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Sex Differences in Neurotrophin Expression After Treadmill Training Following Peripheral Nerve Injury

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Abstract
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Modest daily treadmill training applied following traumatic injury to peripheral nerves results in an enhancement of subsequent axon regeneration. This enhancement is found only in males. Since an increase in brain derived neurotrophic factor (BDNF), neurotrophin 4/5 (NT-4/5), and their common receptor, trkB, in spinal motoneurons has been proposed to underlie this enhancement, the purpose of this study was to evaluate the sex-dependence of treadmill training in inducing the expression of these molecules in axotomized motoneurons. After cutting their sciatic nerves, male and female mice were treadmill trained for one hour daily for two weeks. Histological sections through the L3-L5 regions of the lumbar spinal cords of euthanized mice were processed for the demonstration of BDNF or NT-4/5 mRNA using fluorescent in situ hybridization, or for trkB receptors using immunohistochemistry. Overall BDNF mRNA, NT-4/5 mRNA, and trkB expression was determined by measuring motoneuron mean fluorescence intensity, soma size, and the number of positively labeled motoneurons. Mean fluorescence intensity and soma size of lamina IX motoneurons were determined for both sexes after BDNF mRNA, NT-4/5 mRNA, and trkB assays were performed. Additionally, the mean number of BDNF mRNA, NT-4/5 mRNA, and trkB-expressing motoneurons per spinal cord section was determined for each sex. Males were found to express significantly more BDNF mRNA, NT-4/5 mRNA, and trkB by the recruitment of more BDNF mRNA, NT-4/5 mRNA, and trkB-expressing motoneurons than females. Furthermore, in males, motoneurons from the side of the spinal cord associated with the nerve transection had greater expression of BDNF mRNA at the level of the individual motoneuron.

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Introduction

Peripheral nerve injuries are a common problem that can significantly decrease a patient's quality of life by causing life-long impairment of motor function. A study conducted on 5,777 trauma patients in the United States found that 2.8% of all subjects experienced some form of peripheral nerve injury (Noble et al., 1998). Furthermore, approximately 300,000 cases of peripheral nerve injuries are reported in Europe annually. Functional recovery requires the successful migration of regenerating axons from the proximal to the distal stump, with reinnervation into the target nerve or muscle. Although axons are capable of regenerating considerably after damage, functional recovery is poor. Fewer than 10% of all patients with traumatic peripheral nerve injuries ever recover full function (Frostick et al, 1998). The poor recovery is in part due to the slow speed at which axon regeneration occurs: only as fast as up to 2 mm/day in smaller nerves and up to 5mm/day in larger nerves, as recorded in human subjects (Recknor and Mallapragada, 2006). In light of this problem, one potential target for treating peripheral nerve injuries would be to enhance axon regeneration. In one attempt to do so, Sabatier et al. (2008) found that modest daily exercise in the form of treadmill training resulted in enhanced axon regeneration following peripheral nerve injury in mice. Continuous walking at a moderate treadmill speed (10 m/min) for 1 hr/day over 2 weeks resulted in regenerating axons that were more than twice as long as those of unexercised controls.

Brain-derived neurotrophic factor (BDNF) may play a critical role in this exercise-induced enhancement of axon regeneration. BDNF is a member of the neurotrophin family of growth factors. In neurons, it acts by binding with high affinity to the trkB receptor, causing its phosphorylation and the initiation of a complex

downstream signaling cascade that contributes to axon elongation (Al-Majed et al, 2000). Increased expression of BDNF mRNA has been observed in numerous brain regions, including spinal motoneurons following voluntary exercise (Gomez-Pinilla et al., 2001). Axon regeneration cannot be enhanced by treadmill exercise if BDNF is knocked out selectively in motoneurons (Wilhelm et al., 2011). Thus, an exercise-induced upregulation of motoneuron BDNF has been proposed as the mechanism for the observed enhancement of axon regeneration (Wilhelm et al., 2011). It is also possible that neurotrophin-4/5 (NT-4/5), which shares a high affinity receptor with BDNF, could play a role in assisting axon regeneration. English et al. (2011) found that treadmill training was ineffective in enhancing axon regeneration in NT-4/5 knockout mice.

Recently, Wood et al. (2011) found that a significant enhancement of axon regeneration in cut peripheral nerves was produced by the one hour of continuous daily treadmill exercise described above in male mice but not in females. The goal of this study is to exploit this strikingly different response to the same exercise paradigm in males and females to investigate the dependence on motoneuron BDNF and/or NT-4/5 for the enhancing effects of treadmill training. Since BDNF and NT-4/5 both act by binding with high affinity to the trkB receptor, we were also interested in studying differences in motoneuron trkB expression between exercised male and female mice.

Gomez-Pinilla et al. (2001) has already shown that BDNF mRNA is upregulated in male rats after treadmill training, and Al-Majed et al. (2000) has already shown that motoneuron BDNF mRNA and trkB mRNA are both upregulated in female rats after electrical stimulation. Although relatively little is known about how NT-4/5 mRNA expression changes with exercise, there is reason to believe that it is also upregulated in

the motoneurons following exercise as well, since it has been found to in the spinal white matter after moderate treadmill exercise (Skup et al., 2002). As such, this study focused on comparing motoneuron BDNF mRNA, NT-4/5 mRNA, and trkB levels between males and females after exercise. The results reported here are from experiments designed to examine the hypothesis that increased expression of motoneuron BDNF, NT-4/5, and/or trkB underlies the enhancement of axon regeneration observed in treadmill exercised male mice. The prediction of this hypothesis is that two weeks of modest daily treadmill exercise will result in greater expression of BDNF, NT-4/5 and/or trkB in spinal motoneurons in male mice than in female mice. Motoneuron expression of BDNF and NT-4/5 mRNA in exercised male and female mice was assayed using fluorescent in situ hybridization. Immunohistochemistry was used to study trkB receptor expression in motoneurons from the same spinal cord regions.

Methods

Two experimental groups of mice were used: adult wild type male and female mice whose sciatic nerves have been transected before treadmill training. Anesthesia was administered on all mice before surgery using isoflurane gas. For nerve transection, in anesthetized mice an incision was made into the right hindlimb and then the hamstring muscle in order to access the sciatic nerve, which was severed immediately above the trifurcation of the sural, tibial, and fibular branches and left unrepaired. Incisions in the fascia and skin were closed with absorbable and nonabsorbable sutures, respectively. All mice were treadmill trained continuously for 60 minutes daily at a moderate speed of 10 m/min for 2 weeks. At this slow speed, mice walk comfortably and need little incentive to complete the required amount of treadmill training. At the end of the training period, the animals were euthanized with an overdose of pentobarbital (150 mg/Kg, IP) and then perfused with aldehyde fixatives. L3-L5 sections of the lumbar spinal cord were harvested from the euthanized animals and serial 20 μm thick frozen sections were made in a frontal plane from them.

BDNF and NT4/5 in situ *hybridizations:* Fluorescent in situ hybridization (FISH) was chosen as the preferred means of evaluating BDNF and NT-4/5 expression. Even though there are several antibodies to these molecules available, these antibodies are not particularly effective using immunofluorescence and because questions as to the specificity of the antibodies have been raised (e.g. Skup et al, 2002). Sections to be used for FISH were reacted overnight at 38ºC with digoxygenin (DIG)-conjugated, thousand-base pair antisense riboprobes against the coding region of the BDNF gene. Following this hybridization step, the tissue was reacted with an antibody to DIG followed by an

Alexafluor-conjugated secondary antibody. Spinal cord sections cut from BDNF and NT-4/5 knockout mice were reacted with the BDNF and NT-4/5 RNA riboprobes, respectively, to serve as controls to ensure to specificity of the BDNF and NT-4/5 RNA riboprobes. Images of reacted spinal cord sections were obtained using fluorescence microscopy. Between images, all microscope settings were kept constant.

trkB immunohistochemistry: Several useful antibodies to the full length trkB molecule are available, so trkB expression was studied using immunofluorescence. Sections were incubated overnight in either an anti-trkB primary antibody in incubation solution or incubation solution alone at 4°C. The following day, all sections were incubated for one hour in a secondary antibody, AlexaFluor 594 goat anti-rabbit IgG. Slides that had been incubated only with the secondary antibody served as a control to ensure that the secondary antibody did not bind preferentially to any substance. Fluorescence microscopy was used to obtain images of stained sections. Between images, all microscope settings were kept constant.

Fluorescent in situ hybridization and Immunohistochemistry Quantifications: Spinal motoneurons are found in a distinct region in the ventral horn of the spinal cord (lamina IX). Besides their location in lamina IX, motoneurons were identified on the basis of traits such as a cell nucleus and dendritic processes. It was assumed that all cells identified in the lamina IX region were motoneurons. Only the profiles of these putative motoneurons containing a distinct nucleus were analyzed. When anti-sense riboprobes were applied to spinal cord sections from BDNF or NT-4/5 knockout mice, no positively labeled cells could be seen, thus ensuring the specificity of the anti-sense RNA riboprobes. Additionally, when the fluorophore-conjugated secondary antibody was

applied alone to any tissue without the primary anti-trk-B antibody, no positively labeled cells were visible. This ensured that the fluorophore-conjugated secondary antibody did not bind preferentially to any substance. The amount of BDNF, NT-4/5, or trkB expression was measured in three ways: 1) fluorescence intensity at the level of the individual motoneuron; 2) motoneuron soma size; and 3) counting the number of motoneurons positively labeled for BDNF mRNA, NT-4/5 mRNA, or trkB per spinal cord section.

Using ImageJ software, outlines were traced around all motoneurons identified in lamina IX and fluorescence intensity and soma size were measured. A mean fluorescence intensity and a mean soma area was calculated for each animal, and averages were established across each sex so that comparisons could be made. The average number of motoneurons positively labeled for BDNF mRNA, NT-4/5 mRNA, or trkB in each spinal cord section was also determined for each animal by analyzing 5-10 sections per case while using the criteria described above for motoneuron identification. All quantifications were done under conditions of experimenter blindness. Afterwards, an average was established across each sex so that a comparison could be made.

Statistical Analyses: The unpaired t-test was used for comparisons between normal distributions, while the Mann-Whitney U test was used for comparisons between skewed distributions. Whenever either test resulted in a p-value of less than 0.05, it was concluded that there was a significant difference between the distributions being compared.

Results

All analyses were done on ventral horn motoneurons. Motoneurons were defined as the positively labeled cells (Fig. 1) in the lamina IX region of the ventral horn (Fig. 2B) that were visible at 20x magnification in tissues processed for fluorescent in situ hybridization or immunohistofluorescence. Motoneurons were also identified based on a visible nucleus or dendritic processes. It was assumed that all cells identified in the lamina IX region were motoneurons. Only the profiles of putative motoneurons containing a distinct nucleus were analyzed. When anti-sense riboprobes were applied to spinal cord sections from BDNF or NT-4/5 knockout mice, no positively labeled cells could be seen, thus ensuring the specificity of the anti-sense RNA riboprobes. Similarly, when the fluorophore-conjugated secondary antibody was applied alone to any tissue without the anti-trk-B primary antibody, no positively labeled cells were visible, thus ensuring that the the fluorophore-conjugated secondary antibody did not bind preferentially to any substance (Fig. 2A, "control" column).

Sizes of BDNF, NT-4/5, and trkB-expressing motoneurons

The cross-sectional areas of the somata of all lamina IX motoneurons judged to be labeled with one of the neurotrophin riboprobes or the antibody to trkB were measured. Bimodal distributions of all motoneuron soma areas, ranging from 100 μm^2 to 2000 μm^2, were found for BDNF, NT-4/5, and trkB-expressing motoneurons. The distributions of soma sizes of all three groups of motoneurons were skewed towards smaller cells (Fig. 3A-D) relative to that reported for all mouse motoneurons (Friese et al., 2009). It is assumed in this study that motoneurons smaller than 400 μm^2 are gamma motoneurons and innervate intrafusal fibers within muscle spindles, and motoneurons larger than 400

μm^2 are alpha motoneurons innervating extrafusal muscle fibers. This is the size cutoff used by Friese et al. (2009). The median soma size for trkB-expressing motoneurons was considerably smaller than those of BDNF mRNA and NT-4/5 mRNA-expressing motoneurons (Fig. 4A), suggesting that trkB tends to be expressed after two weeks of treadmill training only in smaller motoneurons. Motoneurons containing trkB in the larger size ranges, such as some of those those found expressing BDNF and NT-4/5 mRNA, were not encountered. The proportion of presumed gamma motoneurons in BDNF and NT-4/5-expressing motoneurons was similar, while there were a greater proportion of gamma motoneurons among trkB-expressing motoneurons (Fig. 4B). However, the three distributions of soma sizes for all BDNF, NT-4/5, and trkB-expressing motoneurons are not significantly different from one another (Mann-Whitney U test, $p>0.05$). Based on these results, it seems likely that similar populations of motoneurons are expressing BDNF, NT-4/5, and trkB following treadmill training.

Comparison of Motoneuron Sizes Between Treadmill-Trained Males and Females

Because motoneuron size distributions were skewed, the Mann-Whitney U test was used to determine whether there were any significant differences between males and female soma size distributions for BDNF, NT-4/5, and trkB-expressing motoneurons. Among the three distributions, no significant difference in soma size could be found between the sexes (Fig. 5A-C, Mann-Whitney U test, BDNF, $p>0.05$; NT-4/5, $p>0.05$; trkB, $p>0.05$).

Males express more BDNF mRNA at the level of the individual motoneuron

After performing fluorescent in situ hybridization assays for BDNF, fluorescence intensities of individual motoneurons were measured using ImageJ software in order to provide an estimate of mRNA expression.

In spinal cord sections assayed for BDNF mRNA expression, no significant difference in motoneuronal fluorescence intensity was found between males and females for both alpha and gamma motoneurons (Fig. 6A-B). There was no significant difference in BDNF mRNA expression at the level of the individual alpha motoneuron between the two sexes (two-tailed t-test, $p>0.05$). Similarly, there was no significant difference between the sexes in BDNF mRNA expression at the level of the individual gamma motoneuron (two-tailed t-test, $p>0.05$). When mean intensity measurements for all positively labeled alpha and gamma motoneurons were taken into account, there was still no significant difference in fluorescence intensities between the two sexes (Fig. 6C; two-tailed t-test, $p>0.05$). When comparing motoneurons between males and females from the side of the spinal cord contralateral to the sciatic nerve transection (Fig. 6D), no signficant difference was found in fluorescence intensity (two-tailed t-test, $p>0.05$). However, when comparing only those motoneurons from the side of the spinal cord ipsilateral to the the nerve transection (Fig. 6E), a significant difference was found between males and females (two-tailed t-test, $p=0.0365$). The average fluorescence intensity of males was 14.71% greater than that of females. These results suggest that treadmill training-induced enhancement of axon regeneration observed in males may partially be explained by a sex-dependent upregulation of BDNF mRNA expression at the level of the individual motoneuron resulting from axotomy.

Exercise leads to a greater recruitment of BDNF mRNA positive motor neurons in males

The number of positively labeled motoneurons was counted on one side of 5 to 10 sections of spinal cord for each animal studied. It is assumed that this selection of motoneurons is a representative sample of all of the motoneurons in the spinal cord that would be scored as positive for each of the three markers. A mean number of positively labeled motoneurons per section was determined for each animal, and an average was established across each sex. This measurement was considered an assay for the number of motoneurons that express the different RNAs/protein after treadmill exercise in the different sexes. After determining the average number of BDNF mRNA positive motoneurons in both treadmill trained males and females, a significantly greater number of labeled motoneurons was found in the males (Fig. 6F; two-tailed t-test, $p=0.0031$). There were approximately 26.40% more BDNF-expressing motoneurons found in each ventral horn region of the males. After treadmill training, males express significantly more BDNF mRNA than females by the recruitment of more BDNF-expressing motoneurons.

Motoneuronal expression of NT-4/5 mRNA is similar in exercised males and females

Motoneuron fluorescence intensities were measured as described before. No significant difference in motoneuronal NT-4/5 mRNA expression was found between treadmill trained males and females at the level of the individual alpha or gamma motoneuron (Fig. 7A-B). There was no significant difference between the sexes in average fluorescence intensities for either alpha or gamma motoneurons (alpha: unpaired two-tailed t-test, $p>0.05$, gamma: unpaired two-tailed t-test, $p>0.05$). After combining

fluorescence intensity measurements for all positively labeled alpha and gamma motoneurons and determining an average across each sex, still no significant difference was found (Fig. 7C; two-tailed t-test, $p>0.05$). When comparing motoneurons between males and females from the side of the spinal cord contralateral to the sciatic nerve transection (Fig. 7D), no signficant difference was found in the fluorescence intensity (two-tailed t-test, $p>0.05$). The same was found when comparing only those motoneurons from the side of the spinal cord ipsilateral to the nerve transection (Fig. 7E; two-tailed t-test, $p>0.05$). This suggests that treadmill training-induced enhancement of axon regeneration in males cannot be explained by sex-dependent differences in NT-4/5 mRNA expression at the level of the individual motoneuron following treadmill training.

Exercise leads to greater recruitment of NT-4/5 mRNA positive motor neurons in males

The number of positively labeled motoneurons per spinal cord section was analyzed as described above so that a comparison could be made between the sexes. There were significantly more positively labeled motoneurons in male spinal cord sections assayed for NT-4/5 mRNA expression in comparison to females (Fig. 7F). There was a significant difference between the sexes (unpaired two-tailed t-test, $p=0.0027$). In treadmill trained males, 19.15% more NT-4/5 mRNA-expressing motoneurons were found than in treadmill trained females. Greater recruitment of NT-4/5 mRNA-expressing motor neurons as a result of treadmill training may contribute to the mechanism for the enhancement of axon regeneration in males.

Motoneurons of exercised males and females express trkB in similar amounts

No significant difference in average gamma motoneuronal fluorescence intensities could be found between the sexes (Fig. 8A; two-tailed t-test, $p>0.05$). Similarly, no significant difference in alpha motoneuronal fluorescence intensities could be found between treadmill trained males and females (Fig. 8B; two-tailed t-test, $p>0.05$). After combining fluorescence intensity measurements of all trkB-expressing motoneurons and establishing an average across each sex, still no significant difference was found (Fig. 8C; unpaired two-tailed t test, $p>0.05$). When comparing motoneurons between males and females from the side of the spinal cord contralateral to the sciatic nerve transection (Fig. 8D), no signficant difference was found in fluorescence intensity (two-tailed t-test, $p>0.05$). The same was found when comparing only those motoneurons from the side of the spinal cord ipsilateral to the nerve transection (Fig. 8E; two-tailed t-test, $p>0.05$). These results show that in comparison to females, males do not benefit from greater trkB receptor expression at the level of the individual motoneuron following treadmill training.

Exercise leads to greater recruitment of trkB positive motor neurons in males

We found significantly more trkB positive motor neurons in treadmill trained males in comparison to females (Fig. 8F, two-tailed t-test, $p=0.0130$). In males, approximately 25.11% more positively labeled motoneurons are found than in females. These results suggest that an upregulation of trk-B-expressing motor neurons may serve as part of a mechanism for the enhancement of axonal regeneration in males.

Discussion

Because enhancement of axon regeneration in cut peripheral nerves is found in continuously treadmill trained males but not females treated with the same exercise program (Wood et al, 2011), and since BDNF (Wilhelm et al, 2011) and NT-4/5 (English et al, 2011) are both strongly implicated in this enhancement, a reasonable presumption might be that continuous treadmill training would cause a significant upregulation of BDNF mRNA, NT-4/5 mRNA, and perhaps also trkB in the spinal motoneurons in males but not females.

The main finding of this study is that after male and female mice are exposed to the same two-week continuous treadmill training paradigm following peripheral nerve injury, significant sex differences in BDNF mRNA, NT-4/5 mRNA, and trkB expression are evident. A significant sex difference in the number of BDNF mRNA-, NT-4/5 mRNA-, and trkB-expressing motoneurons was found. On average, spinal cords of treadmill trained males contained 26.40% more BDNF-mRNA-expressing motoneurons, 19.15% more NT-4/5 mRNA-expressing motoneurons, and 25.11% more trkB-expressing motoneurons than found in females. Furthermore, males produce more BDNF mRNA in individual motoneurons after axotomy. These data are consistent with the conclusion that in treadmill trained males, more BDNF mRNA is expressed at the level of the individual motoneuron, and more BDNF, NT-4/5, and trkB is found after exercise than in females by the recruitment of more motoneurons expressing these neurotrophins and their high affinity receptor.

The extent of BDNF or NT-4/5 mRNA expression in individual motoneurons was studied by measurement of fluorescence intensity. It was assumed that this intensity was

correlated with the extent of expression. In males, mean fluorescence intensity was significantly greater than in trained females, but only in those motoneurons that had been axotomized. Significant sex differences in average fluorescence intensity for NT-4/5 mRNA and trkB protein expression were not found. However, it is not clear that a direct relationship between fluorescence intensity and mRNA expression (for BDNF and NT-4/5) or trkB protein expression exists. If such a relationship existed, one might expect a larger range of fluorescence intensities than was observed. It is possible that, using the reaction conditions employed in this study, the ability to detect neurotrophin mRNA expression was so sensitive that they decreased the range of expression of mRNAs in motoneurons and reduced my ability to detect sex differences in expression. If so, then adjusting the reaction conditions in future studies so that the full range of expression of neurotrophin mRNAs is possible would be very important.

The magnitude of the sex difference in numbers of BDNF-expressing motoneurons following two weeks of treadmill training in mice is compatible with prior reports that exercise increases BDNF expression in the spinal cord of rats (Gomez-Pinilla et al., 2005). Roughly 25% more BDNF-expressing motoneurons were found to express BDNF mRNA in males than females exposed to the same training paradigm, and axotomized motoneurons in males expressed 15% more BDNF mRNA than did those in females. Using both RT-PCR and ELISA , Gomez-Pinilla et al. (2005) reported that, after spinal hemisection at a mid-thoracic region, adult male rats exposed to 28 days of voluntary exercise on a running wheel expressed 27% more BDNF mRNA and 33% more BDNF protein in the lumbar spinal cord in comparison to unexercised control rats. Thus, continuous treadmill training-induced enhancement of axon regeneration found

only in males may partially be due to an overall upregulation of BDNF mRNA by the expression of more BDNF mRNA at the level of the individual motoneuron as well as by the recruitment of more BDNF mRNA-expressing motoneurons in comparison to treadmill-trained females.

Neurotrophin-4/5 is known to share a high affinity receptor with BDNF and is implicated in the enhancement of axon regeneration found after treadmill training. In the present study, significantly more motoneurons were found to express NT-4/5 after continuous treadmill training in males than females. Like BDNF, NT-4/5 expression is at least partially activity-dependent. Skup et al. (2002) found an increase in NT-4/5 protein in the spinal cord white matter after 4 weeks of treadmill training at a moderate speed of 20 cm/s and a daily distance of 1000 m. Whether that increase is similar to the magnitude of the sex difference in NT-4/5 expression following treadmill training is not clear because the average amount of trkB increase after treadmill training was not reported by Skup et al. (2002).

The proposed cellular mechanism by which treadmill training leads to enhanced axon regeneration is an increase in autocrine and/or paracrine neurotrophin signaling in the regenerating axons (English et al, 2011, Wilhelm et al, 2011). Thus, in addition to increases in the numbers of motoneurons expressing neurotrophin ligands following treadmill training, an increase in the number of motoneurons containing the trkB receptor might be anticipated in males but not females. Consistent with this speculation, in the present study, males were found to recruit approximately one fourth more motoneurons to express trkB after treadmill training than females. The activity dependence of the expression of trkB is not known. Skup et al. (2002) studied rat lumbar spinal cord trkB

expression after 4 weeks of treadmill training. They did not report any changes in motoneuron expression, but the number of small cells (size<160 μm^2), which they presumed to be oligodendroglia, doubled in number after exercise. In the present study, similar small cells were not observed, so that it is difficult to compare to the findings of Skup et al (2002). Furthermore, the discrepancy in findings may be attributable to the possible non-specificity of the anti-trk-B antibody used in the Skup et al. (2002) study, a problem raised by the researchers themselves.

Based on analyses of soma sizes, all of the BDNF mRNA-, NT-4/5 mRNA-, and trkB-expressing alpha motoneurons in both sexes are among the smaller cells within the alpha motoneuron size range (Friese et al, 2009). Further, the number of motoneurons that express these markers in the large end of the alpha motoneuron distribution is much smaller than the total number of motoneurons in this size range that contain choline acetyltransferase, a marker for cholinergic motoneurons (Friese et al, 2009). The soma size distribution of all trkB-expressing motoneurons analyzed in the present study is comparable to that reported by Skup et al. (2001) in mice that had been treadmill trained at a moderate speed of 25 cm/s at 1000 m daily for one month. Considering the low to moderate intensity of activity required to perform continuous treadmill training or in the Skup et al. (2002) study, and considering the orderly recruitment of motoneurons by size that is embodied in Henneman's size principle (Henneman and Olsen, 1965), the suggestion that the continuous treadmill training paradigm involves the recruitment of only smaller alpha motoneurons into activity seems reasonable. The observations made in the present study are consistent with an activity-dependent increase in neurotrophin and trkB expression in motoneurons, but only in males.

Approximately half of all BDNF mRNA-, NT-4/5 mRNA-, and trkB-expressing motoneurons analyzed in this study were gamma motoneurons. This proportion is greater than the one third of all lumbar motoneurons judged to be gamma motoneurons in cats by Burke et al. (1977). This is further support for the suggestion that continuous treadmill training preferentially recruits smaller motoneurons and that BDNF mRNA, NT-4/5 mRA, and trkB expression are all activity-dependent.

Based on the number of motoneurons sampled in histological sections assayed for BDNF mRNA and NT-4/5 mRNA expression, there are more BDNF mRNA-expressing motoneurons than NT-4/5 mRNA-expressing motoneurons in the spinal cords of both males and females. This finding is comparable to data reported by Buck et al. (2000), who also found a greater percentage of BDNF mRNA-expressing motoneurons than NT-4/5-expressing motoneurons in the L4-L5 regions of the lumbar spinal cord regions in rats using in situ hybridization.

If continuous treadmill training causes a significantly greater upregulation of BDNF mRNA, NT-4/5 mRNA, and trk-B in males, as hypothesized, then a sex difference in motoneuron BDNF mRNA, NT-4/5 mRNA, and trk-B at the level of the individual motoneuron would be a potential outcome. In this study, only a modest difference in BDNF fluorescence intensity was found between the males and the females. In contrast to this finding, Al Majed et al. (2008) found, using in situ hybridization, that a single application of an hour of continuous electrical stimulation at 20 Hz to the site of a femoral nerve cut dramatically increased motoneuron BDNF and trk-B mRNA expression at the level of the individual motoneurons in rats. Because the immunohistochemistry and fluorescent in situ hybridization protocols used in the present

study were designed to maximize immunofluorescent signaling, it is possible that existing sex differences in BDNF mRNA , NT-4/5 mRNA, and trk-B expression were overshadowed by the extent of the signal intensity. Specifically, a continuous distribution of fluorescent intensities may have been converted into a binary distribution under the protocol used in this study. In a future study, modification of the RNA riboprobe hybridization time as well as the antibody incubation periods could be used to see if such subtle differences could be detected.

This study provides evidence that greater motoneuron BDNF mRNA, NT-4/5 mRNA, and trkB expression underlies the enhancement of axon regeneration found in continuously treadmill trained males but not females. A possible result is the enhancement of BDNF and NT-4/5 signalling in males caused simultaneously by greater expression of both the neurotrophins and their high affinity receptor in response to continuous treadmill training. Wood et al, (2011) found that when castrated male mice are cut at the sciatic nerve and then treadmill trained continuously, they did not experience the same enhancement of axon regeneration found in intact males exposed to the same injury and exercise treatment. Furthermore, Jones et al. (2000) and Brown et al. (1999) found that castrated animals experienced an enhancement of regeneration in the facial muscles and the sciatic nerve, respectively, after treatment with testosterone. Considering those data in the context of the present study, a role for signaling through androgen receptors in regulating the activity dependence of the expression of BDNF, NT-4/5, and trk-B might be considered.

References

Al-Majed, A.A., Brushart, T.M., and Gordon, T. (2000). Electrical stimulation accelerates and increases expression of BDNF and trkB mRNA in regenerating rat femoral motoneurons. *European Journal of Neuroscience*, 12:4381-4390.

Buck, C.R., Seburn, K.L., and Cope, T. (2000). Neurotrophin Expression by Spinal Motoneurons in Adult and Developing Rats. *The Journal of Comparative Neurology*, 416:309-318.

Burke, R.E., Strick, P.L., Kanda, K., Kim, C.C., and Walmsley, B. (1977). Anatomy of medial gastrocnemius and soleus motor nuclei in cat spinal cord. *The Journal of Neurophysiology*, 40:667-680.

English, A.W., Cucoranu, D., Mulligan, A., and Sabatier, M.J., Neurotrophin-4/5 is implicated in the enhancement of axon regeneration produced by treadmill training following peripheral nerve injury. Eur. J. Neurosci. UNDER REVIEW, 2011

Friese, A., Kaltschmidt, J.A., Ladle, D.R., Sigrist, M., Jessell, T., and Arber, S. (2009). Gamma and alpha motor neurons distinguished by expression of transcription factor Err3. *Proceedings of the National Academy of the Sciences*, 106:13588-13593.

Frostick, S. P., Yin, Q. and Kemp, G. J. (1998). Schwann cells, neurotrophic factors, and peripheral nerve regeneration. *Microsurgery.* 18: 397-405.

Gomez-Pinilla, F., Ying, Z., Opazo, P., Roy, R.R. and Edgerton, V.R. (2001). Differential regulation by exercise of BDNF and NT-3 in rat spinal cord and skeletal muscle. *European Journal of Neuroscience*, 13:1078-1084.

Henneman, E., & Olson, C. (1965). Relation between structure and function in the design of skeletal muscles. *Journal of Neurophysiology*, 28(3): 581-598.

Noble, J., Munro, C., Prasad, V., & Midha, R. (1998). Analysis of Upper and Lower Extremity

Peripheral Nerve Injuries in a Population of Patients with Multiple Injuries. *The Journal*

of Trauma: Injury, Infection, and Critical Care, 45(1), 116-122.

Recknor, J.B. & Mallapragada, S.K. (2006). *The Biomedical Engineering Handbook: Tissue*

Engineering and Artificial Organs. New York, NY: Taylor & Francis.

Sabatier, M., Redmon, N., Schwartz, G. & English, A. (2008) Treadmill training promotes axon regeneration in injured peripheral nerves. *Experimental Neurology*, 211: 489-493.

Skup, M., Dwornik, A., Macias, M., Sulejczak, D., Wiater, M., and Czarkowska-Bauch, J. Long-Term Locomotor Training Up-Regulates $TrkB^{FL}$ Receptor-like Proteins, Brain-Derived Neurotrophic Factor, and Neurotrophin 4 with Different Topographies of Expression in Oligodendroglia and Neurons in the Spinal Cord. (2002). *Experimental Neurology*. 176:289-307.

Ying, Z., Roland, R., Edgerton, V.R., and Gomez-Pinilla, F. (2005). Exercise restores levels of neurotrophins and synaptic plasticity following spinal cord injury. *Experimental Neurology*. 193:411-419.

Wilhelm, J.C., Cucoranu, D., Xu, M., Bassell, G.J., and English, A.W. Cooperative roles of BDNF expression in neurons and Schwann cells are modulated by exercise to facilitate nerve regeneration. *The Journal of Neuroscience,* Submitted, 2011

Wood, K., Wilhelm, J.C., Sabatier, M.J., and English, A.W. Sex differences in the effectiveness of treadmill training in enhancing axon regeneration in injured peripheral nerves. *Developmental Neuroscience*, UNDER REVIEW, 2011

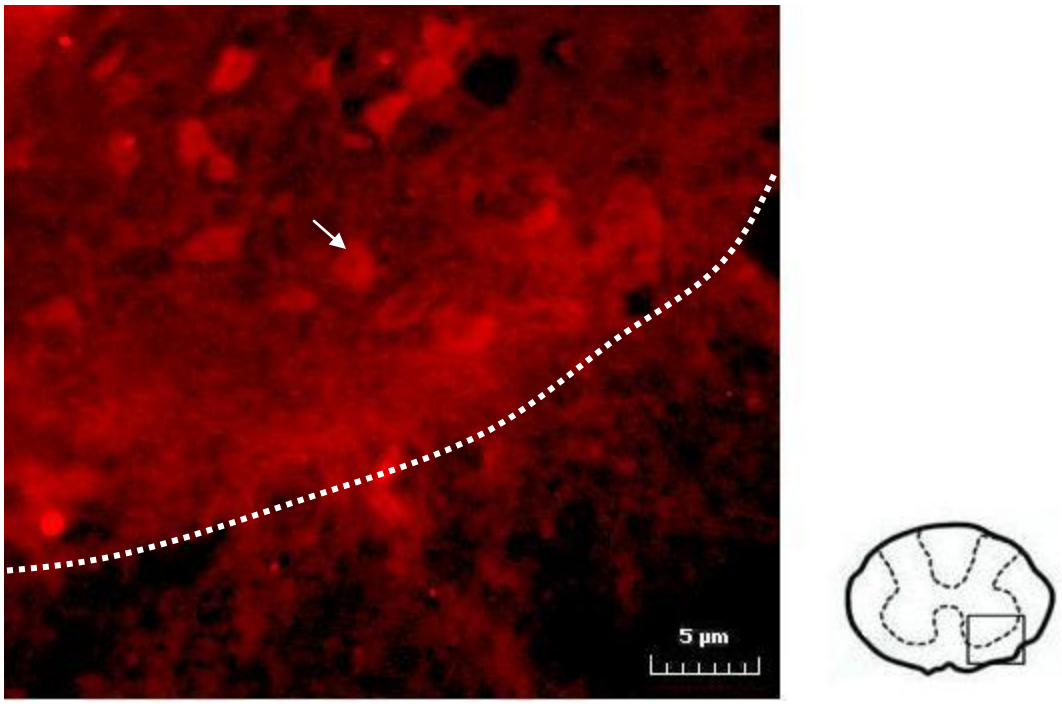

Fig 1. The arrow indicates a putative motoneuron in the lamina IX region of the ventral horn. This tissue section was assayed for BDNF mRNA expression using fluorescent in situ hybridization. The dashed line indicates the boundary of the ventral horn.

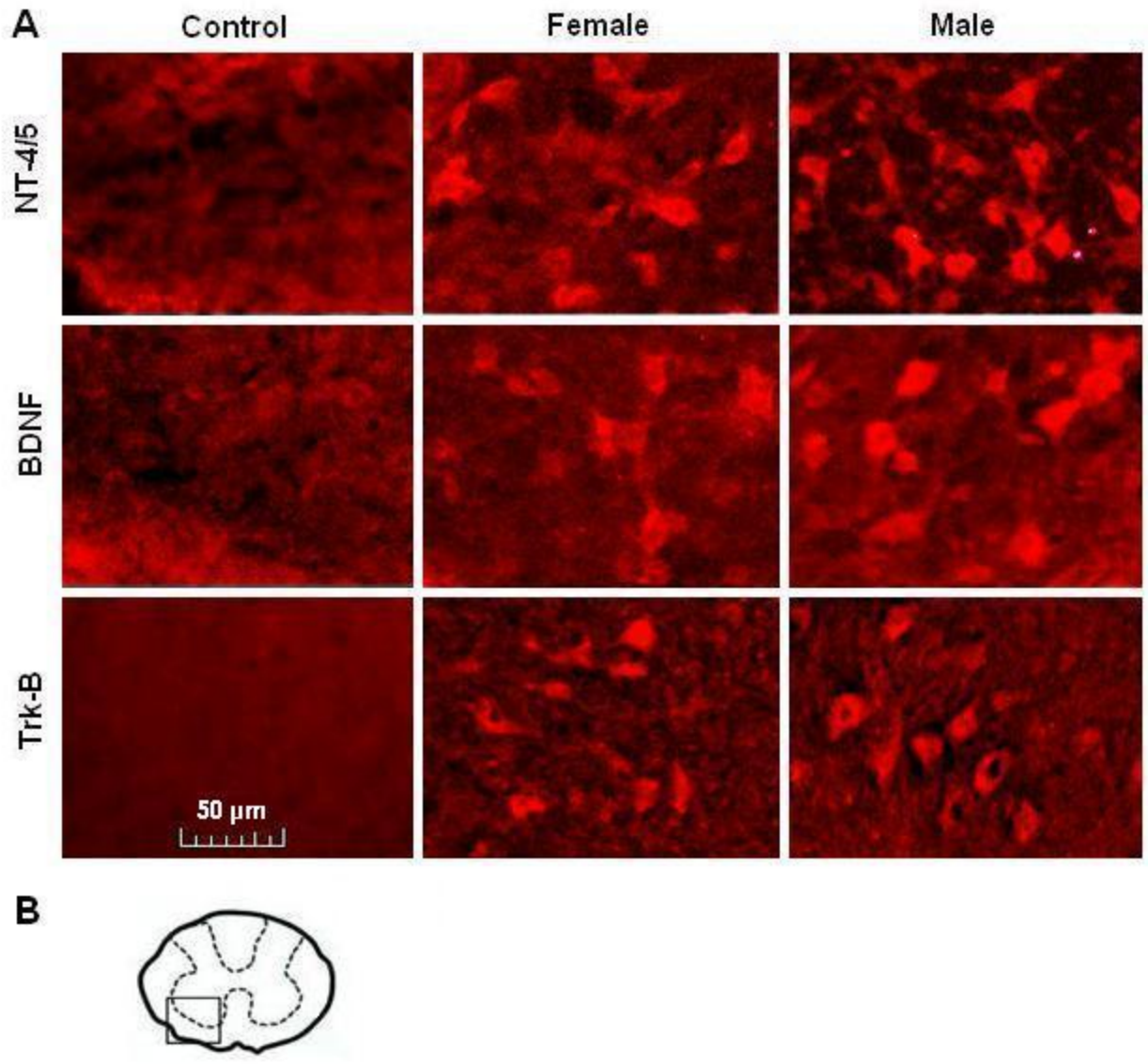

Fig 2. (A) Comparison of tissue sections between male, female, and control tissue sections for for BDNF mRNA, NT-4/5 mRNA, and trkB assays. BDNF and NT-4/5 knockout tissue stained with the anti-sense BDNF and NT-4/5 RNA riboprobes, respectively, served controls to ensure specificity of hybridization. Experimental tissue sections stained with the secondary fluorophore-conjugated antibody alone served as a control to ensure the specificity of the anti-trkB antibody. (B) Illustration of a spinal cord section with a box around the lamina IX region of the ventral horn. The pictures in (A) were taken from this area.

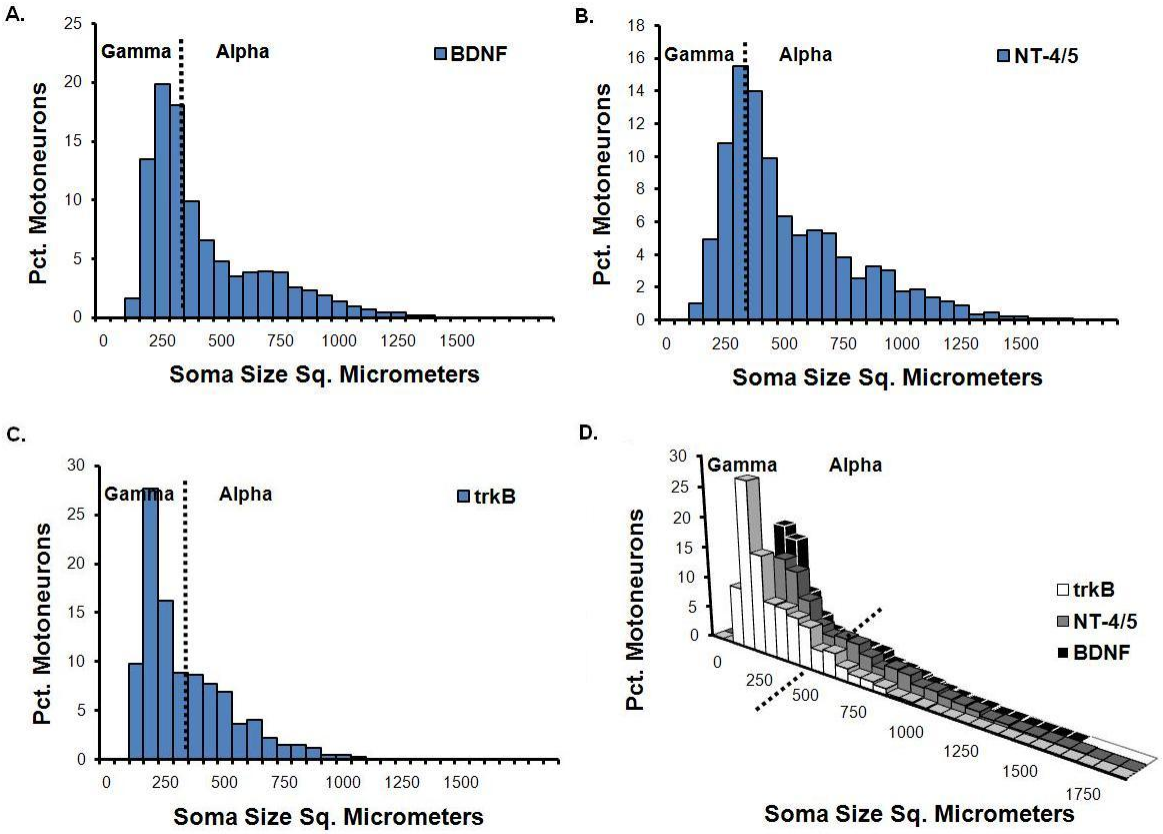

Fig. 3. (A)(B)(C) Histograms showing the percentages of motoneurons falling into the gamma (soma size<400 μm^2)and alpha (soma size>400 μm^2) size categories (n=12, males and females combined). (D) Combined histograms of (A), (B), and (C).

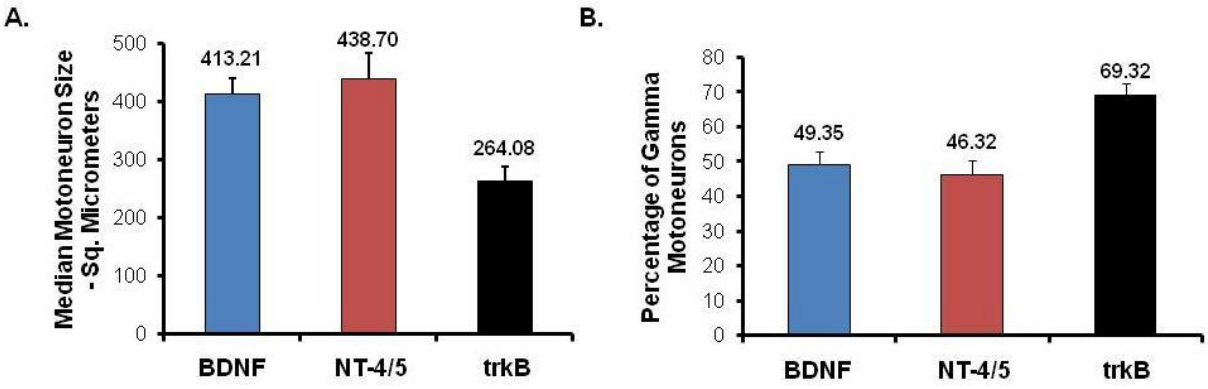

Fig. 4. (A) Median soma sizes (n=12, males and females combined) for BDNF mRNA (413.21±27.78 μm^2), NT-4/5 mRNA (438.70±44.82 μm^2), and trkB-expressing motoneurons (264.08±25.09 μm^2). The median soma size for trkB-expressing motoneurons is considerably smaller than those of the two other populations. (B) Percentage of positively labeled gamma motoneurons in the populations of motoneurons separately assayed for BDNF mRNA, NT-4/5 mRNA, and trkB (n=12, males and females combined). There is a greater percentage of gamma motoneurons in trkB-expressing motoneurons (69.32±3.53%) when compared with the BDNF (49.35±3.78%) and NT-4/5-expressing (46.32±4.31%) motoneuron populations.

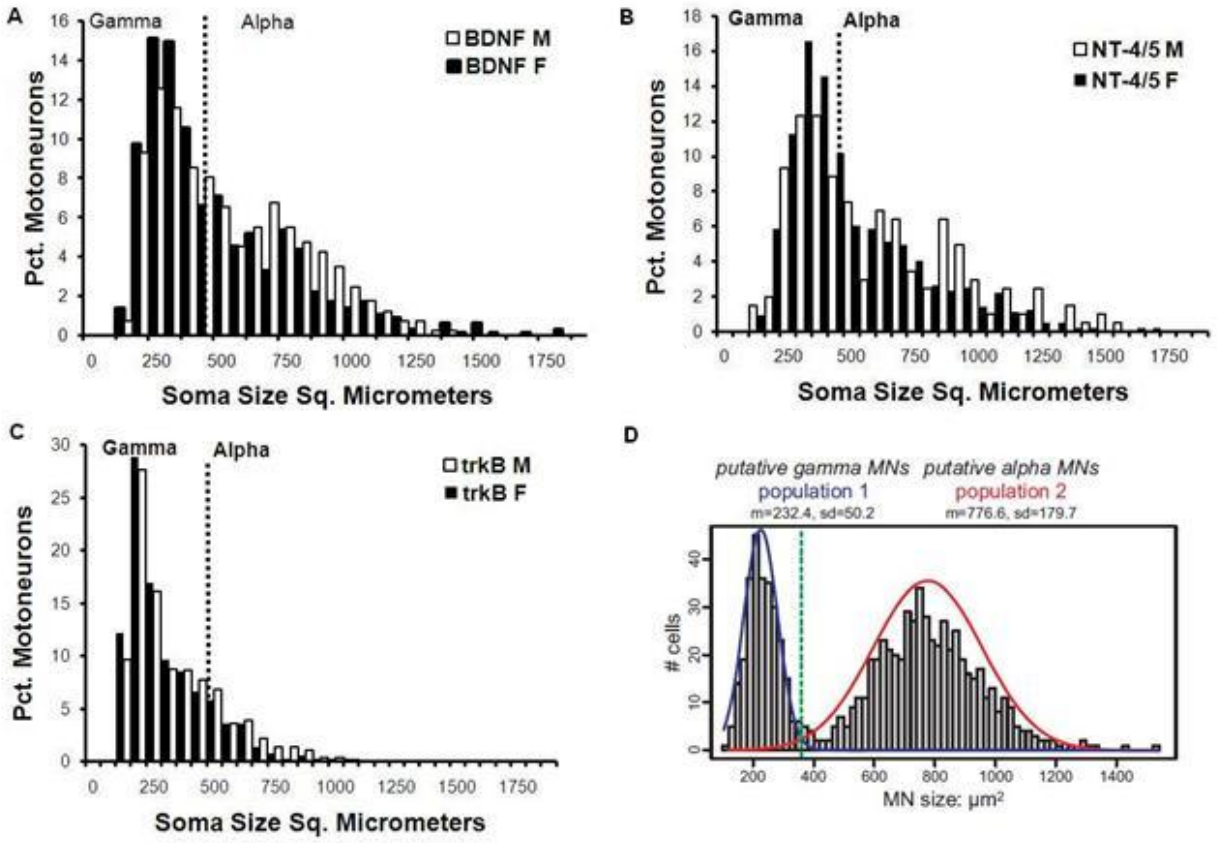

Fig. 5. Histograms showing the percentages of motoneurons in the gamma (soma size<400 µm^2) and alpha (soma size>400 µm^2) size categories. (A) BDNF mRNA-expressing motoneurons (males n=5, females n=5). (B) NT-4/5 mRNA-expressing motoneurons (males n=4, females n=6). (C) trkB-expressing motoneurons (males n=3, females n=6). (D) Soma size distribution for all motoneurons in wild type mice containing choline acetyltransferase, a marker for cholinergic motoneurons (Friese et al., 2009). Soma size distributions in (A), (B), and (C) are skewed towards smaller motoneurons in comparison to the distribution in (D).

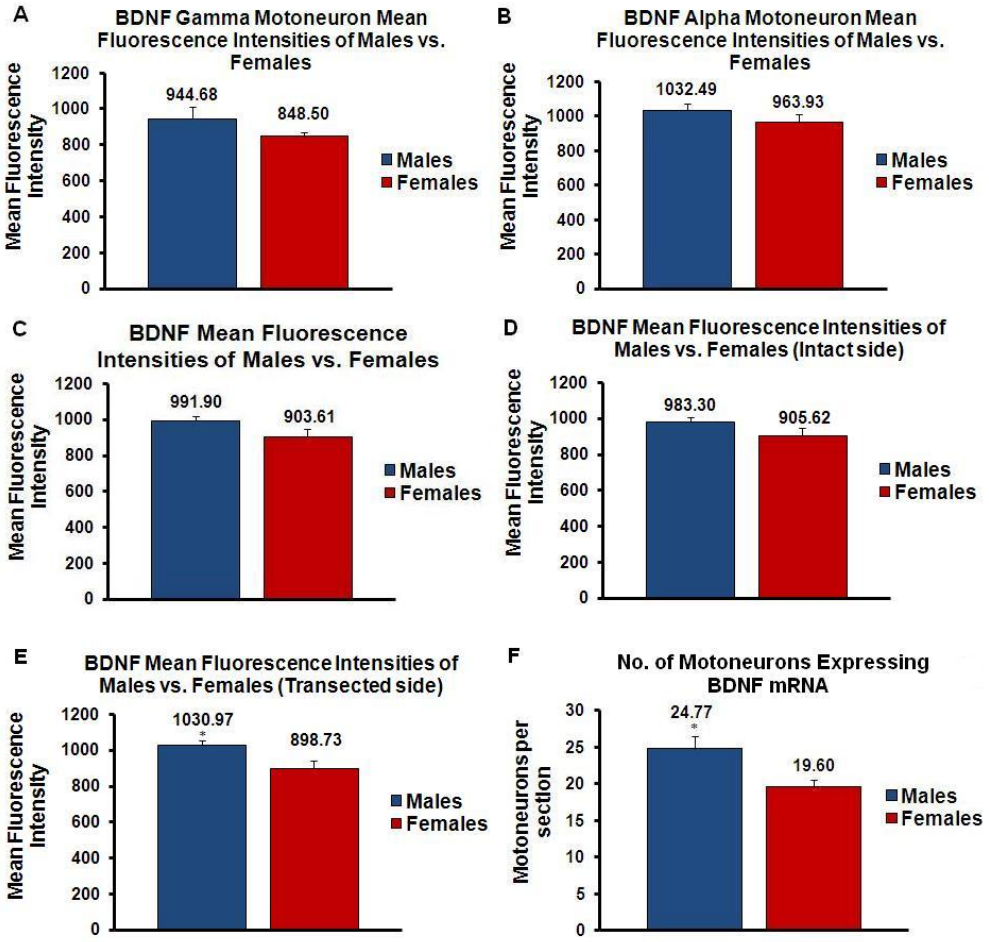

Fig 6. BDNF comparisons between males (n=5) and females (n=5). (A) Mean motoneuron fluorescence intensities of gamma motoneurons for males (944.68±65.58) and females (848.50±21.73). (B) Mean motoneuron fluorescence intensities of alpha motoneurons for males (1032.49±38.98) and females (963.93±38.52). (C) Mean motoneuron fluorescence intensities for males (991.90±43.79) and females (903.61±23.41), taking into account both alpha and gamma motoneurons. (D) Mean motoneuron fluorescence intensities of males (983.30±52.92) and females (905.62±23.15), taking into account only those motoneurons from the intact side. (E) Mean motoneuron fluorescence intensities of males (1030.97±41.05) and females (898.73±23.26), taking into account only those motoneurons from the transected side. (F) Comparison between males (24.77±1.74 motoneurons) and females (19.6±0.97 motoneurons) in the number of motor neurons expressing BDNF mRNA per spinal cord section.

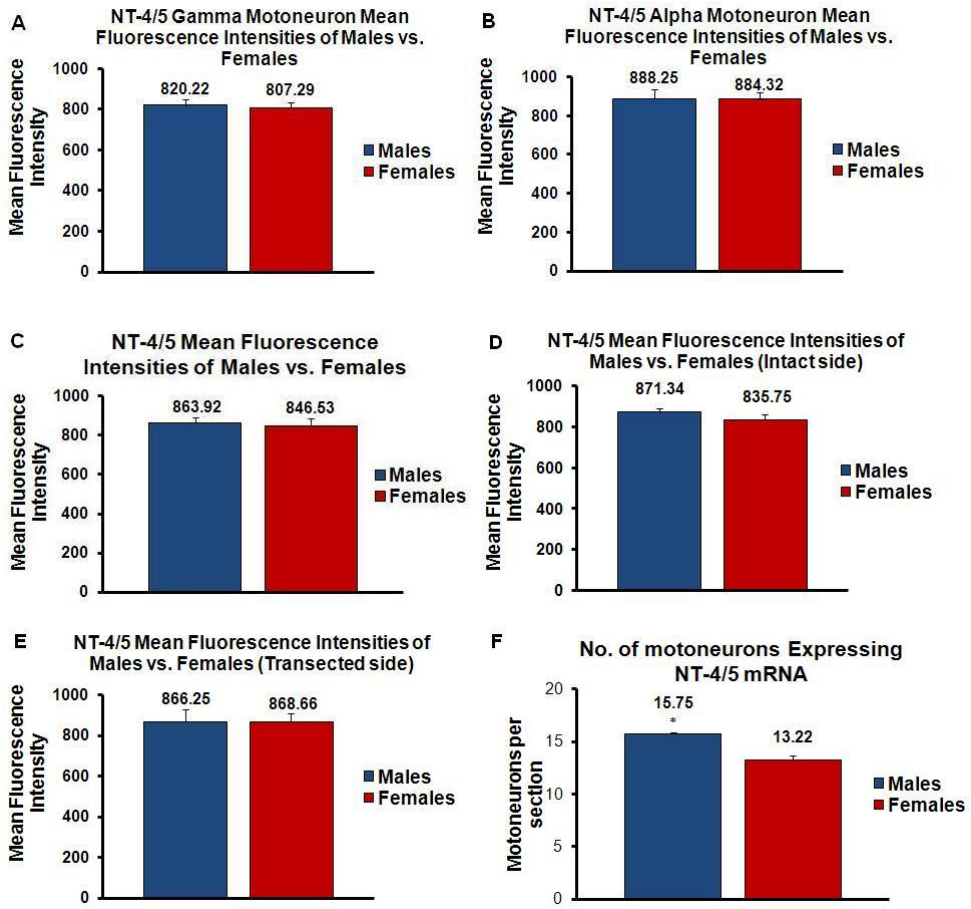

Fig. 7. NT-4/5 comparisons between males (n=4) and females (n=6). (A) Mean motoneuron fluorescence intensities of gamma motoneurons for males (820.22±29.88) and females (807.29±25.10). (B) Mean motoneuron fluorescence intensities of alpha motoneurons for males (888.25±46.05) and females (884.32±33.22). (C) Mean motoneuron fluorescence intensities for males (863.92±38.80) and females (846.53±24.11), taking into account both alpha and gamma motoneurons. (D) Mean motoneuron fluorescence intensities of males (871.34±18.51) and females (835.75±24.16), taking into account only those motoneurons from the intact side. (E) Mean motoneuron fluorescence intensities of males (866.25±62.47) and females (868.66±39.06), taking into account only those motoneurons from the transected side. (F) Comparison between males (15.75±0.07 motoneurons) and females (13.22±0.45 motoneurons) in the number of motor neurons expressing NT-4/5 mRNA per spinal cord section.

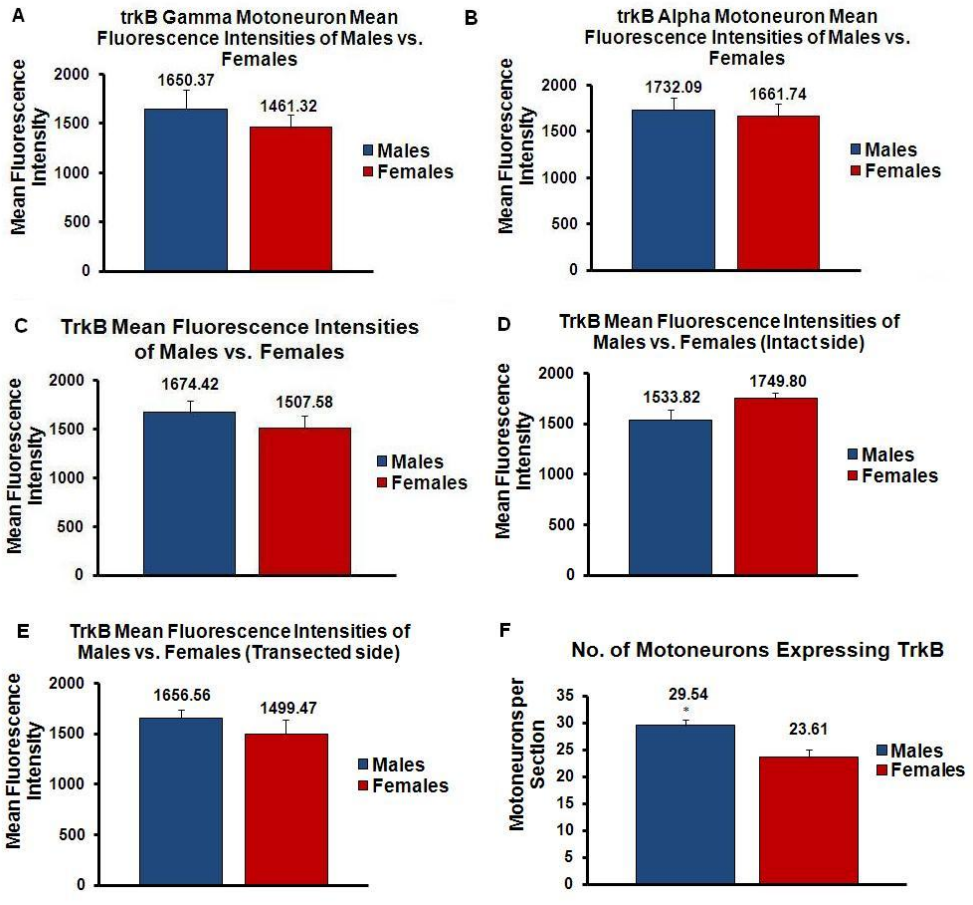

Fig. 8. TrkB comparisons between males (n=3) and females (n=6). (A) Mean motoneuron fluorescence intensities of gamma motoneurons for males (1650.37±189.31) and females (1461.32±126.44). (B) Mean motoneuron fluorescence intensities of alpha motoneurons for males (1732.09±131.62) and females (1661.74±133.82). (C) Mean motoneuron fluorescence intensities for males (1674.42±122.51) and females (1507.58±130.76), taking into account both alpha and gamma motoneurons. (D) Mean motoneuron fluorescence intensities of males (1533.82±103.49) and females (1749.80±58.70), taking into account only those motoneurons from the intact side. (E) Mean motoneuron fluorescence intensities of males (1656.56±82.64) and females (1499.47±133.39), taking into account only those motoneurons from the transected side. (F) Comparison between males (29.54±1.44 motoneurons) and females (23.61±1.00 motoneurons) in the number of motor neurons expressing NT-4/5 mRNA per spinal cord section.