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The effect of treadmill training on synaptic stripping from axotomized motoneurons in mice

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Abstract

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Functional recovery following peripheral nerve injury is poor despite the fact that axons in peripheral nerves can regenerate robustly. A potential cause for poor functional recovery that is not widely appreciated is 'synaptic stripping': the elimination of synaptic inputs from the soma and proximal dendrites of motoneurons in the spinal cord following peripheral nerve injury. The molecular mechanism and signaling pathways of synaptic stripping are currently unknown. However, recent work has provided a case for the dependence on neurotrophins, especially brain derived neurotrophic factor (BDNF). Modest treadmill training has been shown to stimulate neuronal production of BDNF and NT-4/5. In this thesis, I used modest treadmill training in mice to increase BDNF in motoneurons in an attempt to prevent or counter synaptic stripping following a peripheral nerve injury. I found that with treadmill training after a peripheral nerve injury, nearly no synaptic stripping effect was observed compared to untrained mice. I repeated the treadmill training experiments with complete BDNF knockout mice and neuron-specific BDNF knockouts. In complete BDNF knockout mice, treadmill training produced a much smaller effect, and in neuron-specific BDNF knockout mice, treadmill training produced no effect. In fact, the opposite effect was observed resulting in more synaptic stripping after training compared to untrained mice. This thesis provides support for the hypothesis that BDNF plays an integral role in the synaptic reformation caused by treadmill training.

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TABLE OF CONTENTS

Background 1

Methods 5
- Animals and Surgical Methods 5
- Tamoxifen Treatment 7
- Treadmill Training Procedures 7
- Immunohistochemical Analysis 8
- Imaging Analysis 8
- Statistical Analysis 9

Results 10
- Synaptic stripping following sciatic nerve transection in the mouse 10
- Treadmill training prevents synaptic stripping 11
- An astrocyte reaction is absent after treadmill training 11
- Lack of BDNF prevents treadmill training effect 12
- Lack of neuronal BDNF prevents treadmill training effect 13
- Treadmill training effect is dependent upon BDNF 13

Discussion 14

Future Directions 17

References 18

Figures and Legends 22
- Figure 1 22
- Figure 2 23
- Figure 3 24
- Figure 4 25
- Figure 5 26
- Figure 6 27
- Figure 7 28
- Figure 8 29
- Figure 9 30
- Figure 10 31
- Figure 11 32
- Figure 12 33

Background

There are three main problems thought to prevent complete functional recovery after peripheral nerve injury: axons are slow to regenerate, regenerating axons reinnervate at inappropriate locations, and synaptic stripping occurs. In this thesis, I will address synaptic stripping. After an axon lesion to a spinal motoneuron, a retrograde response leads to a removal of synaptic terminals from the motoneuron cell bodies, which decreases their responsiveness to afferent signals. Synaptic stripping has been demonstrated in various animal models including rat (Blinzinger & Kreutzberg, 1968), rabbit (Hamberger et al., 1970), cat (Lindå et al., 1992), and mouse (Oliveira et al., 2008). The use of facial nerves to study synaptic stripping in many previous works supports the hypothesis that a retrograde signal to the motoneuron cell body, rather than signals from sensory inputs, initiates synaptic stripping since facial nerves do not contain sensory axons (Liebermann, 1971; Titmus & Faber, 1990).

The time course for synaptic stripping and subsequent synapse reformation has not been extensively studied. Among the various animal models used to study synaptic stripping, time of onset and recovery are diverse. In many of the studies using cat motoneurons, the progression of synaptic withdrawal increases from 3 weeks, 6 weeks, to 12 weeks (Brannstrom & Kellerth, 1998). However, in mice, synaptic stripping can be seen as soon as one week post-nerve transection (Oliveira et al., 2008). Synapses begin to re-form on the motoneuron somata once target reinnervation to a muscle occurs or a new target is given (Benítez-Temiño et al., 2005). It is currently unknown when synaptic reformation begins to occur after peripheral nerve injury in mice, but it is established that muscle reinnervation following sciatic nerve transection does not occur until at least four

weeks post-axotomy in adult rats (English et al., 2007). Within this time period, a selective stripping of boutons containing spherical vesicles (a marker for excitatory inputs) and a preferential depression of excitatory potentials indicates a good correlation between physiological and morphological aspects of the reaction (Blinzinger & Kreutzberg, 1968; Sumner, 1979). Lindå et al. (2000) further investigated this phenomenon and found that after axotomy of spinal motoneurons, a larger proportion of excitatory glutamatergic synaptic terminals were removed from the soma and proximal dendrites compared to the removal of inhibitory glycinergic and GABAergic terminals. This could be interpreted as a protective mechanism from excitotoxicity after peripheral injury. However, the reformation of synapses onto the motoneuron cell body may lead to unnecessary plastic changes in synaptic input composition around the soma (Oliveira et al., 2008). Thus, synaptic stripping may serve as a neuroprotective precaution and/or may contribute to poor functional recovery following peripheral nerve injury.

The mechanism behind synaptic stripping is unknown, but activated microglia may play an important role in synaptic detachment (Blinzinger & Kreutzberg, 1968; Graeber et al., 1993). Thus, a strong proliferation of microglia is seen soon after a peripheral nerve lesion with a maximum from about day 2 to day 4 post-axotomy (Graeber et al., 1988; Streit & Kreutzberg, 1988). Astrocytes also respond to peripheral axotomy to motoneurons, but with a slower time course. They become hypertrophic and increase their production of glial fibrillary acidic protein (GFAP) (Graeber & Kreutzberg, 1986). In electron micrographic studies, glial projections can be seen interposed between the synaptic terminals and the postsynaptic membrane as well as engulfing the synaptic boutons (Brannstrom & Kellerth, 1998). Research investigating the role of microglia and

astrocytes in synaptic stripping mainly focuses on the major histocompatibility complex (MHC) class I molecules and other immune response molecules (Thams et al., 2008).

Another important factor that may be involved in synaptic stripping is neurotrophic support. Neurotrophins are a family of target-derived molecules that are essential for neuronal survival during development by promoting synaptogenesis and regulating synaptic stability (Vicario-Abejón et al., 2002). In adults, the scope of neurotrophin actions widens to become also mediators of synaptic and morphological plasticity (Vicario-Abejón et al., 2002). It has been proposed that a lack of retrograde neurotrophic support to the motoneuron induced by axotomy gives the initial signal for a profound synaptic stripping effect (Titmus & Faber, 1990). Some recent work has provided compelling support for the dependence of synaptic stability on retrograde neurotrophic support. Davis-Lopez de Carrizosa et al. (2009) showed that prolonged treatment of the proximal stump of a cut eye muscle nerve with brain derived neurotrophic factor (BDNF) and/or neurotrophin-3 (NT-3) completely blocks synaptic stripping.

Gomez-Pinilla et al. (2002) showed that endogenous BDNF expression can be induced in motoneurons in the intact spinal cord after modest exercise, and this change in BDNF expression could be involved in the facilitation of synaptic plasticity (Ying et al., 2005). In fact, very modest exercise in the form of treadmill training produced substantial enhancement of axon regeneration after peripheral nerve injury (Sabatier et al. 2008). Based on additional data from the English lab, such treadmill training also stimulates increased BDNF and NT-4/5 expression in neurons whose axons are regenerating (Wilhelm et al., 2009). Thus, the primary hypothesis of this thesis is that treadmill

training will prevent synaptic stripping following peripheral nerve injury by increasing the endogenous expression of BDNF in motoneurons.

I evaluated the effect of treadmill training after peripheral nerve injury on synaptic stripping from axotomized motoneurons. Treadmill training can be readily applied to both laboratory animals and human subjects. With human subjects, continuous locomotion at a moderate pace is often used. However, laboratory mice use a different pattern of locomotion that involves repeated short interval runs at relatively high speed with rest in between (DeBono et al., 2006). In our lab, this interval training paradigm resulted in a substantial enhancement of axon regeneration following nerve transection and surgical repair of peripheral nerves (Sabatier et al., 2008; English et al., 2009).

In order to test the hypothesized BDNF dependence of the treadmill training effect, I studied mice in which the gene for BDNF was knocked out. In mice in which this gene is knocked out conventionally, homozygous null animals die during the first few postnatal weeks. To avoid this neonatal lethality, I used the Cre-lox system to create conditional BDNF knockout mice. Two different models of Cre recombinase expression to eliminate the coding region of the BDNF gene in floxed (BDNF f/f) adult (> 2 months old) mice were used. In one model, BDNF is knocked out in all cells using a tamoxifen-inducible Cre recombinase promoter. In the other model, the BDNF gene is knocked out using a neuron-specific expression of Cre. I found that treadmill training prevented synaptic stripping following peripheral nerve injury, and a diminished effect was seen in BDNF knockout mice. Increased synaptic stripping was observed after treadmill training in the neuron-specific BDNF knockout mice.

Methods

Animals and Surgical Methods

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. Experiments were conducted on adult (>2 months old) mice weighing 17g-48g. All mouse genotypes used in the following experiments were on a C57BL/6J background. This study exploited the use of mouse genetics to evaluate whether BDNF is necessary for any effect of treadmill training on synaptic stripping following peripheral nerve injury. Three different mice were used. Two of the groups feature a mouse termed single-neuron labeling with inducible Cre-mediated knockout (SLICK) (Young et al. 2008). In these animals, yellow fluorescent protein (YFP) is expressed under the control of the *thy-1* promoter and it is found in a portion of all motoneurons and dorsal root ganglion (DRG) neurons. The name applied to these mice reflects the fact that the YFP+ neurons also express a tamoxifen-inducible Cre recombinase that can be used to generate conditional BDNF knockout mice. Conventional BDNF knockout mice die during the first postnatal week (Ernfors et al., 1994) so techniques for knocking out the gene selectively were used to avoid this lethality. If SLICK mice are bred with transgenic mice in which loxP sites are inserted flanking the coding region of the BDNF gene (floxed BDNF mice), then effective induction of Cre expression in their offspring (SLICK::BDNF f/f) by treatment with the synthetic estrogen, tamoxifen, will result in the excision of this floxed sequence, and a selective knockout of BDNF in YFP+ neurons will be achieved. These mice will be used to investigate the role of neuronal BDNF in synaptic stripping. This genotype will be

referred to as SLICK knockout. In non-tamoxifen (vehicle) treated SLICK::BDNF f/f mice, no effect on the BDNF gene was found; these mice will be genetically identical to wild type mice. These mice will serve as controls and will be referred to as wild-type throughout. In the third mouse model used, the same Cre-lox system was used to knock out the BDNF gene in all cells, not just neurons. Floxed BDNF mice were bred with mice in which tamoxifen-inducible Cre is expressed under the control of a ubiquitous promoter. When the offspring of these mice are treated with tamoxifen, Cre is expressed in all cells and BDNF is knocked out in all Cre-expressing cells. This genotype will be referred to as BDNF knockout.

All mice were subjected to one survival surgery while anesthetized with isoflurane. The sciatic nerves were cut bilaterally in the mid thigh; a section of the nerve was removed to avoid reinnervation and the proximal segment of the cut nerve was left disconnected from their distal segments. Spinal motoneurons were labeled by application of Dextran (MW 1000) conjugated to a red fluorophore (either Alexafluor 594 or Alexafluor 555) onto the proximal stumps of the cut nerves. In some mice, the sciatic nerves were left intact, as a control, and the motoneuron cell bodies were labeled by injection of the cholera toxin B subunit conjugated with the aforementioned Alexafluors (1µl) into the gastrocnemius and tibialis anterior muscles. These mice were considered the sham treatment group. One third of the mice in each of the three genotype groups were subject to daily treadmill training, beginning two to four days after surgery, and lasting for two weeks. One third of the mice in each of the three genotype groups served as unexercised controls after surgery. The final third of the mice in each group were left intact, as described above, and unexercised to form a baseline control for comparison

with the experimental mice. Mice from each genotype were randomly assigned to a treatment. All surgeries were controlled for the amount of time under anesthesia to avoid nuisance variables that may exist between the nerve injection and nerve cut and soak procedures. Based on periodic observations, nerve cut surgeries did not affect daily activity in the cage.

Tamoxifen Treatment

All mice were genotyped from tail DNA prior to selection for treatment. Tamoxifen was administered orally to adult Cre-mediated BDNF knockout mice three times a week for one week. SLICK::BDNF f/f mice were treated with tamoxifen for two rounds of three times a week with two weeks in between. Dosages were calculated based on the weight of the mouse at 0.75mg/20g, and the tamoxifen was dissolved in a 10mg/mL solution of sunflower seed oil. All mice were not used for surgery until two weeks following the last day of tamoxifen treatment to allow the knockout procedure to go to completion. Tamoxifen treatment does not affect BDNF levels in mice (Wilhelm et al., 2009)

Treadmill Training Procedures

An interval treadmill training paradigm was used (20m/min, 4x2min/5min rest, 5days/week) because it resembles the natural mouse locomotion patterns observed by DeBono et al. (2005). Two to three recovery days after surgery, one third of the mice from each genotype group was subjected to treadmill training for a duration of two weeks. The mice were placed in individual lanes on the treadmill that were completely

enclosed, and they were given 2-3 minutes to acclimate. The mice were monitored during training. Based on periodic observations, treadmill training did not affect normal activity in the cage

Immunohistochemical Analysis

Animals were deeply anesthetized with pentobarbital (150mg/kg, i.p.) and transcardially perfused with 100ml of normal saline followed by 4% paraformaldehyde. Spinal cord segments L3-L5 were harvested and cryoprotected in 20% sucrose. Spinal cords were sectioned on a cryostat (Reichert-Jung) in the transverse plane at 20µm. Standard direct immunohistological procedures were performed to detect the following antigens: synaptic vesicle protein 2 (SV2) (Harvard Medical School; 1:40) and glial fibrillary acidic protein (GFAP) (Axell; 1:500). Biotinylated anti-mouse and –rabbit secondary antibodies with streptavidin conjugated to Alexafluor 647 were used to detect signal. Sections were mounted on Superfrost Plus slides and coverslipped with Entellan (Electron Microscopy Sciences). Sections were viewed with a Zeiss confocal microscope (LSM-510, Zeiss).

Imaging Analysis

The experimenter was blinded to the experimental grouping of the source of tissues on slides while capturing images and collecting data. The slides were covered with a coded label that was not decoded until after data analysis. Twenty to forty high magnification (40x) images of individual labeled motoneuron cell bodies at a confocal slice thickness of 1 µm were retrieved from both sides of the spinal cord for each animal.

These optical sections were made through a region of the motoneuron soma containing a visible nuclear shadow (Fig. 1). Images were processed to minimize light scattering using 2D blind deconvolution, but no other image processing methods were applied. For each motoneuron selected for study, a region of interest was created about the perimeter of the cell and intersecting SV2 immunopositive synaptic terminals on the soma and proximal dendrites. Only the channel containing the motoneuron was on during measurements to avoid bias from synaptic boutons not attached to the soma. A plot profile was created measuring the light intensity along the perimeter or the region of interest for each motoneuron. Then, the percentage that the perimeter measurement was above a user-defined threshold was calculated (Fig. 1). Mean values of the percentage of soma synaptic coverage were determined for each mouse. All measurements were made using ImageJ software.

Synaptic stripping is associated with a robust glial cell reaction (Graeber & Kreutzberg, 1986; Sabha et al., 2008). To measure the astrocyte reaction, I measured the mean intensity of GFAP immunoreactivity in a region of interest in lamina IX surrounding the pool of labeled motoneurons. Measurements of fluorescence intensity were corrected for background fluorescence in regions of interest selected from sections reacted without application of the primary antibody for GFAP. Fifteen to twenty measurements were averaged for each side of the spinal cord for each animal.

Statistical Analysis

Each side of the spinal cord of each animal was treated as an independent subject to minimize the use of animals. Each treatment group contained two mice for n=4 sides

of the spinal cord. Significance ($p<0.05$) of differences between all groups were made using analysis of variance (Statistica 6.0). When the omnibus test for significance was met, paired post-hoc testing (Fishers least significant differences) was applied. When $n<4$ (Fig. 5 only) a two-sample Student's t-test was used for significance of differences ($p<0.05$).

Results

Using an interval training paradigm, we tested the hypothesis that treadmill training would prevent synaptic stripping from motoneuron somata that normally follows sciatic nerve transection. With BDNF knockout mice, we tested the hypothesis that BDNF is a crucial factor involved in the treadmill training effect.

Synaptic stripping following sciatic nerve transection in the mouse

Synaptic stripping from motoneurons following sciatic nerve transection can be seen in images of spinal cord sections reacted with an antibody to the synaptic vesicle protein, SV2 (Fig. 2). The average percent ($\pm$SEM) coverage of motoneuron somata and proximal dendrites by SV2+ synaptic boutons is shown in Fig. 3 for sham (0d) (mean=55.46$\pm$2.11 (SEM)), one week post-axotomy (7d) (30.43$\pm$1.44), and two weeks post-axotomy (14d) (26.74$\pm$1.56) (n=4 for all groups). A significant stripping effect was found at both one week and two weeks following sciatic nerve transection ($p<0.00001$, 1 week; $p<0.0000001$, 2 weeks). All subsequent data and graphs express this reduction in synaptic coverage as a percent change from control (sham) calculation and are presented in that format. A reduction of approximately 50% in synaptic coverage surrounding the

motoneuron cell bodies and proximal dendrites was found post-axotomy ($p<0.0000001$, 2 weeks, Fig. 4). This reduction in synaptic coverage is similar to what others have reported in the same mouse strain (Sabha et al., 2008). No significant difference was found between one week and two weeks post-axotomy ($p=0.38$, Fig. 3). Therefore, all experiments other than controls were performed using a two week survival period post-axotomy to better fit the treadmill training paradigm.

Treadmill training prevents synaptic stripping

Treadmill training following sciatic nerve transection resulted in synaptic coverage similar to that noted in controls (Figs. 2, 4). The percent change in synaptic coverage vs. control is shown in figure 4 for two weeks post-axotomy (untrained) (51.78 ± 2.80) and two weeks post-axotomy (trained) (-1.15 ± 5.73) ($n=4$ for all groups). The differences between these two groups were statistically significant ($p<0.00001$). No significant difference was found between mice two weeks post-axotomy with treadmill training and sham controls ($p=0.88$).

An astrocyte reaction is absent after treadmill training

A notable feature of synaptic stripping is the glial reaction that occurs in the spinal cord after axotomy, in which reactive GFAP-positive astrocytes surround the axotomized motoneurons (Graeber & Kreutzberg, 1986; Sabha et al. 2008). Confocal images from sections of the spinal cord that were reacted with an antibody to GFAP are shown in figure 5. The mean intensity of GFAP immunoreactivity (mean gray value) in regions of interest in the sciatic motor nucleus is shown in Fig. 6 for the following

conditions in wild-type mice: sham (0d) (mean=3064.8±373.5), one week post-axotomy (7d) (mean=8036.9±198.4), two weeks post-axotomy (14d untrained) (12283.9±112.5), and two weeks post-axotomy with treadmill training (14d trained) (2748.0±389.3) (n=2 for all groups). A significant increase in GFAP immunoreactivity was observed one week and two weeks post-axotomy ($p<0.001$, one week; $p<0.0001$, two weeks, Fig. 5). No significant difference was found between the sham group and the treadmill trained group ($p=0.60$, Fig. 5).

Lack of BDNF prevents treadmill training effect

The synaptic coverages of motoneurons in BDNF-KO mice with intact sciatic nerves as well as two weeks post-axotomy with and without treadmill training are shown as selected confocal images in figure 7. The percent change in synaptic coverage vs. wild-type controls is shown in Fig. 8 for the following treatments respectively: sham (-55.65±3.60), two weeks post-axotomy (untrained) (-72.06±7.18), and two weeks post-axotomy (trained) (-29.26±5.57) (n=4 for all groups). In complete BDNF knockouts, there was a significant reduction in synaptic coverage in mice with intact sciatic nerves compared to wild-type mice ($p<0.0000001$). The reduction relative to intact wild-type mice increased further after axotomy ($p<0.000000001$) and this decrease is significantly different from BDNF-KO sham ($p<0.04$). However, in treadmill trained mice, synaptic coverage increased significantly relative to the genotype's control condition ($p<0.01$), but remained significantly below wild-type control ($p<0.001$).

Lack of neuronal BDNF prevents treadmill training effect

The synaptic coverage of motoneurons in SLICK-KO mice with intact sciatic nerves as well as two weeks post-axotomy with and without treadmill training are shown as selected confocal images (Fig. 9). The percent change in synaptic coverage vs. wild-type controls is shown in Fig. 10 for sham (-26.09$\pm$2.43), two weeks post-axotomy (untrained) (-21.78$\pm$8.67), and two weeks post-axotomy (trained) (-42.93$\pm$3.91) (n=4 for all groups). In neuron-specific BDNF knockouts, there was a significant reduction in synaptic coverage in mice with intact sciatic nerves compared to wild-type mice ($p<0.001$). After axotomy, synaptic coverage is not significantly changed from that of sham-treated mice of the same genotype ($p=0.58$), but significantly less than the wild-type sham group ($p<0.01$). Surprisingly, after treadmill training synaptic coverage decreased dramatically beyond the reduction seen in axotomized untrained SLICK-KO mice vs. wild-type controls ($p<0.00001$). This change was significantly different from the SLICK-KO sham group and untrained group ($p<0.04$ and $p<0.01$, respectively).

Treadmill training effect is dependent upon BDNF

The synaptic coverage of motoneurons in each genotype that received treadmill training are shown to compare the effects of genotype on the result from treadmill training (Fig. 11). The percent change in synaptic coverage vs. wild-type controls is shown in Fig. 12 for all three genotypes that received treadmill training. Wild-type (trained) (-1.15$\pm$5.73), BDNF-KO (trained) (-29.26$\pm$5.57), and SLICK-KO (trained) (-42.93$\pm$3.91) (n=4 for all groups). The synaptic coverage in both BDNF-KO models was significantly less compared to treadmill trained wild-type ($p<0.001$, BDNF-KO;

$p<0.00001$, SLICK-KO). No significant difference was found between KO groups (trained) ($p=0.08$). Both KO groups were significantly different from the wild-type trained group ($p<0.001$ and $p<0.00001$, respectively).

Discussion

The main finding of my thesis is that treadmill training following peripheral nerve injury prevents synaptic stripping. Treadmill training after a peripheral nerve injury has also been shown to enhance axon regeneration (Sabatier et al., 2008), and to do so without an increase in the misdirection of regenerating axons (English et al, 2009). Since synaptic stripping might be a source of poor functional recovery following peripheral nerve injury, these combined effects of treadmill training have potential for improved functional recovery after injury.

Based on the results presented above, new insights as to the cellular basis for synaptic stripping following peripheral nerve injury and its restoration following reinnervation may be forthcoming. Axotomy-induced synaptic stripping is thought to be a result of deprivation of motoneurons from target-derived molecules that contribute to synaptic stability (Titmus & Faber, 1990). Stripping occurs in axotomized facial motoneurons, where afferent axons forming synapses on those motoneurons are spared (Liebermann, 1971). Along with the withdrawal of some inhibitory boutons (Lindå et al., 2000), these results are consistent with stripping being an effect mediated through the motoneurons and not the synaptic inputs to the motoneurons. A compelling case can be made that neurotrophins, such as BDNF, are among the molecules that are reduced or absent in the axotomized motoneurons. Davis-Lopez de Carrizosa et al. (2009) showed

that by application of BDNF to the proximal stumps of cut nerves, they were able to block synaptic stripping. Treadmill exercise is known to increase the expression of neurotrophins, such as BDNF in motoneurons (Gomez-Pinilla et al., 2002). Thus I hypothesized that treadmill training acts to retain synapses on axotomized motoneurons by stimulating the production of BDNF and that this production replaces the lost target-derived source of BDNF.

This hypothesis was tested using the conditional BDNF knockout mice. This is first study to provide a case of synaptic stripping in genetic knockout mice, and it is the first to demonstrate a synaptic plasticity effect of treadmill training in the spinal cord. If BDNF is required for the maintenance of synaptic inputs onto motoneurons, then one might predict that significant synaptic stripping would occur in BDNF knockout mice. It is clear from the BDNF-KO results that it has an important role in maintaining synaptic connections and is involved in the mechanism behind the treadmill training effect. The fact that there is less synaptic coverage in intact BDNF-KO animals compared to wild-type controls suggests the importance of BDNF from all sources. However, there does seem to be an effect of treadmill training in these mice. The difference between the 28% reduction found after training and the 55% reduction found in intact BDNF-KO mice suggests that something about the training induces new synapse formation. The role of other factors is also suggested by the increase in synaptic stripping from 55% in intact BDNF-KO mice to 71% found two weeks post-axotomy. Since the motoneuron is already deprived of all sources of BDNF, there must be other factors that were removed after axotomy that were necessary for synaptic maintenance. A likely source of such an effect might be neurotrophic factors other than BDNF. Davis-Lopez de Carrizosa et al. (2009)

showed that neurotrophin-3 application to the proximal stumps of cut nerves reduced synaptic stripping from motoneurons and that the nature of this reduction was different than that noted when they treated cut nerves with BDNF. It would be interesting to evaluate the involvement of the other neurotrophins in synaptic maintenance.

In the experiments with intact SLICK-KO mice, a smaller but significant reduction in synaptic coverage, relative to wild type mice, was found when compared to the reduction found in BDNF-KO mice. This difference reflects the effect of motoneuron-specific BDNF elimination in these mice. In the BDNF-KO mice, all of the cells, including afferent neurons and glial cells, such as astrocytes, are deficient in BDNF. In the SLICK-KO mice the effect is only in the motoneurons and a relatively small number of other neurons. The difference in reduction of coverage between BDNF-KO (55%) and SLICK-KO (25%) is evidence that BDNF from other sources than the motoneurons such as glia might have an important role in maintaining synaptic inputs. The fact that there was no significant change associated with axotomy in SLICK-KO mice likely means that any stripping related to target deprivation of BDNF was already exhausted. There was no stripping that resulted from depriving BDNF null motoneurons from their targets. The increased stripping that resulted from treadmill training in these mice was unexpected. It is difficult to interpret without knowing what else might be going on in these motoneurons. A change in the homeostatic balance of neurotrophins, unique in these mice, may have resulted in a negative response increasing the stripping by some unknown mechanism. Overall, it seems like there are two sources of synaptic stripping: intrinsic to the motoneurons that is represented here as target derived BDNF;

and stripping resulting from sources outside of the motoneurons, such as afferent neurons and glial cells

Future Directions

Our data supports our hypothesis that treadmill training, acting through an upregulation of BDNF and possibly other neurotrophins, prevents synaptic stripping. However, further analysis is required to study glial or other sources of BDNF and other neurotrophic factors that may be responsible for the treadmill training effect seen in this paper. Conditional BDNF knockout mice with BDNF knocked out in glial cells would be useful in future analyses as well as other neurotrophin knockout mice.

Overall, the effect of blocking synaptic stripping by treadmill training can be seen as a means by which functional recovery following peripheral nerve injury could be enhanced. However, the prevention of synaptic stripping from axotomized motoneurons by treadmill training might not be so beneficial if stripping functions as a substrate for adaptive synaptic plasticity. Further analysis to look at any possible synaptic reformations or rearrangements after treadmill training is necessary to provide more insight to the potential enhancement or reduction in functional recovery by synaptic stripping. For example, Alvarez et al. (2004) has shown that after rhizotomy, a preferential removal of VGLUT1 positive terminals (believed to belong to group Ia spindle afferents) were removed over VGLUT2 positive terminals. Further characterization of the types of synapses before and after treadmill training on the soma of axotomized motoneurons would also be helpful in determining more functional outcomes from the treadmill training effect discussed in this paper.

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Figures and Legends

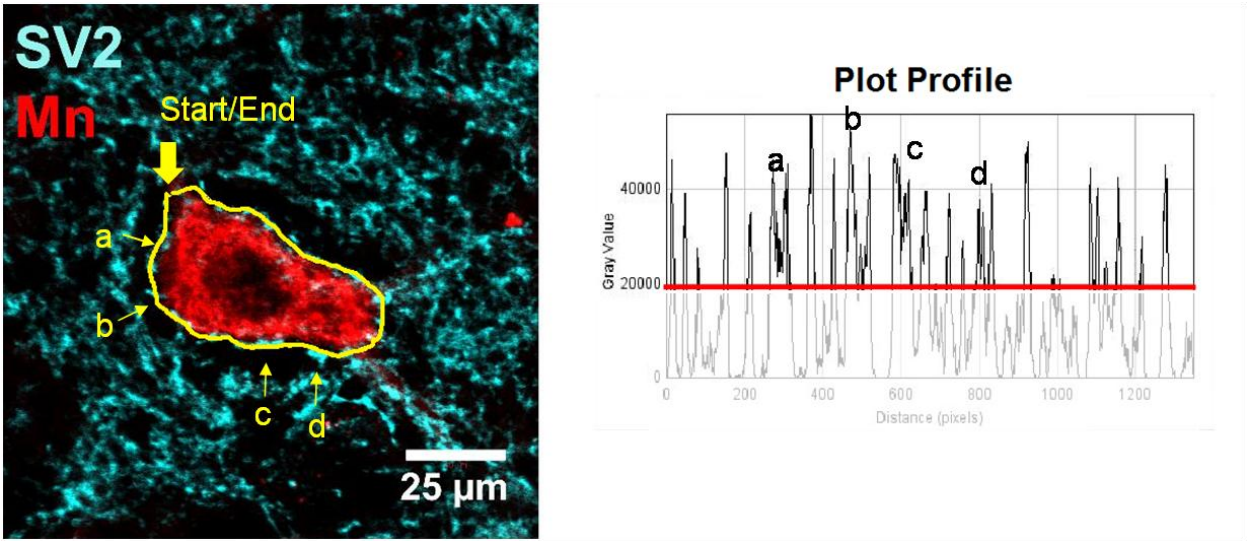

Figure 1. Single motoneuron measurement diagram is shown with profile plot obtained with Image J. Letters a-d indicate synaptic boutons that are intersected by the yellow perimeter line and correlate with the labeled spikes on the profile plot. The red line across the profile plot represents the user-defined threshold at 20000 gray value. Spikes above this line are counted as synaptic terminals on the motoneuron.

Synaptic stripping following sciatic nerve transection in the mouse

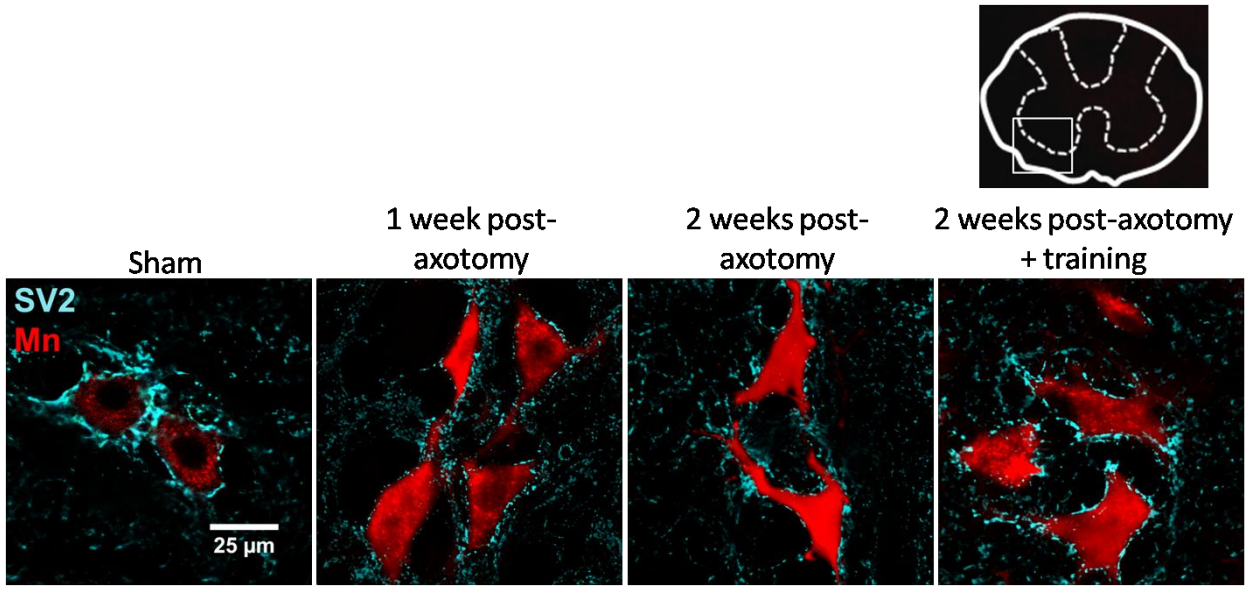

Figure 2. Single motoneuron profiles are shown with red cell bodies and cyan synaptic boutons. Images were captured under 40x objective magnification and 2x optical zoom. Order from left to right: sham (0d), one week post-axotomy (7d), two weeks post-axotomy (14d), two weeks post-axotomy with treadmill training (14d trained).

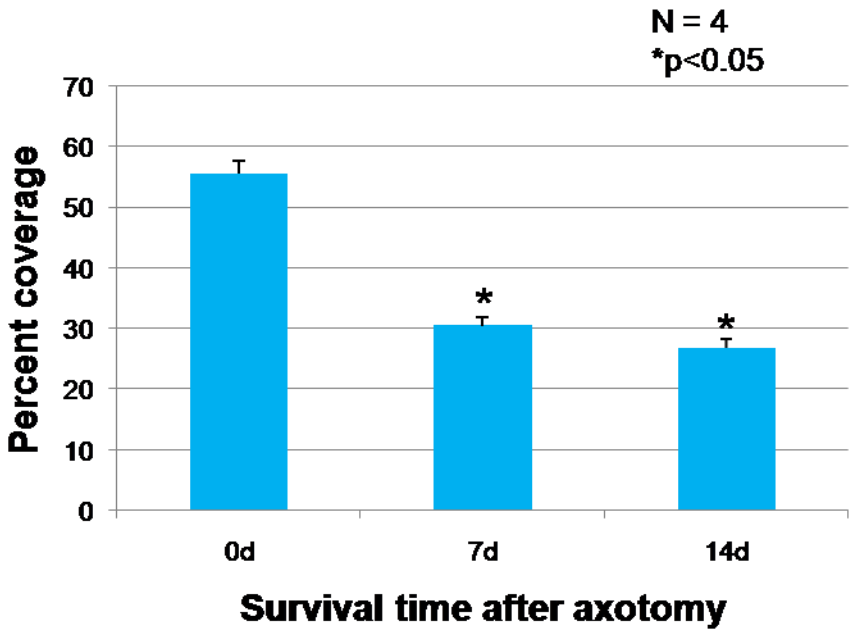

Figure 3. The mean percent coverage of synaptic boutons surrounding motoneuron somata and proximal dendrites (±SEM) is shown in wild-type mice for the following conditions: sham (0d), one week post-axotomy (7d), and two weeks post-axotomy (14d) (n=4 for all groups). Asterisks on error bars indicate a significant difference with wild-type sham group.

Treadmill training blocks synaptic stripping

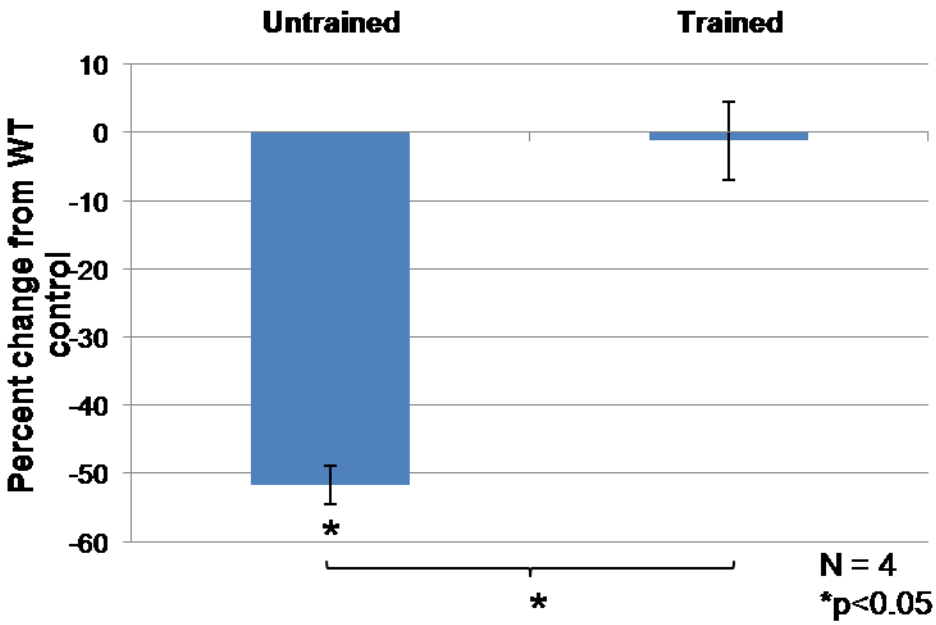

Figure 4. The mean percent change in synaptic coverage vs. control (±SEM) in wild-type mice is shown for two weeks post-axotomy (untrained) and two weeks post-axotomy (trained) (n=4 for all groups). The apparent absence of a bar for the trained group is due to a very small percent change from controls. Asterisks on error bars indicate a significant difference with wild-type sham group. Asterisks on brackets indicate significance between groups within the graph.

An astrocyte reaction is absent after treadmill training

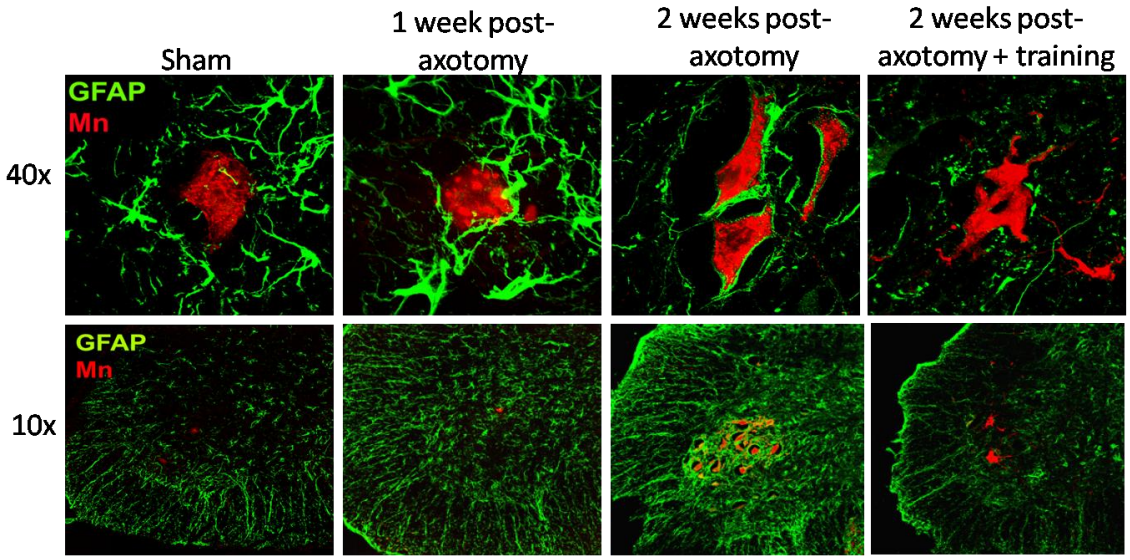

Figure 5. Single motoneuron profiles are shown with red cell bodies and green astrocytes. In the first row, images were captured under 40x objective magnification and 2x optical zoom. In the second row, images were captured under 10x objective magnification. Order from left to right: sham (0d), one week post-axotomy (7d), two weeks post-axotomy (14d), two weeks post-axotomy with treadmill training (14d trained).

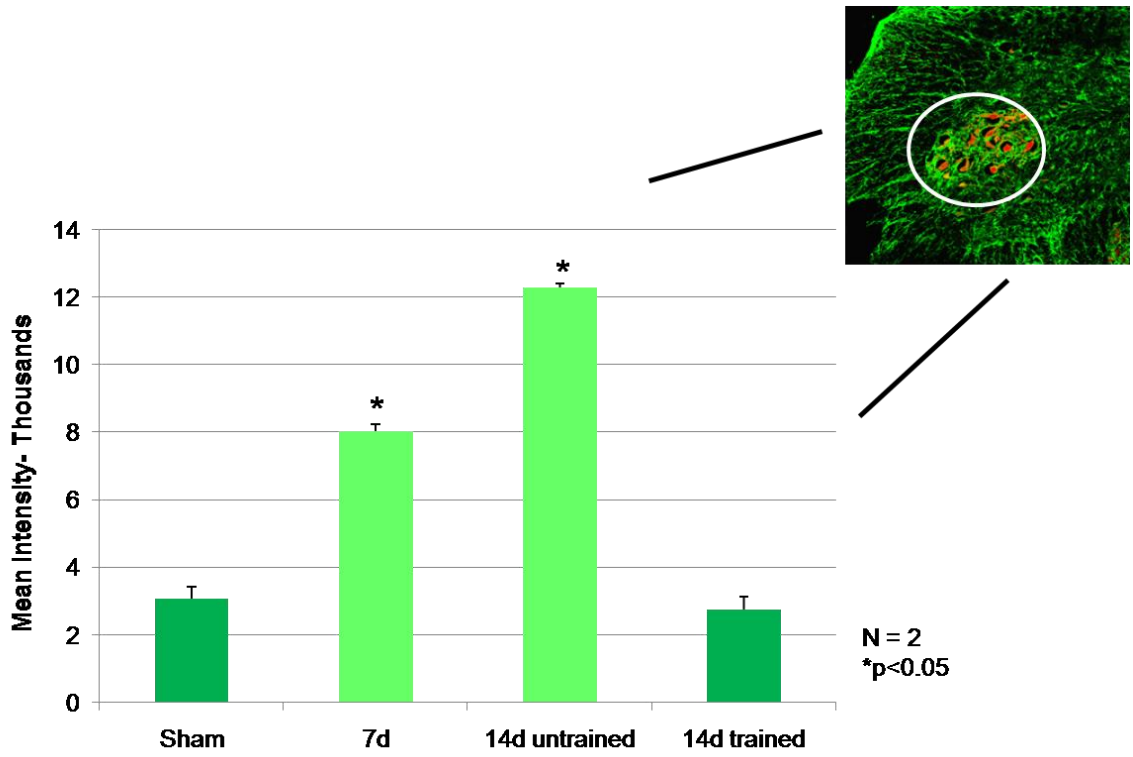

Figure 6. The mean intensity of GFAP immunoreactivity (mean gray value) (±SEM) represents each bar for the following conditions in wild-type mice: sham (0d), one week post-axotomy (7d), two weeks post-axotomy (14d untrained), and two weeks post-axotomy with treadmill training (14d trained) (n=2 for all groups). The diagram in the top-right depicts the method through Image J that was performed to obtain the values in the graph. Asterisks indicate a significant difference from sham group.

Lack of BDNF prevents treadmill training effect

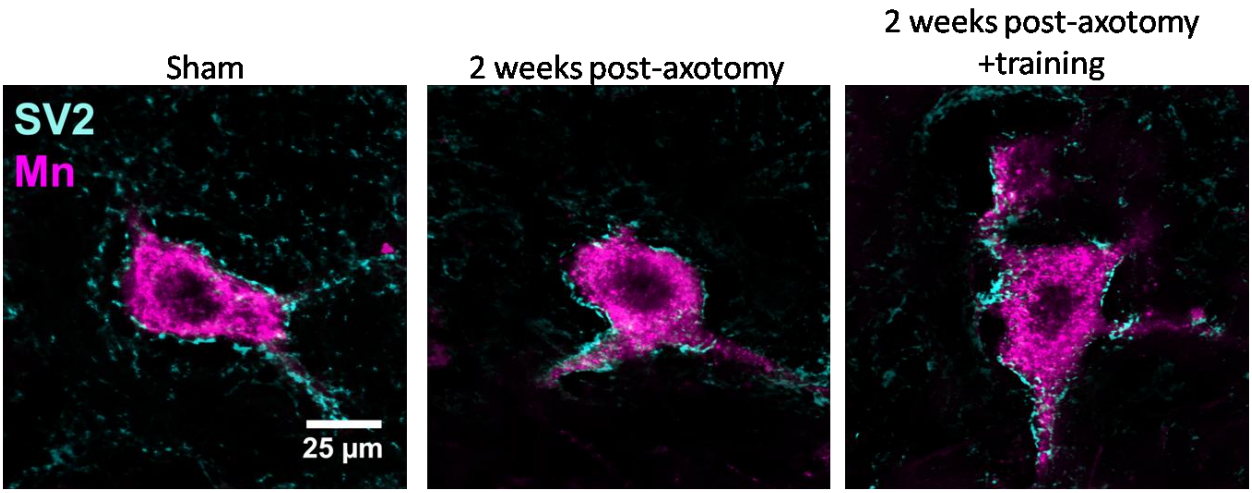

Figure 7. Single motoneuron profiles from BDNF knockout mice are shown with purple cell bodies (indicating complete BDNF knockout) and cyan synaptic boutons. Images were captured under 40x objective magnification and 2x optical zoom. Order from left to right: sham (0d), two weeks post-axotomy (14d untrained), two weeks post-axotomy with treadmill training (14d trained).

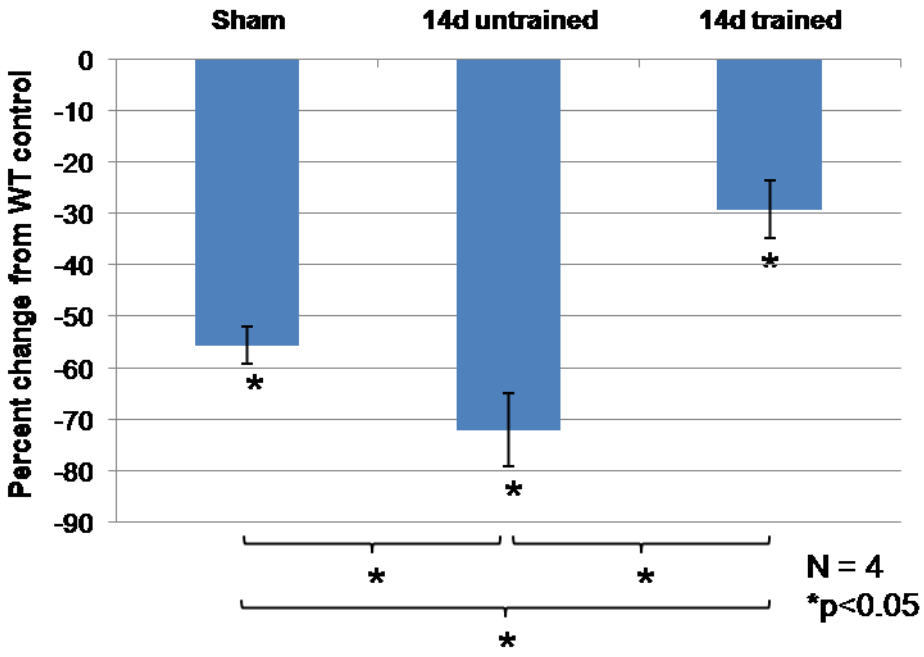

Figure 8. The mean percent change in synaptic coverage vs. wild-type controls (±SEM) is shown for BDNF-KO mice in the following treatments respectively: sham, two weeks post-axotomy (untrained), and two weeks post-axotomy (trained) (n=4 for all groups). Asterisks on error bars indicate a significant difference with wild-type sham group. Asterisks on brackets indicate significance between groups within the graph.

Lack of neuronal BDNF prevents treadmill training effect.

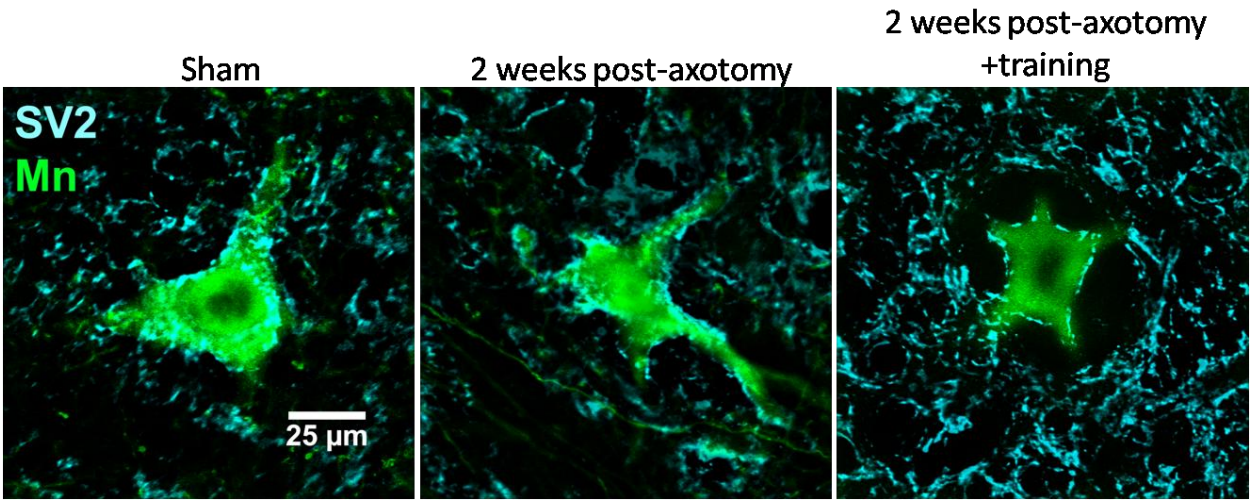

Figure 9. Single motoneuron profiles from SLICK knockout mice are shown with red cell bodies and cyan synaptic boutons. Images were captured under 40x objective magnification and 2x optical zoom. Order from left to right: sham (0d), two weeks post-axotomy (14d untrained), two weeks post-axotomy with treadmill training (14d trained).

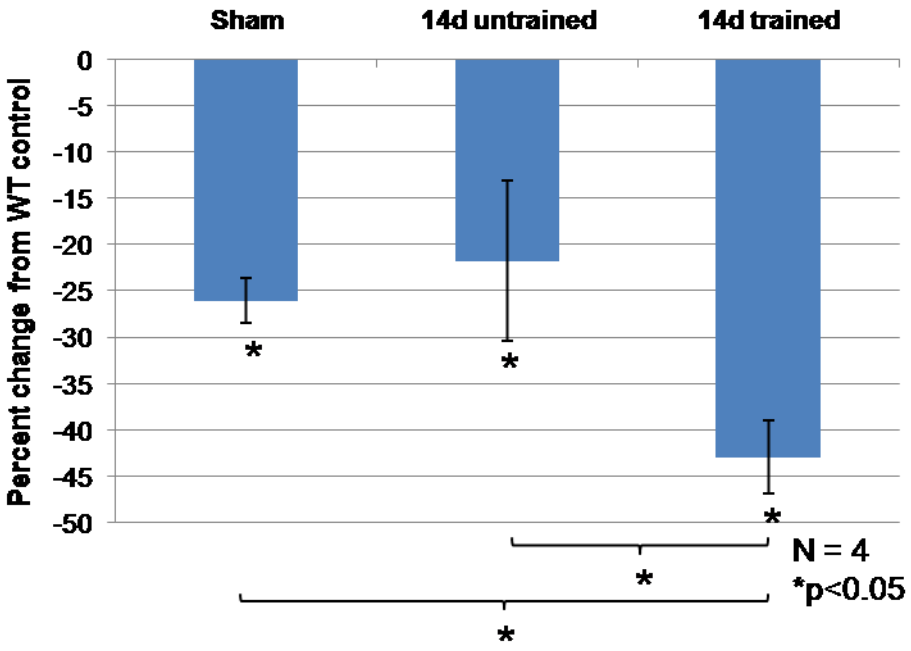

Figure 10. The mean percent change in synaptic coverage vs. wild-type controls (±SEM) is shown for SLICK-KO mice in the following treatments respectively: sham, two weeks post-axotomy (untrained), and two weeks post-axotomy (trained) (n=4 for all groups). Asterisks on error bars indicate a significant difference with wild-type sham group. Asterisks on brackets indicate significance between groups within the graph.

Treadmill training effect is dependent upon BDNF.

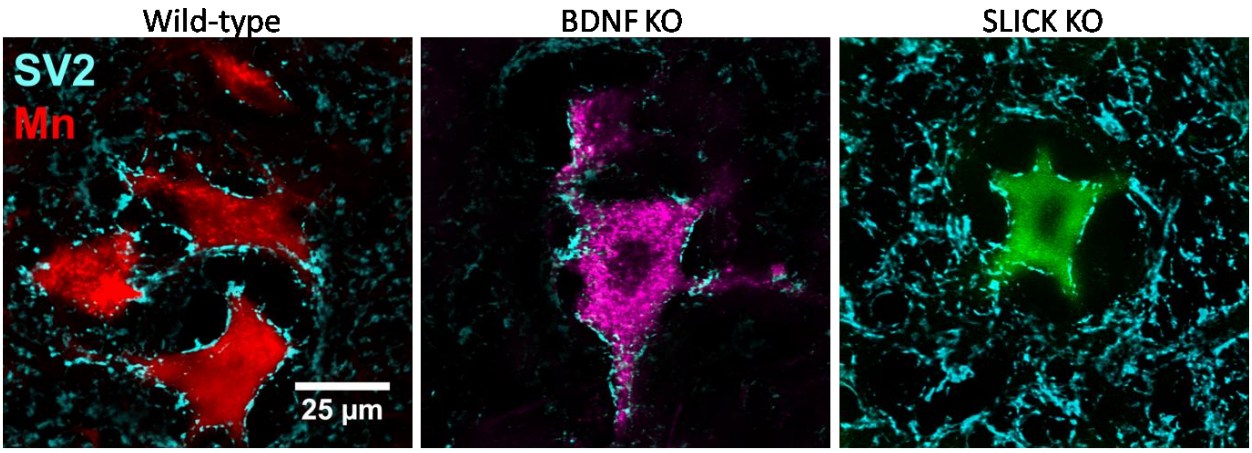

Figure 11. Single motoneuron profiles from wild-type, complete BDNF-KO, and SLICK BDNF-KO mice, respectively that received treadmill training, are shown with red, purple, and green cell bodies according to genotype and cyan synaptic boutons. Images were captured under 40x objective magnification and 2x optical zoom.

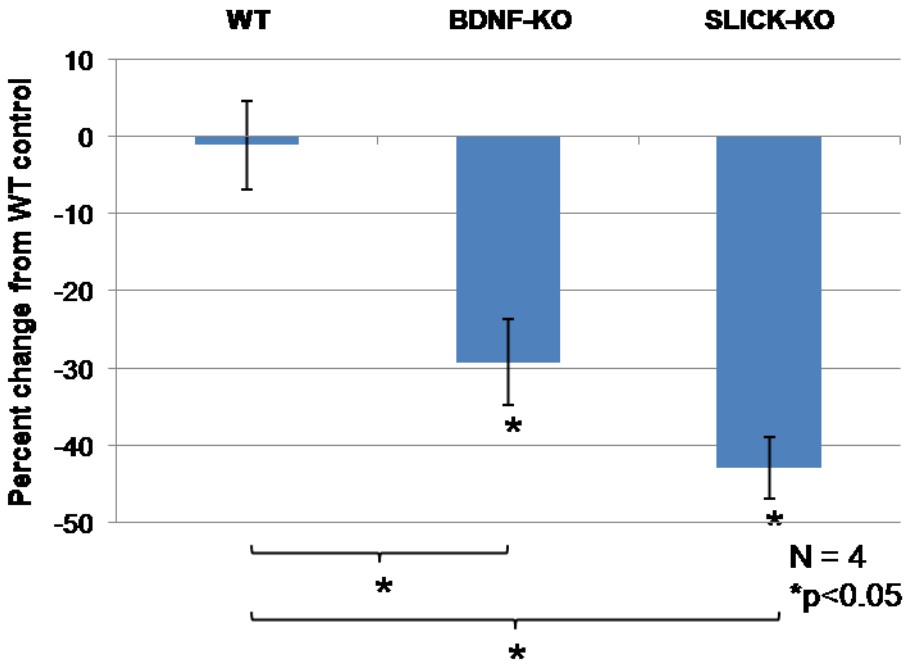

Figure 12. The mean percent change in synaptic coverage vs. wild-type controls (±SEM) is shown for all three genotypes that received treadmill training. Wild-type (trained), BDNF-KO (trained), and SLICK-KO (trained) (n=4 for all groups). The apparent absence of a bar for the wild-type trained group is due to a very small percent change from wild-type controls. Asterisks on error bars indicate a significant difference with wild-type sham group. Asterisks on brackets indicate significance between groups within the graph.