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Cooperative roles of BDNF expression in neurons and Schwann cells are modulated by exercise to facilitate nerve regeneration

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Abstract

After peripheral nerve injury, neurotrophins play a key role in the regeneration of damaged axons which can be augmented by exercise, although the distinct roles played by neurons and Schwann cells are unclear. In this study, we evaluated the requirement for the neurotrophin, brain derived neurotrophic factor (BDNF), in neurons and Schwann cells, for the regeneration of peripheral axons after injury. Common fibular or tibial nerves in *thy-1-YFP-H* mice were cut bilaterally and repaired using a graft of the same nerve from transgenic mice lacking BDNF in Schwann cells (BDNF^{-/-}) or wild-type mice (WT). Two weeks post-repair, axonal regeneration into BDNF^{-/-} grafts were markedly less than WT grafts, emphasizing the importance of Schwann cell BDNF. Nerve regeneration was enhanced by treadmill training post-transection, regardless of the BDNF content of the nerve graft. We further tested the hypothesis that training-induced increases in BDNF in neurons allow regenerating axons to overcome a lack of BDNF expression in cells in the pathway through which they regenerate. Nerves in mice lacking BDNF in YFP⁺ neurons (SLICK) were cut and repaired with BDNF^{-/-} and WT nerves. SLICK axons lacking BDNF did not regenerate into grafts lacking Schwann cell BDNF. Treadmill training could not rescue the regeneration into BDNF^{-/-} grafts if the neurons also lacked BDNF. Both Schwann cell- and neuron-derived BDNF are thus important for axon regeneration in cut peripheral nerves.

Introduction

Injured peripheral axons have the ability to regenerate, but the regenerative process is slow and generally functional recovery is very poor. One reason for decreased regeneration could be due to a decline in neurotrophic support in tissue surrounding the regenerating axons over time (reviewed in (Gordon, 2009)). To facilitate regeneration, treatment approaches have included manipulation of neurotrophins. In particular, brain derived neurotrophic factor (BDNF) has been shown to be important in the growth of regenerating axons into peripheral nerve grafts (Zhang et al., 2000; English et al., 2005).

After peripheral nerve injury, BDNF mRNA is upregulated in axotomized motoneurons as well as Schwann cells distal to the injury (Funakoshi et al., 1993). The upregulation of BDNF mRNA in axotomized motoneurons is transient and last only a few days (Gordon, 2009). The increase in BDNF in cells in the distal stump is much longer lasting. Thus, much research has focused on the role of BDNF at the distal stump in facilitating axon regeneration after nerve injury. Because traditional genetic BDNF knockout mice die shortly

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after birth (Jones et al., 1994; Ernfors et al., 1995; Conover and Yancopoulos, 1997), most research has focused on the pharmacologic manipulation of BDNF. Application of trophic factors such as BDNF and NT4/5 in pharmacologic doses at the site of injury has been shown to enhance the regeneration of axons into the distal stump (English et al., 2005). Reducing neurotrophin signaling through systemic infusion of BDNF antibodies (Zhang et al., 2000) significantly limits the regenerative elongation of axons.

Although the upregulation of BDNF mRNA in axotomized motoneurons is brief, it may provide a source of neurotrophic support to regenerating axons prior to the increase in BDNF in distal Schwann cells (Gordon, 2009). Increasing motoneuron-derived BDNF may enhance axon regeneration. Experiments examining the potential for motoneuron-derived BDNF to promote axon regeneration have been indirect. Treadmill training (Gomez-Pinilla et al., 2001) and electrical stimulation of axons in the proximal stump (Al-Majed et al., 2000) enhance BDNF mRNA expression in motoneurons after nerve cut. This increase in BDNF likely underlies the significant enhancement of axon elongation into a peripheral nerve graft after two weeks of treadmill training (Sabatier et al., 2008).

Until recently, the technology required to examine the differential contributions of Schwann cell-derived and motoneuron-derived BDNF has not been available. In the current study, we manipulated the expression of BDNF genetically in a cell-type specific manner, removing BDNF selectively from Schwann cells or motoneurons. We hypothesize that removing BDNF from the Schwann cells surrounding the regenerating axons will impair the growth of the regenerating axons. In addition, we propose a novel model in which motoneuron-derived BDNF regulates treadmill training-mediated elongation of axons after nerve cut. Preliminary reports of these findings have been made (Wilhelm et al., 2009).

Materials and Methods

Animals and surgical methods

All procedures were approved by the Institutional Animal Care and Use Committee of Emory University and conformed to the Guidelines for the Use of Animals in Research and the Society for Neuroscience. All mouse genotypes used in the following experiments were on a C57BL/6J background that were backcrossed for at least 6 generations. Experiments were conducted on adult (>2 months old) male and female mice weighing 18g-48g (mean = 22g).

Under isofluorane anesthesia, the common fibular (CF) and tibial (TIB) nerves were exposed and cut approximately 1 mm distal to their branching from the sciatic nerve. A segment of the CF or tibial nerve (8-10 mm-long) was harvested from a wild type (WT) or BDNF lacking donor mouse that did not contain a YFP transgene. The proximal end of the donor graft was apposed to the proximal segment of the cut nerve of the matching nerve of the host mouse on a small rectangle of silastic film (Dow Corning, Midland, MI) and secured using approximately 5 μ l of fibrin glue containing thrombin, fibrin, and fibronectin (de Vries et al., 2002; MacGillivray, 2003). In eight nerves BDNF (2.0 μ g) was added to the fibronectin portion of the fibrin glue at the time of the repair. The distal end of the donor graft was not secured to the host's distal stump to prevent any influence by potential target derived molecules. All surgeries were performed bilaterally, so that each limb contained one nerve repaired with a wild type graft and one nerve repaired with a BDNF lacking graft. The graft types were also balanced across nerves (TIB or CF) such that each mouse had one TIB repaired with a KO graft and the contralateral TIB repaired with a WT graft. Additionally, conditions were counterbalanced across animals so that equal numbers of mice had left limb TIB nerves repaired with KO grafts and WT grafts. No significant differences were found in the length of regenerating axon profiles based on the type of nerve grafted (CF vs. TIB), the

limb used (left vs. right), or the sex of the host mouse. Therefore, data from all CF and TIB nerves were pooled together for analysis regardless of host sex or limb used.

Two different host models were used in the above transection paradigm to allow us to manipulate the BDNF expression in the regenerating axons (Table 1). The H strain of *thy-1-YFP* mice (*thy-1-YFP-H* (Feng et al., 2000)) was used in experiments in which hosts had wild type BDNF expression levels. In these mice a subset of motor and sensory axons in peripheral nerves is marked completely by yellow fluorescent protein (YFP) (Feng et al, 2000). Marked axons in these mice are assumed to represent a sample of all of the axons in the peripheral nerves (Groves et al, 2005). Founders of these mice were obtained from the Jackson Laboratories (Bar Harbor, ME). The second host model used lacked BDNF in YFP⁺ neurons. We used single-neuron labeling with inducible Cre-mediated knockout (SLICK) mice (Young et al., 2008), in which YFP is expressed in neurons in a manner similar to that in the *thy-1-YFP-H* mice with a representative subset of axons containing YFP. In these mice, the YFP⁺ sensory and motor neurons also express a tamoxifen-inducible Cre-recombinase. When bred with transgenic mice expressing loxP-flanked BDNF genes, selected offspring (e.g., SLICK::BDNF^{f/f}) were treated with tamoxifen (see below) and BDNF was knocked out in the YFP⁺ neurons. Host mice lacking either one (SLICK::BDNF^{f/w}) or both (SLICK::BDNF^{f/f}) BDNF alleles were used. The founders of the A strain of SLICK mice (B6.Cg-Tg(CAC-cre/Esr1*)5AmcJ; stock no. 004682) and of the *thy-1-YFP-H* mice (B6.Cg-Tg(Thy1-YFP)2Jrs/J; stock no. 003782) were obtained from The Jackson Laboratory. The founders of our colony of floxed BDNF mice were a generous gift of Dr. Michael Sendtner (Universität Würzburg).

Several different mouse models were used for the collection of graft tissues to allow us to manipulate the environment into which the axons could attempt to regenerate (Table 1). Graft tissue was collected from strains of non-YFP expressing mice. Graft tissue containing BDNF was collected from a non-YFP expressing littermate of the host mouse or a transgenic mouse ((B6.129(Cg)-*Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo*/J) (Jackson Laboratories, stock 007676). When graft tissue from these transgenics, termed tomato, was used, a red background was observed because tomato mice express red fluorescence in all tissues unless Cre-recombinase is expressed (Muzumdar et al., 2007). Once Cre-recombinase is expressed in a cell the red fluorescent protein gene is excised and green fluorescent protein is produced. Only non-Cre-expressing tomato mice were used as graft donors. We found no significant difference in the lengths of axons growing into grafts from wild type littermates or tomato mice; therefore, data from these two groups were combined and labeled WT.

Because traditional homozygous BDNF knockout mice die shortly after birth (Jones et al., 1994; Ernfors et al., 1995; Conover and Yancopoulos, 1997), graft tissue lacking BDNF was collected from conditional BDNF knockout mice created using Cre-lox technology. Cre-recombinase was expressed under one of two different promoters. Mice in which a ubiquitous tamoxifen-inducible Cre-recombinase was expressed (strain B6.Cg-Tg(Thy1-Cre/ESR1, -EYFP)AGfng/J; stock no. 007606; The Jackson Laboratory) (Hayashi and McMahon, 2002) were bred to floxed BDNF mice in which loxP sites were inserted around *bdnf* exon 8 to create inducible BDNF null mice (Rauskolb et al., 2010). Offspring from these matings were treated with tamoxifen to induce ubiquitous Cre expression and excision of the BDNF gene in all tissues (tam-Cre::BDNF^{f/f} Tx). After tamoxifen administration, tam-Cre::BDNF^{f/f} Tx mice had an average weight of 38 grams; whereas the average weight for all other mouse genotypes was 22 grams. This weight gain may be due to a reduction in BDNF signaling in the hypothalamus (Unger et al., 2007). Nerve size did not appear to be altered by the elevated weight of these donor mice. Transgenic mice containing a construct that expresses Cre recombinase under the control of an exogenous promoter for ciliary

neurotrophic factor (CNTF-Cre::BDNF^{f/f}) were bred to the same floxed BDNF mice described above to produce offspring in which BDNF is knocked out selectively in CNTF-expressing cells. Mice with only one floxed BDNF allele (tam-Cre::BDNF^{f/w} Tx and CNTF-Cre::BDNF^{f/w}) were used to examine the effects of reduced BDNF. Transgenic mice not treated with tamoxifen (tam-Cre::BDNF^{f/f} UT and tam-Cre::BDNF^{f/w} UT) were used as additional controls. The lack of BDNF in tam-Cre::BDNF^{f/f} Tx mice was confirmed using fluorescent in situ hybridization (FISH) (Figure 1C). To evaluate the expression of Cre in CNTF-Cre mice, we used a Cre-recombinase reporter mouse, the tomato mouse described above, as a measure of Cre-expression. The presence of GFP+ Schwann cells in the sciatic nerve of CNTF-Cre::tomato mice (Figure 1 D-E) confirms that Cre-recombinase is expressed in a cell-type specific manner and, by analogy, that BDNF is knocked out selectively in CNTF-Cre::BDNF^{f/f} mice. RT-PCR was performed on the distal stumps of transected TIB and CF nerves from CNTF-Cre::BDNF^{f/f} and WT mice two weeks post-transection to further assess the BDNF content of the nerves of these mice. In nerves from WT mice, a 25.0 ± 7.3 (SEM) fold increase was found in BDNF mRNA, relative to that in nerves from CNTF-Cre::BDNF^{f/f} mice. These RT-PCR results combined with the data from the reporter mice support our assumption that BDNF is highly reduced in CNTF-expressing Schwann cells in CF and TIB nerves of CNTF-Cre::BDNF^{f/f} mice.

Tamoxifen administration

Powdered tamoxifen (Sigma-Aldrich) was dissolved in a solution consisting of 10% ethanol (Sigma-Aldrich) and 90% sunflower oil (Sigma-Aldrich) and heated to 37°C. The tamoxifen was stored at 4°C for three days. Adult tam-Cre::BDNF and SLICK mice were administered this tamoxifen solution orally, at a dose of 0.75mg/20g BW per day for three days. The SLICK mice were administered a second round of tamoxifen two weeks after the original dosing because a second dose has been reported to be necessary to achieve complete knockout in these animals (Young et al., 2008). All mice were used in experiments a minimum of two weeks after final tamoxifen administration.

Treadmill training

Beginning the third day post-nerve transection and repair, mice were treadmill trained for five days a week for two weeks using either an interval or continuous paradigm (Sabatier et al., 2008). We have previously reported a sex difference in the elongation of axons in response to treadmill training (Wood et al., 2011). In these experiments, we used the training paradigm appropriate to each sex that has been shown to enhance axon regeneration. Thus, male mice were trained continuously for one hour at a slow speed of 10 m/min (continuous training), and female mice performed four repetitions at a faster speed of 20 m/min for two minutes with a 5-minute rest (interval training). There was no statistically significant difference in the values obtained from males and females, thus, the data from both sexes were combined.

Tissue collection

Animals were deeply anesthetized with pentobarbital (150 mg/kg, i.p.) and transcardially perfused with 0.9% saline and 4% paraformaldehyde. Sciatic nerves and spinal cord segments L3-L5 were harvested and cryoprotected for 3 days in 20% sucrose. Both CF and TIB nerves were mounted onto slides using VectaShield and stored at 4°C. Fixed spinal cord segments (L3-L5) were collected to analyze the amount of BDNF in the tissue. The spinal cord segments were cut (transverse sections; 20µm thick) on a cryostat (Reichert-Jung) and stored at -50°C.

Axon length analysis

The confocal imaging and measurements of regenerating axon profiles were performed as previously described (English et al., 2005; Groves et al., 2005). Briefly, the nerves were imaged at a low magnification using a Zeiss LSM510 confocal microscope (Oberkochen, Germany). Stacks of serial optical sections were (10 μ m thick) were stitched together using Adobe Photoshop to reconstruct the entire length of the repaired nerve and graft. The lengths of YFP⁺ axon profiles were measured using Image Pro Plus or ImageJ software.

Fluorescence In Situ Hybridization (FISH)

The expression of BDNF mRNA was examined using FISH as previously described with modifications (Muddashetty et al., 2007). BDNF riboprobes of 745bp corresponding to nt485-1229 of the mouse BDNF (GenBank accession number EF125685) were generated from linearized pcDNA3-BDNF plasmid by *in vitro* transcription with DIG RNA Labeling Mix (Roche). T7 and SP6 RNA polymerases were used to obtain both antisense and sense-labeled probes, respectively. Riboprobes were alkaline hydrolyzed to reduce the size. The spinal sections were prehybridized in hybridization buffer (4X SSC, 50% deionized formamide, 1X Denhardt's solution, 10% dextran sulphate, 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml yeast t-RNA) at 55 °C for 1 hr before they were incubated with DIG-labeled antisense and sense riboprobes at 55 °C overnight. The hybridized DIG-probe labeled sections were detected using a Cy3-conjugated mouse anti-DIG antibody (1:200; Jackson ImmunoResearch, West Grove, PA). Images were acquired on a Zeiss LSM510 confocal microscope (Oberkochen, Germany) using a 20x objective.

RT-PCR

The dissected sciatic nerves were subjected to RNA extraction using TRIzol® reagent (Invitrogen). Reverse transcription was done with random hexamer primers by Superscript III First-Strand Synthesis System (Invitrogen). Real-time PCR for BDNF mRNA from the sciatic nerves was performed as previously described (Donnelly et al., 2011; Willis et al., 2011). In brief, the reverse-transcribed RNA was amplified using Prism7000 Sequence Detection System (Applied Biosystems) with QuantiTect SYBR Green PCR Kits (QIAGEN). The following primers were used for real-time PCR amplification: mouse BDNF (forward: 5'-CGGCGCCCATGAAAGAAGTA-3'; reverse: 5'-AGACCTCTCGAACCTGCCCT-3') and mouse 18S rRNA (forward: 5'-TTAACGAGGATCCATTGGAG-3'; reverse: 5'-GGCCTGCTTTGAACACTCTA-3'). Relative cycle threshold quantification ($\Delta\Delta$ Ct) was performed using 18S rRNA as endogenous control to obtain Δ Ct and then Δ Ct from the knockout nerve tissue being normalized to the one from wildtype to obtain $\Delta\Delta$ Ct. Fold change was expressed by $2^{-\Delta\Delta$ Ct}.

Statistical analysis

Data are expressed as the average \pm SEM. Differences in the number of axons as well as the median length of axon profiles were assessed using a one-way analysis of variance (ANOVA) with Fisher's Least Significant Differences post hoc analysis ($\alpha < 0.05$) where appropriate. A Student's t-test was used to assess differences based on the sex of the host, the nerve cut and repaired, and the limb used. Because axon profile lengths are not normally distributed (English et al., 2005), the differences between axon profile length populations were examined using the Kolmogorov-Smirnov two sample test. Using this non-parametric method, the probability that two distributions measured could be samples drawn from the same population is determined. Its use requires no prior assumptions about the distributions of the data.

Results

Axon regeneration is reduced significantly in grafts lacking BDNF

The CF and TIB nerves of *thy-1-YFP-H* mice were cut and repaired with 8-10 mm long grafts harvested from the corresponding nerve in a mouse lacking both BDNF alleles in all cells (tam-Cre::BDNF^{f/f} Tx), a mouse missing one BDNF allele in all cells (tam-Cre::BDNF^{f/w} Tx), or a mouse with both BDNF alleles in all cells (WT, tam-Cre::BDNF^{f/f} UT, or tam-Cre::BDNF^{f/w} UT). Two weeks post-repair, host mice were euthanized, and the regeneration of YFP⁺ axon profiles was analyzed using confocal microscopy. We found that the lengths of axon profiles from grafts lacking BDNF (tam-Cre::BDNF^{f/f} Tx) are significantly decreased, as demonstrated by a significant shift in the distribution to the left of data from *thy-1-YFP-H* mice whose cut axons regenerated into grafts from WT mice (p 0.003, Figure 2A). Regenerating axons grow readily into (BDNF-containing) grafts from WT donors, but most axons growing into grafts from BDNF^{-/-} donors stop just distal to the surgical repair site. We also analyzed the median lengths of axon profiles growing into each graft type (Figure 2B). The average median length of axon profiles from nerves repaired with grafts from tam-Cre::BDNF^{f/f} Tx mice was significantly shorter than that in nerves repaired with grafts from WT, tam-Cre::BDNF^{f/f} UT, or tam-Cre::BDNF^{f/w} UT mice (p 0.0006) or from tam-Cre::BDNF^{f/w} Tx mice (p 0.0001). No significant difference was found in the distributions of axon profile lengths or in the median lengths of axon profiles growing into WT, tam-Cre::BDNF^{f/f} UT, or tam-Cre::BDNF^{f/w} UT, or tam-Cre::BDNF^{f/w} Tx grafts. The lack of difference in axon growth into WT and BDNF^{f/w} environments is not surprising because this effect has been reported in previous papers where traditional BDNF heterozygotes were used as graft donors (English et al., 2005). Thus, we feel that these data are strong evidence that BDNF in the environment surrounding regenerating axons (the graft) is necessary for elongation of axon profiles after nerve cut and repair.

To isolate the cellular source of BDNF important for axon regeneration into a graft, we generated transgenic mice that lack BDNF only in CNTF-Cre-recombinase expressing cells. Grafts from CNTF-Cre::BDNF^{f/f} mice lack BDNF in CNTF⁺ Schwann cells. Similar to the results with the tamoxifen-inducible BDNF^{f/f} mice, the distribution of axon profile lengths growing into CNTF-Cre::BDNF^{f/f} grafts is significantly shifted to the left (Figure 2C) compared to WT (p 0.004) or heterozygous CNTF-Cre::BDNF^{f/w} grafts (p 0.01). No difference was found in the median length of axon profiles or in the population distributions of axons growing into WT or CNTF-Cre::BDNF^{f/w} grafts. The median length of axon profiles growing into CNTF-Cre::BDNF^{f/f} grafts was significantly shorter than those growing into WT (p 0.0001) or CNTF-Cre::BDNF^{f/w} grafts (p 0.002). Axons did not grow very far into the grafts lacking BDNF in Schwann cells (Figure 3E). When considered together with the tam-Cre::BDNF^{f/f} data presented above, we conclude that Schwann cell-derived BDNF is critical for the elongation of axons into nerve grafts after nerve injury.

Exogenous BDNF enhances axon regeneration

To examine whether the lack of growth into BDNF lacking grafts could be reversed by the exogenous administration of BDNF, we performed a series of experiments in which recombinant human BDNF was added at the time of surgery to the fibrin glue used to secure the repaired nerve. If BDNF is important for the elongation of axons into a graft after nerve injury, then we hypothesized that axons would grow into BDNF-lacking grafts if the neurotrophin was added to the repair site. We found that adding a presumed pharmacologic dose of BDNF to the fibrin glue at the time of repair enhanced the growth of axons, shifting the distribution of WT axon profile lengths significantly to the right compared to data from animals repaired similarly but without BDNF treatment (p 0.04, Figure 3A). This shift to the right was similar to that found when BDNF was added to grafts from transgenic mice

lacking BDNF ($p = 0.0001$). This trend towards longer axons was also reflected in a very large increase in the median axon profile lengths of axons growing into BDNF-treated WT grafts (Figure 3B; $p = 0.0001$) and BDNF-treated CNTF-Cre::BDNF^{f/f} grafts ($p = 0.0001$). There is no significant difference in the lengths of axon profiles growing into WT grafts treated with BDNF and CNTF-Cre::BDNF^{f/f} grafts treated with BDNF. The dramatic elongation of axons after the addition of BDNF is similar to data reported previously (English et al., 2005) regarding the addition of BDNF to wild type grafts. In both the current and previously published experiments, the addition of BDNF produces axons that grow much longer than similarly repaired nerves without treatment. Thus, the addition of BDNF at the time of nerve cut results in elongation of axon profiles even in the absence of Schwann cell derived BDNF.

Treadmill training enhances axon regeneration

Previous studies have shown that BDNF is upregulated after exercise (Gomez-Pinilla et al., 2002). To determine if the source of the increased BDNF is Schwann-cell derived, we performed a set of experiments in which we treadmill-trained *thy-1-YFP-H* mice with cut CF and TIB nerves repaired with CNTF-Cre::BDNF^{f/f} or WT grafts. All host mice ran 5 days a week for 2 weeks on a level-incline treadmill beginning 2 days post-nerve transection. The cumulative population of axon profile lengths for trained WT ($p = 0.03$) and CNTF-Cre::BDNF^{f/f} ($p = 0.0002$) mice was significantly shifted to the right indicating a larger number of long axons compared to untrained mice (Figure 3C). No difference was found in the length of axon profiles growing into either graft type in trained hosts. The median axon profile lengths in trained WT ($p = 0.0001$) and trained CNTF-Cre::BDNF^{f/f} ($p = 0.0001$) are significantly longer than untrained controls (Figure 3D). Thus, treadmill training results in an enhancement of axon regeneration despite the lack of Schwann cell derived BDNF from the graft which was shown earlier to stimulate axon regeneration. The enhanced growth of axons into grafts lacking BDNF found in trained mice could be due to the upregulation of factors other than BDNF in the graft or an upregulation of BDNF in the regenerating neurons themselves.

Treadmill training upregulates BDNF mRNA in motoneurons

To test the hypothesis that treadmill training upregulates BDNF expression in motoneurons, we used FISH to detect BDNF mRNA in lamina IX cells of the spinal cord. FISH was performed on tissue from spinal segments L3-L5 from trained and untrained WT and tam-Cre::BDNF^{f/f} Tx mice whose CF and TIB nerves were cut and repaired. Significantly more BDNF mRNA signal was found in intact and trained WT mice (Figure 1B) compared to intact and untrained WT mice (Figure 1A). The distribution of the cells labeled as BDNF mRNA positive also changed with training. More small cells surrounding lamina IX were labeled after training in WT mice. No fluorescence was found in either trained or untrained tam-Cre::BDNF^{f/f} Tx mice (Figure 1C), consistent with the idea that the transgenic mice are BDNF null in all tissues. The FISH data, considered together with the finding that axons elongate into BDNF-lacking grafts when the hosts are treadmill trained, are evidence that the training paradigm used may be adequate to upregulate motoneuron BDNF to levels sufficient to overcome any lack of BDNF in Schwann cells in the pathway taken by the axons.

Treadmill-training induced axon regeneration is dependent on neuron-derived BDNF

To test the hypothesis that neuronal BDNF is sufficient to produce training-mediated axon elongation, we created a transgenic mouse in which BDNF was null in a subset of axons that also contain YFP. Using these SLICK::BDNF^{f/f} mice as hosts, we were able to measure the length of BDNF-lacking, YFP⁺ axon profiles growing into BDNF rich (WT) or BDNF null (tam-Cre::BDNF^{f/f} Tx) grafts (Figure 4). Marked axons of SLICK::BDNF^{f/f} hosts grow into

WT grafts in which Schwann cells produce BDNF. Axons from SLICK::BDNF^{f/f} mice marked by YFP do not grow very far into grafts lacking Schwann cell BDNF. The entire population of YFP⁺ SLICK::BDNF^{f/f} axons growing into grafts from tam-Cre::BDNF^{f/f} Tx mice is shifted significantly to the left of the population of YFP⁺ SLICK::BDNF^{f/f} axons growing into WT grafts (Figure 4A; $p = 0.04$). Thus, a lack of BDNF in both the neurons and the graft leads to an increase in short versus long axons growing into the graft. Hosts that lacked only one BDNF allele (SLICK::BDNF^{f/w}) were used as controls and grew in patterns similar to the BDNF-rich, *thy-1 YFP-H* hosts. Similar to the *thy-1 YFP-H* hosts, untrained SLICK::BDNF^{f/w} hosts did not grow as far into BDNF lacking grafts (BDNF^{f/f}) compared to WT grafts ($p = 0.04$), again demonstrating the importance of Schwann cell derived BDNF in axon regeneration. However, axons in treadmill trained SLICK::BDNF^{f/w} hosts did successfully grow into BDNF-lacking grafts compared to untrained SLICK::BDNF^{f/w} hosts (Figure 4D; $p = 0.0005$). Axons in treadmill trained SLICK::BDNF^{f/f} hosts grew longer into WT grafts than did axons from untrained SLICK::BDNF^{f/f} mice ($p = 0.0001$), with a larger overall population of long axons (Figure 4C; $p = 0.05$). These findings are similar to those of axons in *thy-1-YFP-H* hosts growing into wild type and BDNF null grafts. However, unlike axons from treadmill trained *thy-1-YFP-H* host mice, axons in treadmill trained SLICK::BDNF^{f/f} hosts do not grow into grafts lacking Schwann cell BDNF. The significant shift left in the cumulative histogram for these axons confirms a larger percentage of very short regenerating axons (Figure 4C; $p = 0.01$). There is no significant difference among treadmill trained SLICK::BDNF^{f/f} hosts growing into WT grafts and treadmill trained SLICK::BDNF^{f/w} hosts growing into WT or BDNF^{f/f} Tx grafts. Thus, in the absence of Schwann cell-derived BDNF in the pathway taken by regenerating axons, increased expression of neuron-derived BDNF is necessary to drive optimal regeneration. We conclude that both axonal and Schwann cell sources of BDNF are necessary for axon regeneration.

BDNF content in the regenerating axons themselves or in the cells of the distal stump does not affect regenerative sprouting

In addition to the elongation of axons, we investigated whether the lack of BDNF in the graft or axons affects regenerative sprouting. We assessed the number of YFP⁺ axons whose lengths were measured in the grafts as a ratio of the total number of YFP⁺ axons in the proximal stump. This sprouting index was used as a global estimate of the number of branches per axon (Sabatier et al, 2008). We found no significant difference in the sprouting index regardless of the graft type or host model used (data not shown). Thus, the lack of BDNF production by cells in the segment of nerve distal to the transection or by the axons themselves did not affect the number of regenerative sprouts forming from the segment of the nerve proximal to the transection.

Discussion

In this paper we found that BDNF derived from either Schwann cells or motoneurons can facilitate axon regeneration. Two weeks following nerve transection and repair, the lengths of profiles of regenerating axons growing into grafts lacking BDNF in Schwann cells were strikingly shorter than those growing into wild type grafts. The addition of recombinant human BDNF at the site of repair restored axon regeneration in the absence of Schwann cell-derived BDNF and enhanced the length of regenerating axons into both wild type and BDNF null grafts dramatically. Thus, we concluded that BDNF derived from Schwann cells in the pathway surrounding regenerating axons is important for their elongation after nerve transection. If the host animal is treadmill trained after injury, then motoneuron-derived BDNF can enhance axon regeneration irrespective of the pathway surrounding the axons. Two weeks of daily treadmill training after nerve transection results in enhanced axon

elongation, likely due to an increased expression of BDNF mRNA in motoneuron cell bodies. Selectively knocking out BDNF in neurons blocked the enhancing effect of treadmill training on axon elongation into grafts from Schwann cells lacking BDNF. We infer from these data that motoneuron-derived BDNF mediates the effect of treadmill training on elongation of regenerating axons after nerve injury.

We propose a model of axon regeneration in peripheral nerves in which two distinct mechanisms involving BDNF released from Schwann cells and neurons may be involved. Following peripheral axotomy, Schwann cells in the nerve segment distal to the injury synthesize and secrete BDNF into the Schwann cell basal laminae (Funakoshi et al., 1993). Regenerating axons entering endoneurial tubes in the distal segment initially use this Schwann cell-derived BDNF to begin elongation. Spinal motoneurons contain BDNF and *trkB* mRNAs (Al-Majed et al., 2000; Buck et al., 2000). After axotomy, spinal motoneurons increase the expression of BDNF relatively quickly, but expression levels decrease within one week (Funakoshi et al., 1993). Schwann cells in the distal stump, however, can synthesize and secrete BDNF for two months or longer (reviewed in (Gordon, 2009)). Since the transformed Schwann cells in the pathway of regenerating axons do not contain a full-length *trkB* receptor (Frisen et al., 1993), the stimulation of axon elongation is via a classic retrograde signaling pathway from the Schwann cells via *trkB* receptors on the regenerating axons. Treadmill training after nerve transection results in an increased production of neuronal BDNF. We propose a second neurotrophin signaling mechanism whereby neuronal expression of BDNF produced by treadmill training enhances the elongation of axons through the cut site using an autocrine signaling mechanism. This second neurotrophin mechanism of elongation is sufficient to overcome a lack of BDNF in the distal stump.

Using Cre-lox gene deletion technology, we created mice in which BDNF was greatly reduced in CNTF-expressing Schwann cells. When tissue from these animals was used to repair nerves after transection, axon regeneration was inhibited. In previous studies a transient upregulation of BDNF in the motoneuron cell bodies (Funakoshi et al., 1993) as well as skeletal muscles (Madison et al., 2009) has been reported after nerve transection; however, clearly these sources of BDNF are not sufficient to overcome the lack of BDNF in these grafts. Contributions from Schwann cells in the proximal stump also do not seem to play an important role in BDNF-mediated axonal elongation as experiments in which both axons and grafts lacked BDNF resulted in little growth despite the presence of WT levels of BDNF in the Schwann cells in the proximal stump. Using RT-PCR analysis of CNTF-Cre::BDNF^{f/f} nerves, we show that a small amount of BDNF mRNA may be present in the distal stumps of transected CF and TIB nerves. The source of the BDNF in this tissue is unknown. The most likely explanations are that it could be due to incomplete deletion of the *bdnf* gene by Cre-recombinase in CNTF-expressing Schwann cells (Nagy, 2000) or to cells not expressing CNTF (and Cre). Regardless of the source, the remaining BDNF is 25 fold lower than levels found in WT nerves and is insufficient to encourage axon growth into the CNTF-Cre::BDNF^{f/f} nerve graft. Thus, we conclude that Schwann cell-derived BDNF in the pathway taken by regenerating axons acting on receptors on those axons must be the primary mechanism by which axons regenerate in cut nerves. When animals are treadmill trained, however, a more pronounced and prolonged production of BDNF by neurons is sufficient to allow for autocrine signaling to occur independent of Schwann cell-derived BDNF.

Consistent with previous findings, we found that treadmill training produced longer axon profiles (Sabatier et al., 2008). Regardless of the presence or absence of BDNF in Schwann cells in the distal stump, these axon profiles grew almost as long as axons growing into grafts treated with BDNF at the time of surgical repair. This elongation of axons likely is due to the upregulation of BDNF mRNA in motoneuron cell bodies after training. Removing

BDNF from neurons results in axons that can no longer grow into grafts lacking Schwann cell derived-BDNF when the host is treadmill trained. This finding that motoneuron upregulation of BDNF can mediate axon elongation is supported by similar findings in experiments using electrical stimulation after nerve cut. Electrically stimulating the proximal stump of a cut nerve produces a large increase in motoneuron BDNF expression and a concomitant enhancement of axon elongation (Al-Majed et al., 2000). When the cut nerve is repaired with an acellular graft lacking neurotrophins, electrically stimulated axons can still regenerate (English et al., 2007). Thus, with training or electrical stimulation, the upregulation of BDNF in motoneurons is sufficient to promote regeneration of axons even in the absence of Schwann cell BDNF.

We found that treadmill training enhances the elongation of BDNF-lacking axons growing into wild type grafts but not into BDNF-lacking grafts. One explanation of this finding is that the training also could increase the expression of trkB receptors on the regenerating axons. Such an increase in trkB expression would make the regenerating axons more sensitive to Schwann cell-derived BDNF, thereby increasing the length of axons. Consistent with this idea, axon regeneration in trkB^{+/-} mice is limited (Boyd and Gordon, 2001; MacGillivray, 2003; Lee et al., 2004), where the regenerating axons would be presumed to be less sensitive to the growth promoting effects of Schwann cell-derived BDNF.

Other mechanisms also exist that could account for the changes seen with treadmill training. The activation of motoneurons during the training could upregulate BDNF enough to cause a shift in the balance between growth promotion and growth inhibition, allowing axons to overcome growth inhibitory molecules such as chondroitin sulfate proteoglycans at the surgical repair site. Once the axons enter the distal stump, BDNF-secreting Schwann cells or the axons themselves could create a gradient to facilitate the growth towards target tissues. The upregulation of BDNF by treadmill training may enhance this process by releasing more BDNF at the growth cones and activating trkB receptors in an autocrine fashion.

It is likely that complex interactions between regenerating axons and the regenerative environment exist. Previously, parsing apart the contributions of each to the regenerative process has been difficult and interpretation complicated. Using emerging genetic technologies to manipulate the cellular sources of neurotrophins and other growth molecules has led to a better understanding of the important role of each of these in axonal regeneration. Utilizing exercise to increase neuronal BDNF could help to improve axon regeneration and patient outcome after traumatic nerve injury.

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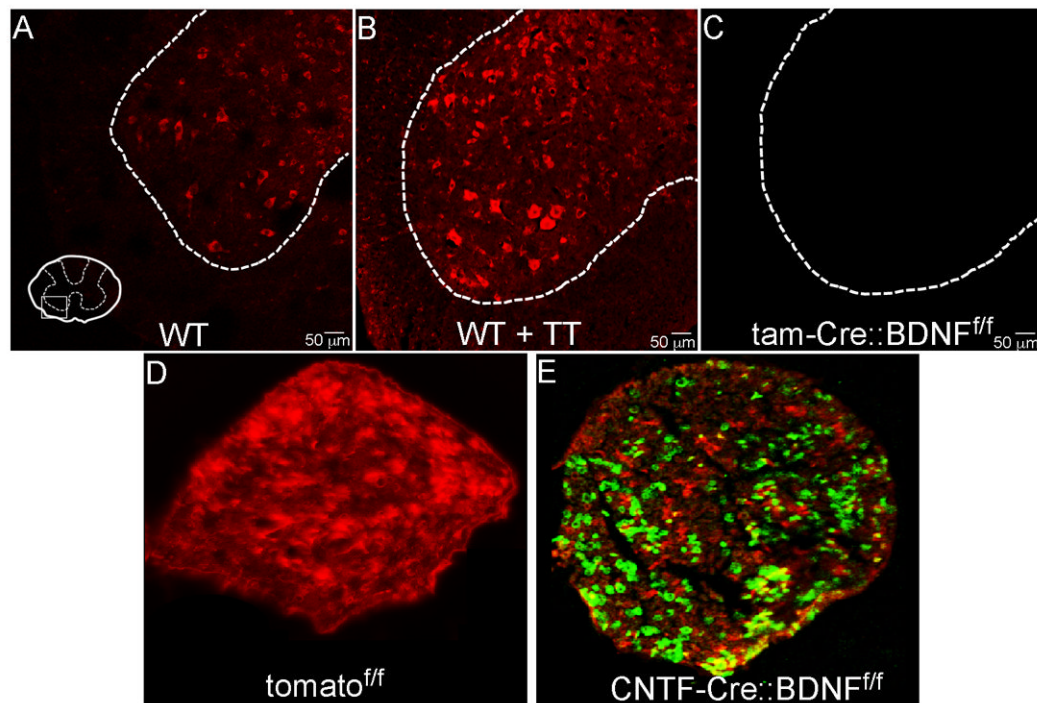


Figure 1.

BDNF mRNA is not present in cells of the transgenic knockouts used as nerve graft donors. A) Based on results of fluorescence in situ hybridization (FISH), BDNF mRNA is present in the cells in the ventral horn of the spinal cord of untreated wild type mice. B) Two weeks of treadmill training resulted in increased labeling of BDNF mRNA positive cells in wild type mice. C) No BDNF mRNA was detected in tissues collected from tam-Cre::BDNF^{f/f} mice, regardless of training condition. D) BDNF allele deletion for the CNTF-Cre::BDNF^{f/f} mice was assessed using a Cre reporter mouse termed tomato. All cells in the sciatic nerve of the tomato mouse are tomato fluorophore positive. E) After expression of Cre-recombinase, Cre+ cells express green fluorescent protein. The presence of green fluorescence in presumptive Schwann cells indicates that Cre-recombinase is active in cells of the sciatic nerve of CNTF-Cre expressing mice. These data support the idea that Cre activity in CNTF⁺ cells in CNTF-Cre::BDNF^{f/f} mice removes BDNF from CNTF⁺ cells.

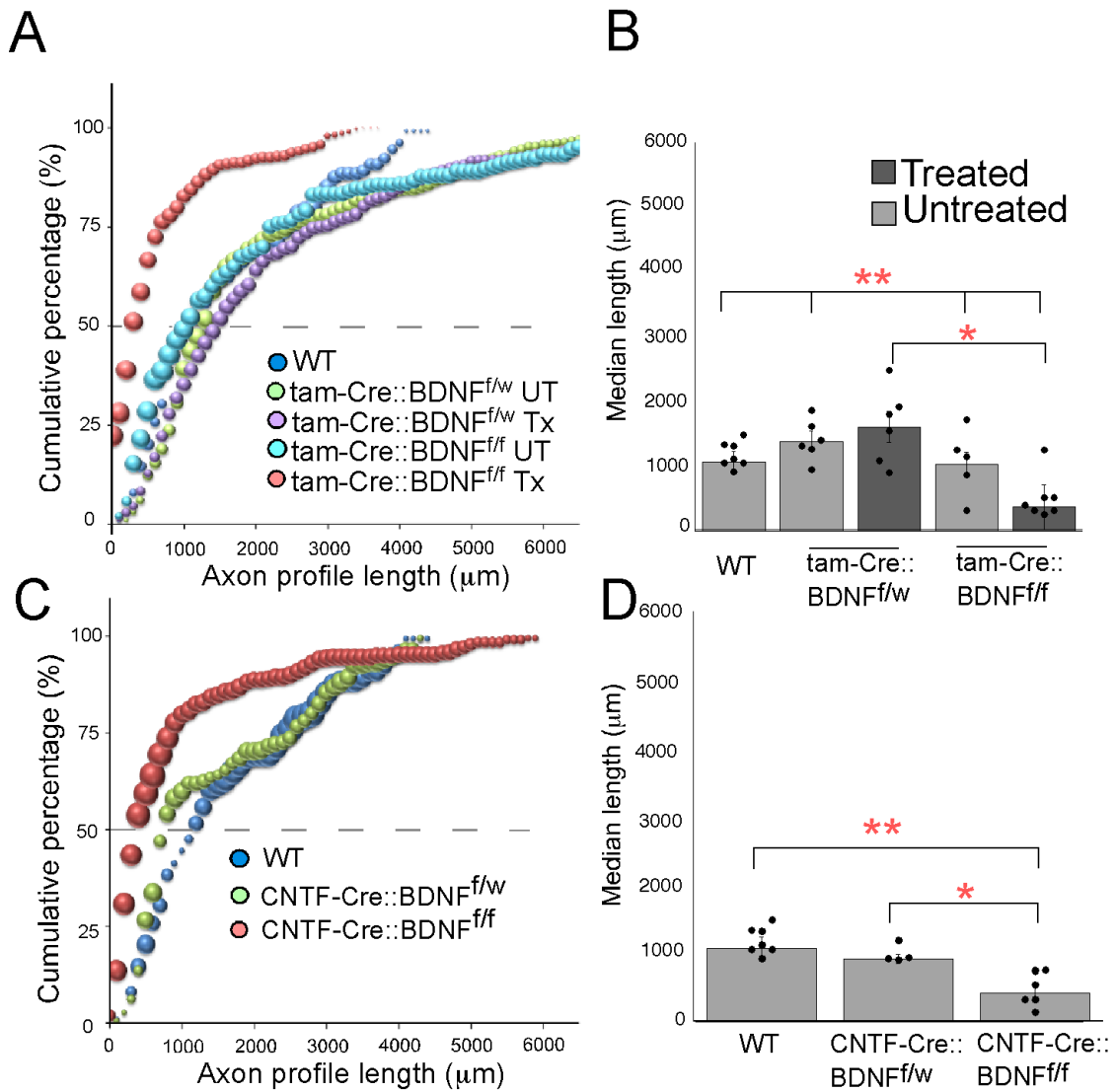


Figure 2. Profiles of axons growing into grafts lacking BDNF (*tam-Cre::BDNFf/f* treated and *CNTF-Cre::BDNFf/f*) are significantly shorter than controls. **A, C**, The cumulative frequency histogram of profile lengths of axons growing into *tamCre::BDNFf/f*-treated (**A**) and *CNTF-Cre::BDNFf/f* (**C**) grafts are significantly shifted to the left compared with controls, indicating an overall decrease in profile lengths. For all cumulative histograms, the size of each symbol is proportional to the SEM of that data point and the dashed line indicates the 50th percentile. **B**, The median length of axon profiles growing into *tam-Cre::BDNFf/f*-treated grafts are significantly reduced compared to those growing into WT and untreated grafts (***p* .0006) and *tam-Cre::BDNFf/w Tx* grafts (**p* 0.0001). **D**, The median length of axon profiles from *CNTF-Cre::BDNFf/f*-treated grafts are significantly reduced compared with the lengths of regenerating axon profiles growing into grafts containing BDNF (***p* 0.0001 compared to WT, **p* 0.002 compared to *CNTF-Cre::BDNFf/w*). For all bar graphs, circles represent individual data points and error bars represent SEM.

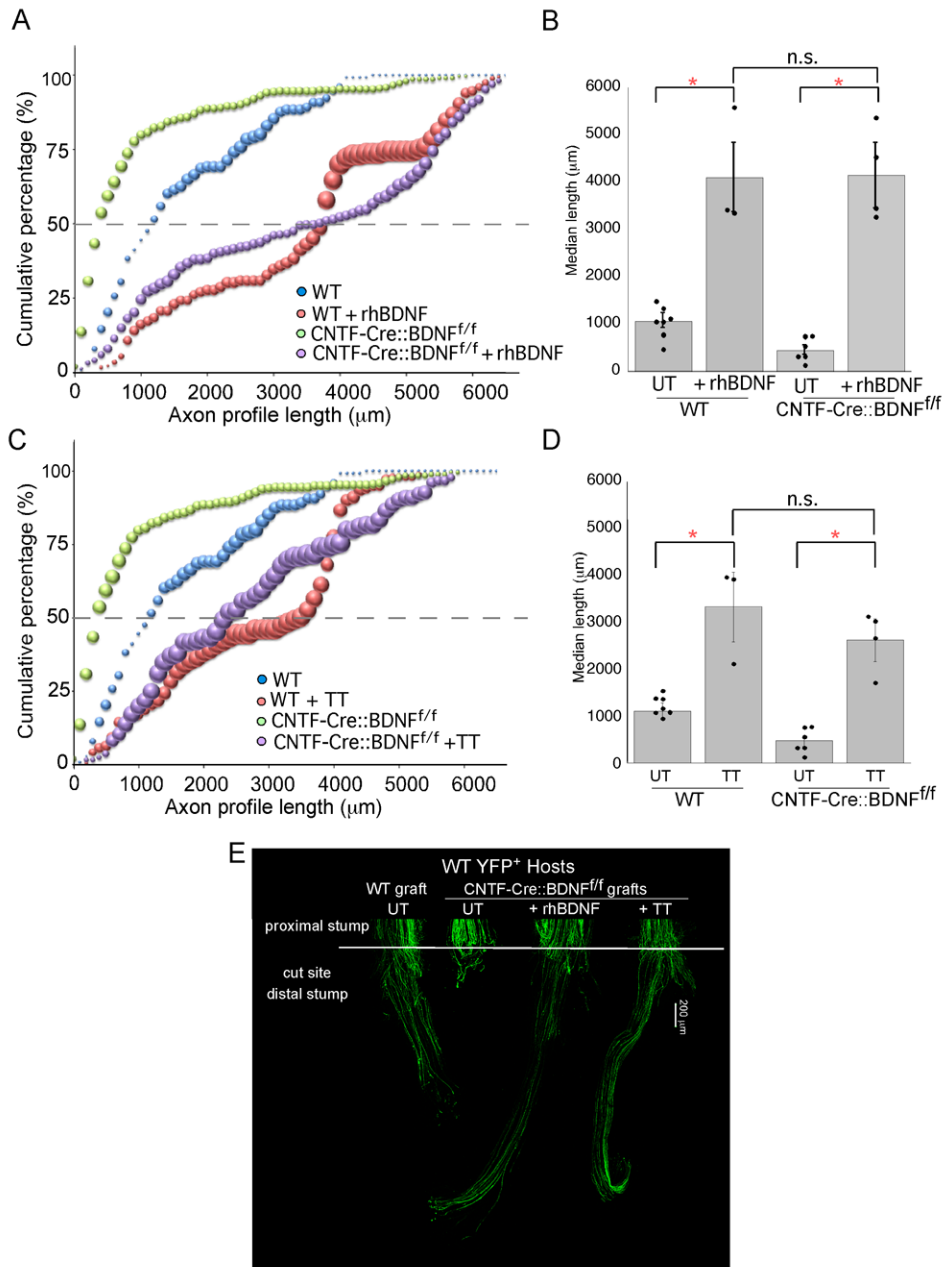
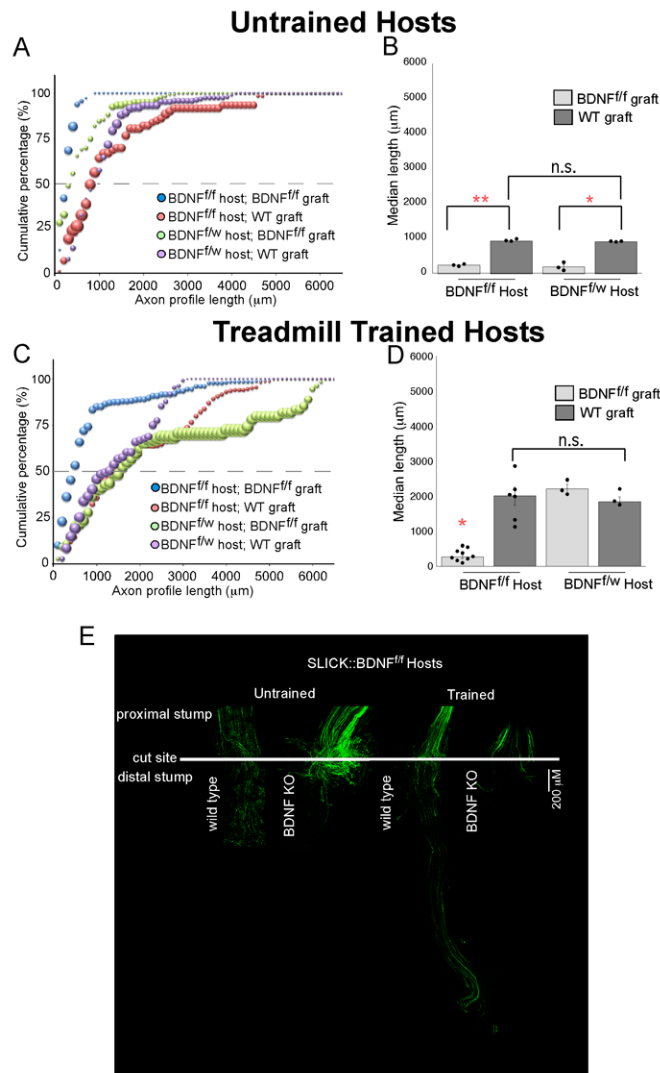


Figure 3. Two weeks of treadmill training or the administration of BDNF to the surgical repair site significantly enhances axon profile lengths. A) The cumulative histogram of profile lengths of axons growing into wild type or CNTF-Cre::BDNF^{f/f} grafts treated with rhBDNF is shifted significantly to the right of those from mice whose nerves were repaired with either untreated wild type or CNTF-Cre::BDNF^{f/f} grafts, suggesting that their axons are significantly longer. B) The median axon profile lengths are significantly longer into BDNF-treated wild type and CNTF-Cre::BDNF^{f/f} grafts compared to controls (* p < 0.0001). C) The cumulative histograms of the lengths of axon profiles in wild type and CNTF-Cre::BDNF^{f/f} mice are shifted significantly to the right of those of control mice after treadmill training, indicating a significant increase in the lengths of all regenerating axon

profiles. D) The median axon profile length of wild type and CNTF-Cre::BDNF^{fl/fl} is significantly increased after treadmill training (* p < 0.0001). E) Low magnification images of representative nerves from *thy-1-YFP* hosts two weeks after nerve repair. The white bar across the nerve indicates the site of surgical repair.

**Figure 4.**

Axons lacking BDNF do not significantly regenerate into BDNF-lacking grafts regardless of the training. A) The cumulative histograms of axon profile lengths of SLICK::BDNF^{f/f} and SLICK::BDNF^{f/w} axons growing into tam-Cre::BDNF^{f/f} Tx grafts are shifted significantly to the left of those of controls, suggesting that these axons are significantly shorter than those growing in WT grafts ($p = 0.04$). B) The median axon profile lengths are significantly shorter into BDNF^{f/f} grafts compared to WT (* $p = 0.009$; ** $p = 0.0001$). C) The cumulative histogram of the lengths of axon profiles of YFP⁺ SLICK::BDNF^{f/f} axons is significantly shifted to the left when nerves are repaired with BDNF^{f/f} grafts and hosts are treadmill trained for two weeks post-repair ($p = 0.01$). D) The median axon profile length of SLICK::BDNF^{f/f} axons growing into BDNF^{f/f} grafts remains significantly decreased after treadmill training compared to SLICK::BDNF^{f/f} axons growing into a WT graft ($p = 0.0001$). E) Low magnification images of representative nerves from trained and untrained SLICK::BDNF^{f/f} hosts two weeks after nerve repair. The white bar across the nerve indicates the site of surgical repair.

Table 1

Transgenic mice were used to assess the roles of BDNF in axon regeneration. Multiple transgenic mice with floxed BDNF genes were used to manipulate the presence of BDNF in neurons and Schwann cells.

Genotype	Treatment tamoxifen (Tx) or saline (UT)	Host mouse or nerve donor	BDNF Alleles
WT, tomato	Tx or UT	Donor	Systemic BDNF +/+
<i>thy-1-YFP-H</i>	UT	Host	Systemic BDNF +/+
Tam-Cre::BDNF ^{f/f}	Tx	Donor	Systemic BDNF -/-
Tam-Cre::BDNF ^{f/f}	UT	Donor	Systemic BDNF +/+
Tam-Cre::BDNF ^{f/w}	Tx	Donor	Systemic BDNF +/-
Tam-Cre::BDNF ^{f/w}	UT	Donor	Systemic BDNF +/+
CNTF-Cre::BDNF ^{f/f}	UT	Donor	CNTF ⁺ cells BDNF -/-, other cells BDNF+/+
CNTF-Cre::BDNF ^{f/w}	UT	Donor	CNTF ⁺ cells BDNF +/-, other cells BDNF+/+
SLICK::BDNF ^{f/f}	Tx	Host	YFP ⁺ neurons BDNF -/-, other cells BDNF +/+
SLICK::BDNF ^{f/w}	Tx	Host	YFP ⁺ neurons BDNF +/-, other cells BDNF +/+