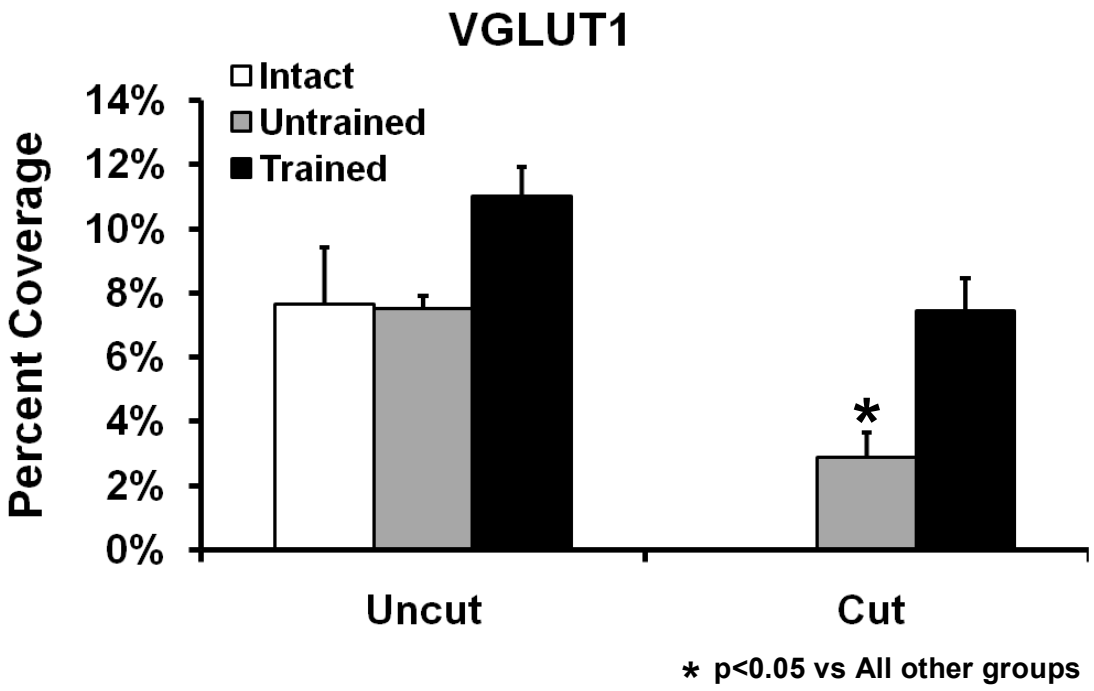

Figure 5. Effects of delayed treadmill training on the mean percent coverage (±SEM) by VGLUT1 immunopositive excitatory synaptic terminals contacting motoneuron somata and proximal dendrites is shown for the different groups studied. Cut and Uncut refer to the two sides of each animal. Trained animals began treadmill training one week after nerve transection.

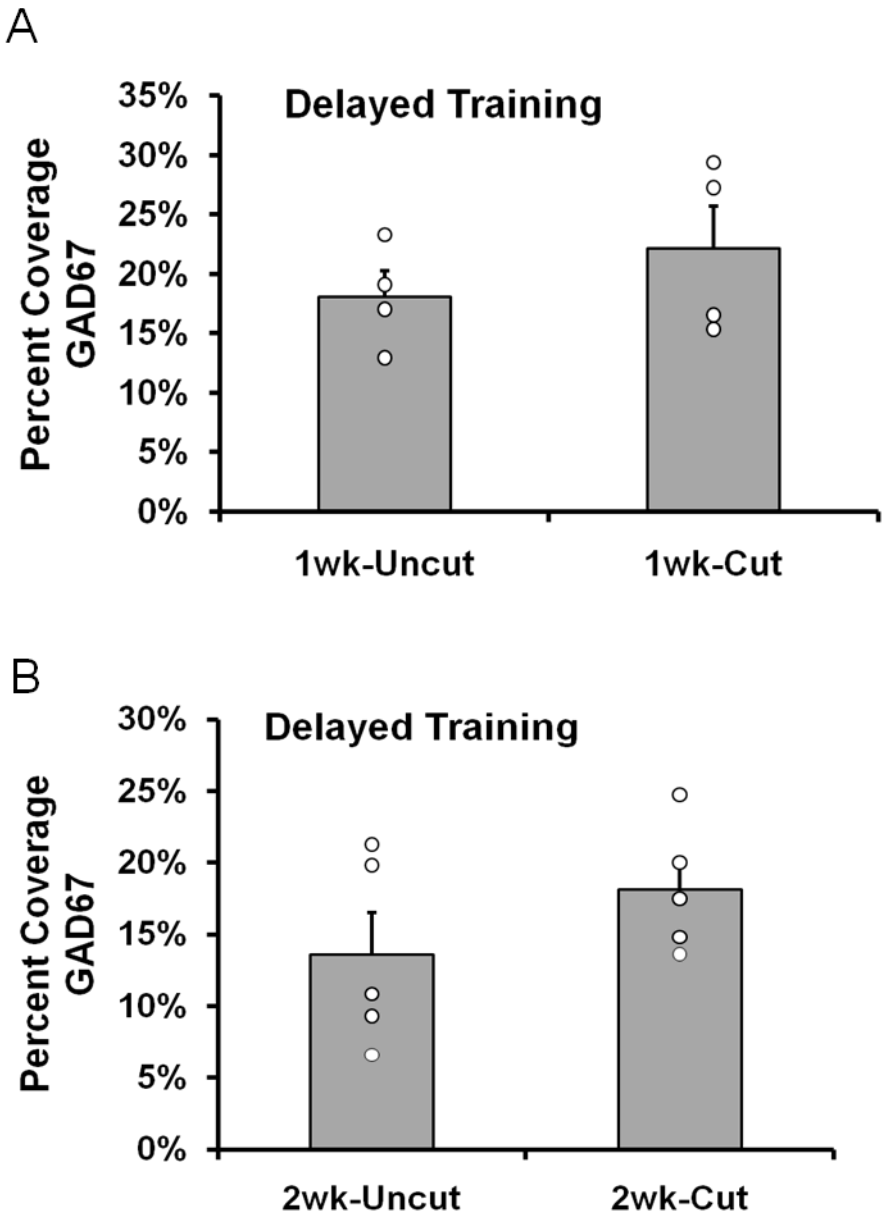

Figure 6. Effect of delayed treadmill training on GAD67 immunopositive inhibitory synapses. The mean percent synaptic coverage (±SEM) by GAD67+ terminals for one week (A) and two weeks (B) of treadmill training. Cut and Uncut refer to the two sides of these animals.

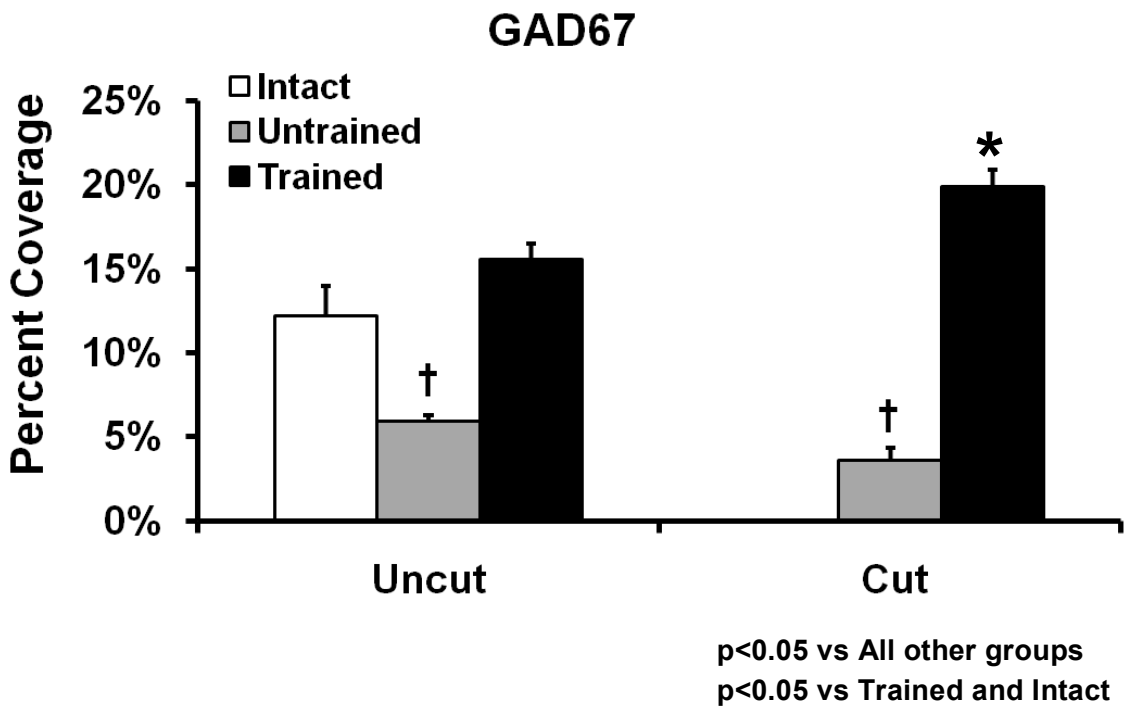

Figure 7. Effects of delayed treadmill training on the mean percent coverage (±SEM) of GAD67 immunoreactive inhibitory synaptic terminals contacting motoneuron somata and proximal dendrites is shown for the different groups studied. Cut and Uncut refer to the two sides of each animal. Trained animals began treadmill training one week after nerve transection.