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Nicholas J. Thompson April 12, 2013

Enhancement of Peripheral Nerve Regeneration due to Treadmill Training and Electrical Stimulation is Dependent on Androgen Receptor Signaling

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Abstract

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By Nicholas J. Thompson

Moderate exercise in the form of treadmill training and brief electrical nerve stimulation (ES) both enhance axon regeneration after peripheral nerve injury. Androgens were suspected to be involved in the enhancement of axon regeneration due to treadmill training because of a sex difference previously identified, in which different regimens of exercise lead to dissimilar results of axon regeneration between male and female mice (Wood *et al.*, 2012). We treated mice with the androgen receptor blocker, flutamide, prior to either exercise or ES, to evaluate the role of androgen receptor signaling in these methods of enhancing axon regeneration. The common fibular (CF) and tibial (TIB) nerves of *thy-1-YFP-H* mice, in which axons in peripheral nerves are marked by yellow fluorescent protein (YFP), were transected and repaired using CF and TIB nerve grafts harvested from non-fluorescent donor mice. Silastic capsules filled with flutamide were implanted subcutaneously to release the drug continuously. Exercised mice were treadmill trained five days/week for two weeks, starting on the third day post transection. For ES, the sciatic nerve was stimulated continuously for one hour prior to nerve transection. After two weeks, lengths of YFP+ axon profiles were measured from harvested nerves. Both exercise and ES enhanced axon regeneration, but this enhancement was blocked completely by flutamide treatments. Signaling through androgen receptors is necessary for the enhancing effects of treadmill exercise or ES on axon regeneration in cut peripheral nerves.

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Introduction

Peripheral nerves have the capacity for regeneration, but functional recovery after peripheral nerve injury is rare (Frostick *et al.*, 1998; Scholz *et al.*, 2009). A primary cause of the poor outcome following injury is slow axon regeneration (Gordon *et al.*, 2009). To facilitate axon growth, several methods of enhancing axon regeneration after injury have been identified and investigated. Among these enhancing procedures are daily exercise in the form of treadmill training (Seo *et al*., 2006; Ilha *et al.*, 2008; Sabatier *et al.*, 2008; Asensio-Pinilla *et al.*, 2009; Udina *et al.*, 2011) and brief electrical stimulation of the injured nerve at the time of transection (Al-Majed *et al*., 2000b), both of which enhance axon regeneration following axotomy.

A sex difference in the enhancement of peripheral nerve regeneration due to treadmill training has been identified (Wood *et al*., 2012). Continuous training, an hour of daily slow walking, enhances axon regeneration in male mice, but not in female mice. Conversely, interval training, a series of interrupted short sprints, is effective in female mice, but not male mice. Androgens are implicated to play a role in this sex difference, as castration eliminates the enhancement effect of continuous training in male mice. Additionally, females naturally make testosterone, which can be converted into estradiol with the enzyme, P450 aromatase. When female mice are treated with a pharmacological dose of anastrazole, an aromatase inhibitor, axon regeneration improves significantly (Wood *et al*., 2012). Based on these results, one might suggest that androgens

could play a principal role in the enhancement of axon regeneration due to exercise for both male and female mice. One goal of this project is to evaluate the androgen-dependence of the effect of exercise in enhancing axon regeneration in cut peripheral nerves in male and female mice.

Brain-Derived Neurotrophic Factor (BDNF) is also important for axon regeneration after peripheral nerve injury. Both Schwann cell- and neuron-derived BDNF are facilitators of axon growth post-axotomy (Funakoshi *et al.*, 1993), but BDNF of neuronal origin is required for the enhancement effect of exercise (Wilhelm *et al.*, 2012). Consistent with these findings is the observation that prolonged treatments of castrated male rats with testosterone propionate results in a prolonged increase in BDNF mRNA levels in facial motoneurons (Sharma *et al.*, 2010).

Brief electrical stimulation, for as little as one hour at the time of surgical repair of a cut nerve, results in a marked enhancement of axon regeneration (Al-Majed *et al.*, 2000b), but it also causes a sharp but short-lived increase in motoneuron BDNF mRNA expression, both in gonadally intact female rats (Al-Majed *et al.*, 2000a) and in castrated male rats (Sharma *et al.*, 2010). Very little evidence exists to suggest that the effects of brief electrical stimulation might depend on androgens. It is possible that the enhancement of axon growth following nerve injury due to electrical stimulation relies solely on a sudden increase in activity of the nerve, which in turn increases expression of BDNF without androgen involvement. A second goal of this study was to investigate

whether the effectiveness of brief electrical stimulation in the enhancement of axon regeneration following peripheral nerve injury is dependent on androgen receptor signaling.

Materials and Methods

Mice and surgical procedures – All experimental methods were approved by the Institutional Animal Care and Use Committee of Emory University. Axon regeneration was studied in the H strain of *thy-1-YFP* mice (Feng *et al.*, 2000). In this strain, yellow fluorescent protein (YFP) completely fills a subset of axons in the peripheral nervous system, making regenerated axons visible with confocal microscopy. All mice were anesthetized using 1% isoflurane. The common fibular and/or tibial nerves, two branches of the sciatic nerve, were transected and repaired bilaterally in each mouse using a 10-15 mm long segment of the same nerve harvested from a strain matched donor mouse (Figure 1A). Nerves and grafts were secured with fibrin glue (MacGillivray, 2003). The YFP+ axons in the proximal segment of the host mouse were allowed to regenerate into the donor graft for two weeks, after which the mouse was euthanized and perfused. Nerves and grafts were harvested and imaged at low magnification (10X) using confocal microscopy. Stacks of optical sections were obtained through contiguous and overlapping microscope fields, extending over the full extent of each nerve and graft, and these were stitched together using Adobe Photoshop. The result was a complete reconstruction of the repaired nerve and graft. Representative confocal images of reconstructed repaired nerves and grafts are shown in Figure 2. Lengths of YFP+ axon profiles were measured from the resulting reconstructions using ImageJ. Median axon profile lengths were determined in each nerve and the significance of differences in average medians

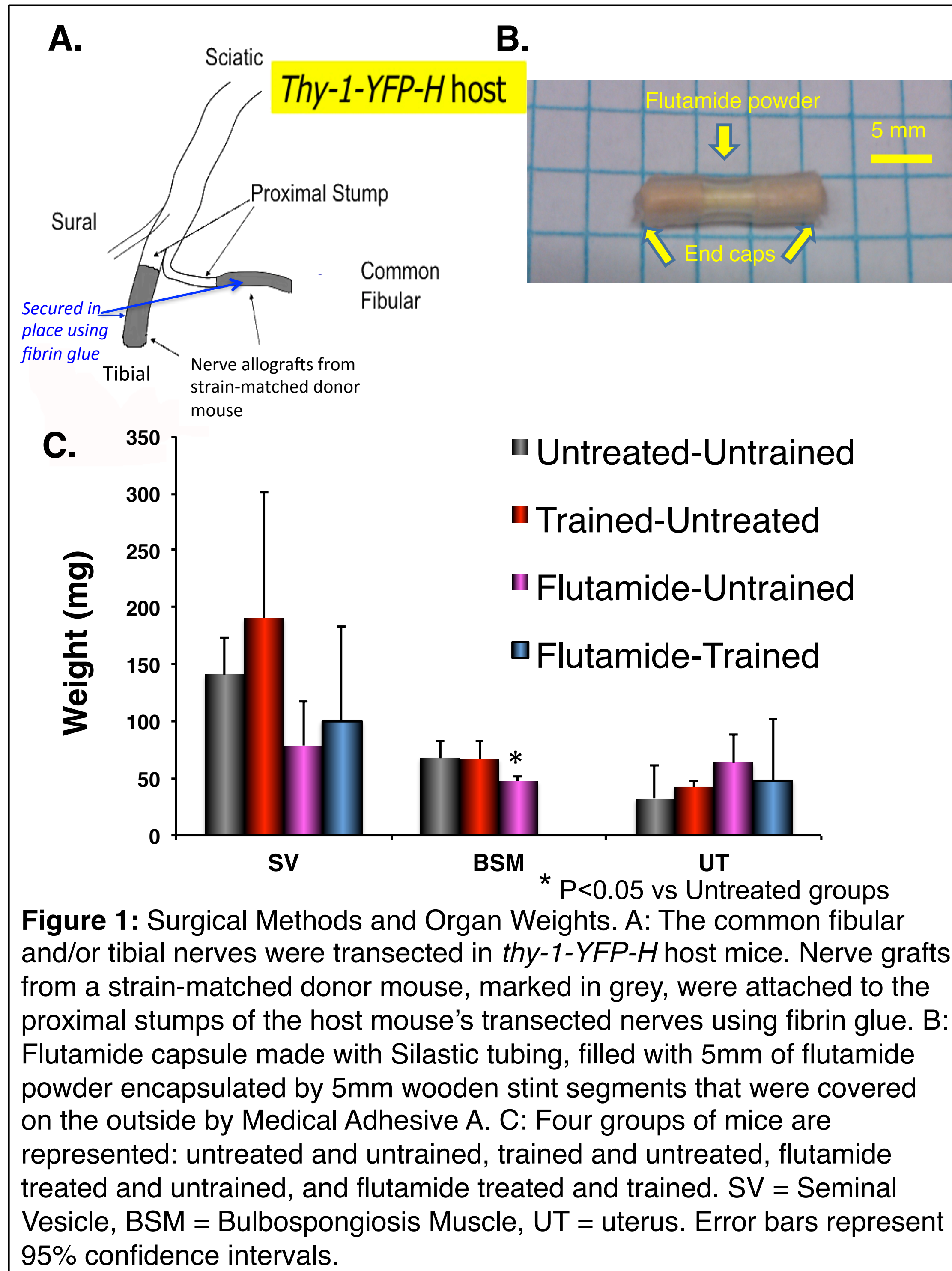

Figure 1: Surgical Methods and Organ Weights. A: The common fibular and/or tibial nerves were transected in *thy-1-YFP-H* host mice. Nerve grafts from a strain-matched donor mouse, marked in grey, were attached to the proximal stumps of the host mouse's transected nerves using fibrin glue. B: Flutamide capsule made with Silastic tubing, filled with 5mm of flutamide powder encapsulated by 5mm wooden stint segments that were covered on the outside by Medical Adhesive A. C: Four groups of mice are represented: untreated and untrained, trained and untreated, flutamide treated and untrained, and flutamide treated and trained. SV = Seminal Vesicle, BSM = Bulbospongiosis Muscle, UT = uterus. Error bars represent 95% confidence intervals.

between groups was compared using a one-way analysis of variance (ANOVA) with post-hoc (Fisher's least significant difference, LSD) paired testing, where appropriate.

Treatments - Treadmill exercise and brief electrical stimulation were employed in different groups of mice to enhance axon regeneration after injury. For treadmill exercise, males and females were trained using different protocols, as described elsewhere (Wood *et al.*, 2012). Continuous training, or slow walking at 10 m/min for 1 hour per day, was used for male mice. Interval training, or 4 repetitions of short sprints at 20 m/min for 2 minutes followed by 5 min of rest, was used for female mice. All mice were exercised on a level treadmill five days/week for 2 weeks, beginning on the 3rd day post transection. For electrically stimulated mice, a bipolar cuff electrode (Stein *et al.*, 1977) was placed around the sciatic nerve in the mid-thigh. Short (0.1 ms) pulses were delivered to the nerve via this cuff at a rate of 20 Hz continuously for one hour prior to nerve transection. Stimulus intensity was set at twice the minimum voltage needed to evoke a twitch in the gastrocnemius muscles.

To assess the importance of androgen receptor signaling in the enhancement of axon regeneration after injury, flutamide, an androgen receptor antagonist, was employed. The androgen receptor antagonist was applied in a sustained release dosage form via flutamide capsules (Jones *et al.*, 2012). Capsules composed of 15mm long Silastic tubing (1.57 mm i.d.; 3.18 mm o.d.; Dow Corning Corp., Midland, MI) were packed with 5mm of flutamide powder (2-methyl-*N*-[4-nitro-3-(trifluoromethyl)phenyl]-propanamide; Sigma Aldrich, Seelze, Germany) and flanked on each side by 5mm wooden stint segments. The ends of the capsule were then covered externally by Medical Adhesive A (Figure 1B).

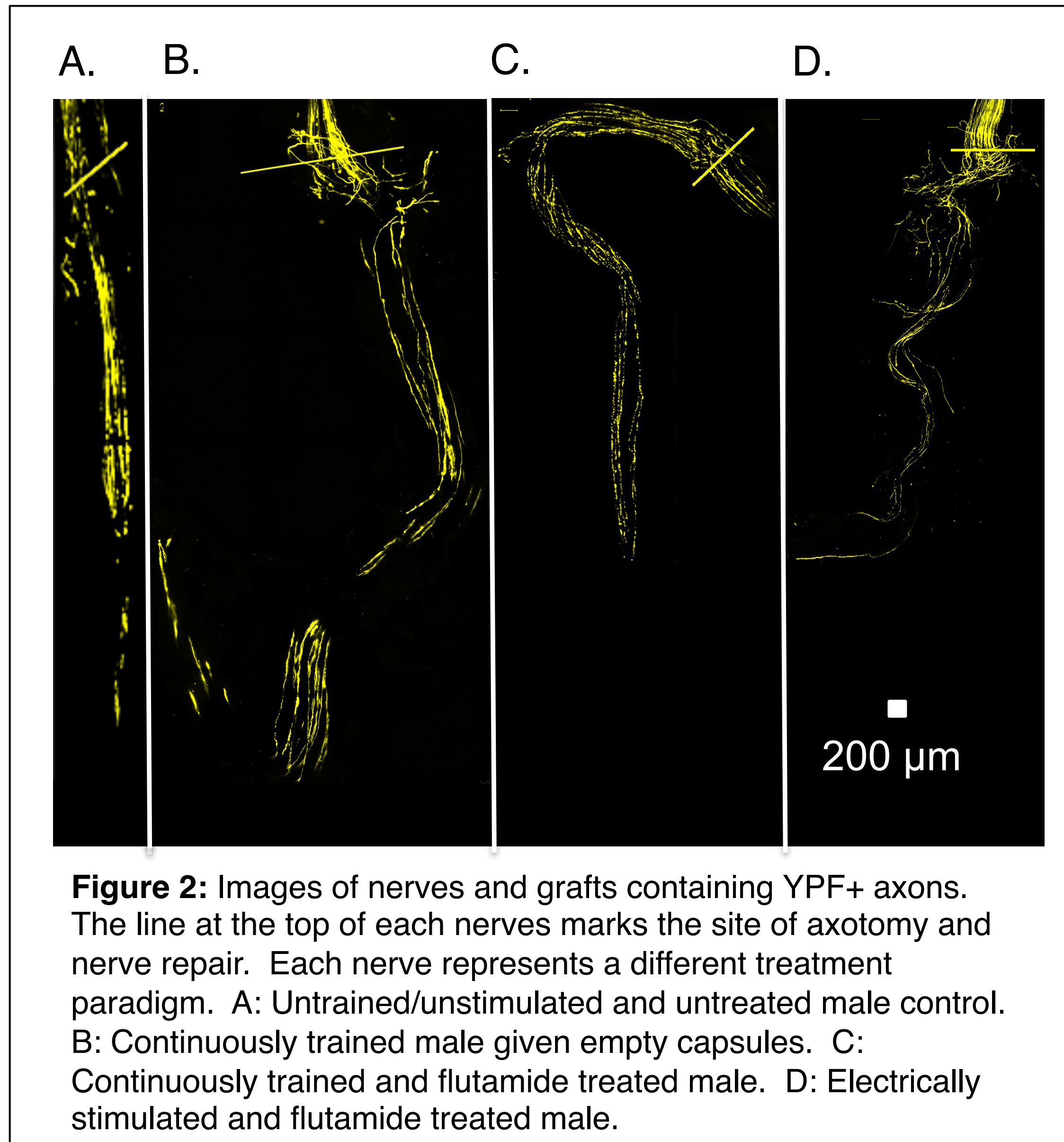

Figure 2: Images of nerves and grafts containing YPF+ axons. The line at the top of each nerves marks the site of axotomy and nerve repair. Each nerve represents a different treatment paradigm. A: Untrained/unstimulated and untreated male control. B: Continuously trained male given empty capsules. C: Continuously trained and flutamide treated male. D: Electrically stimulated and flutamide treated male.

Capsules were soaked in normal saline solution at 37°C for 24 hours prior to implantation to prime the flutamide powder for release. Two such flutamide capsules were implanted in each mouse subcutaneously three days prior to the onset of treadmill training or electrical stimulation to ensure disruption of androgen receptor signaling at the time of the enhancement procedure. Control mice received similar capsules that were not filled with flutamide. Flutamide

treatment efficacy was measured using seminal vesicle and bulbospongiosis muscle weights in males and uterus weights in females.

Four experimental mouse groups were studied: flutamide treated and continuously trained males, flutamide treated and interval trained females, flutamide treated and electrically stimulated males, and flutamide treated and electrically stimulated females. Four additional, control groups were used to assess the effect of flutamide treatment and capsule implantation: males flutamide treated with no enhancement procedure, females flutamide treated with no enhancement procedure, males continuously trained and implanted with blank capsules, and females interval trained and implanted with blank capsules.

Results

Flutamide treatments disrupt androgen receptor signaling - The effectiveness of sustained release flutamide treatments was first evaluated by comparing the weights of seminal vesicles and bulbospongiosis muscles in males and of uterus weights in females in treated and untreated animals. The results of this comparison are shown in Figures 1C. As demonstrated in Figure 1C, seminal vesicle and bulbospongiosis muscle weights were overall lower in flutamide treated males compared to untreated males, and seminal vesicle weights were lower in untrained males compared to trained males. Significance was found with bulbospongiosis weight between flutamide treated males and untreated males. In females, uterus weights showed no consistent trend between either flutamide or exercise.

Flutamide treatments block the enhancing effects of exercise – Inhibition of androgen receptor signaling via flutamide treatment eliminated the enhancement of axon regeneration produced by continuous treadmill training in male mice. The distribution of axon profile lengths measured in flutamide treated male mice that were exposed to two weeks of daily continuous treadmill exercise is shown in Figure 3A. Flutamide treated, continuously trained males had a similar axon profile distribution compared to untreated, untrained males and castrated, continuously trained males. However, the distributions of all three of these groups were distinct from that of continuously trained, untreated males.

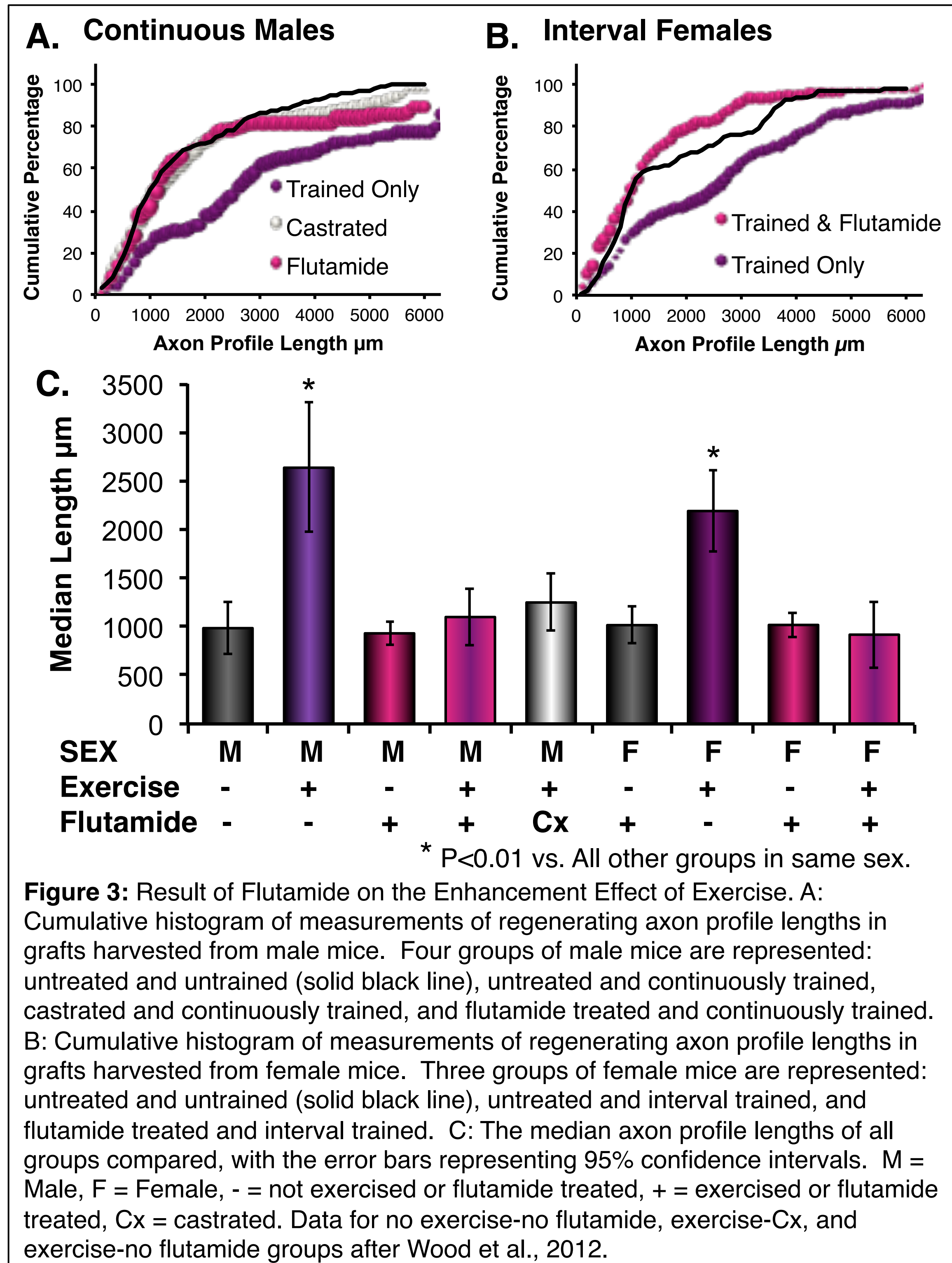

Figure 3: Result of Flutamide on the Enhancement Effect of Exercise. A: Cumulative histogram of measurements of regenerating axon profile lengths in grafts harvested from male mice. Four groups of male mice are represented: untreated and untrained (solid black line), untreated and continuously trained, castrated and continuously trained, and flutamide treated and continuously trained. B: Cumulative histogram of measurements of regenerating axon profile lengths in grafts harvested from female mice. Three groups of female mice are represented: untreated and untrained (solid black line), untreated and interval trained, and flutamide treated and interval trained. C: The median axon profile lengths of all groups compared, with the error bars representing 95% confidence intervals. M = Male, F = Female, - = not exercised or flutamide treated, + = exercised or flutamide treated, Cx = castrated. Data for no exercise-no flutamide, exercise-Cx, and exercise-no flutamide groups after Wood et al., 2012.

The median axon profile lengths of these groups are shown in Figure 3C. These data were subjected to one-way ANOVA. The resulting omnibus test was

significant ($F_{5,21}$=24.664, p<0.0000004). Based on paired, post-hoc (Fisher's least significant difference, LSD) testing, lengths of profiles of YFP+ regenerating axons in flutamide treated and continuously trained male mice were not significantly different from those found in untrained and untreated males (p=0.5496). They were significantly shorter than axon profile lengths measured in continuously trained mice given either blank capsules (p<0.000002) or no capsules (Sabatier *et al.*, 2008) (p<0.000005) (Figure 2C). Regenerating axon profile lengths in flutamide treated and untrained males were not significantly different from untrained and untreated males (LSD, p=0.7923). Thus, signaling through the androgen receptor is necessary for the enhancement of axon regeneration due to continuous training in male mice, but is likely not involved in the mechanism that leads to the basal rate of axon regeneration after injury seen in untrained and untreated males.

In Figure 3B, the distribution of axon profile lengths measured in flutamide treated and control female mice that were exposed to two weeks of daily interval treadmill exercise is presented. For comparison, analogous data are included from untreated, untrained female controls (Sabatier *et al.*, 2008). The distribution of flutamide treated, interval trained females was similar to that of untreated, untrained controls, but distinct from interval trained, untreated females.

Median axon profile lengths for these groups of female mice are shown in Figure 3C. Flutamide treatment eliminated the enhancement effect of interval training in female mice (ANOVA, $F_{4,18}$,=11.889, p<0.00007). Lengths of profiles

of YFP+ regenerating axons from female mice that were interval trained and given blank sustained release were significantly longer ($p<0.01$) than untrained females, but this enhancement effect was eliminated in interval trained female mice also treated with flutamide (LSD, $p=.8212$).

Flutamide treatment blocks the effect of brief electrical stimulation – As little as one hour of electrical stimulation (ES) applied to peripheral nerves at the time of their surgical repair has been shown to produce a marked enhancement of axon regeneration (Al-Majed *et al.*, 2000b). To investigate whether this effect of ES required androgen receptor signaling, the effect of flutamide treatment on the enhancing effects of ES was evaluated.

Blocking androgen receptor signaling with flutamide eliminated the enhancing effect of electrical stimulation. As no significant sex difference was found in untreated, electrically stimulated mice, data from males and females were pooled in electrically stimulated groups in Figure 4. The distribution of axon profile lengths measured in flutamide treated mice that were exposed to one hour of electrical stimulation is shown in Figure 4A. For comparison, distributions from untreated, unstimulated mice and electrically stimulated, untreated mice were included (English *et al.*, 2007). The distribution of flutamide treated, electrically stimulated mice was similar to that of untreated, untrained controls, but distinct from electrically stimulated, untreated mice. Differences in median axon profile lengths for these groups of mice, as well as flutamide treated, unstimulated controls, are significant (ANOVA, $F_{7,22}=12.074$, $p<0.000004$) (Figure 4B). In

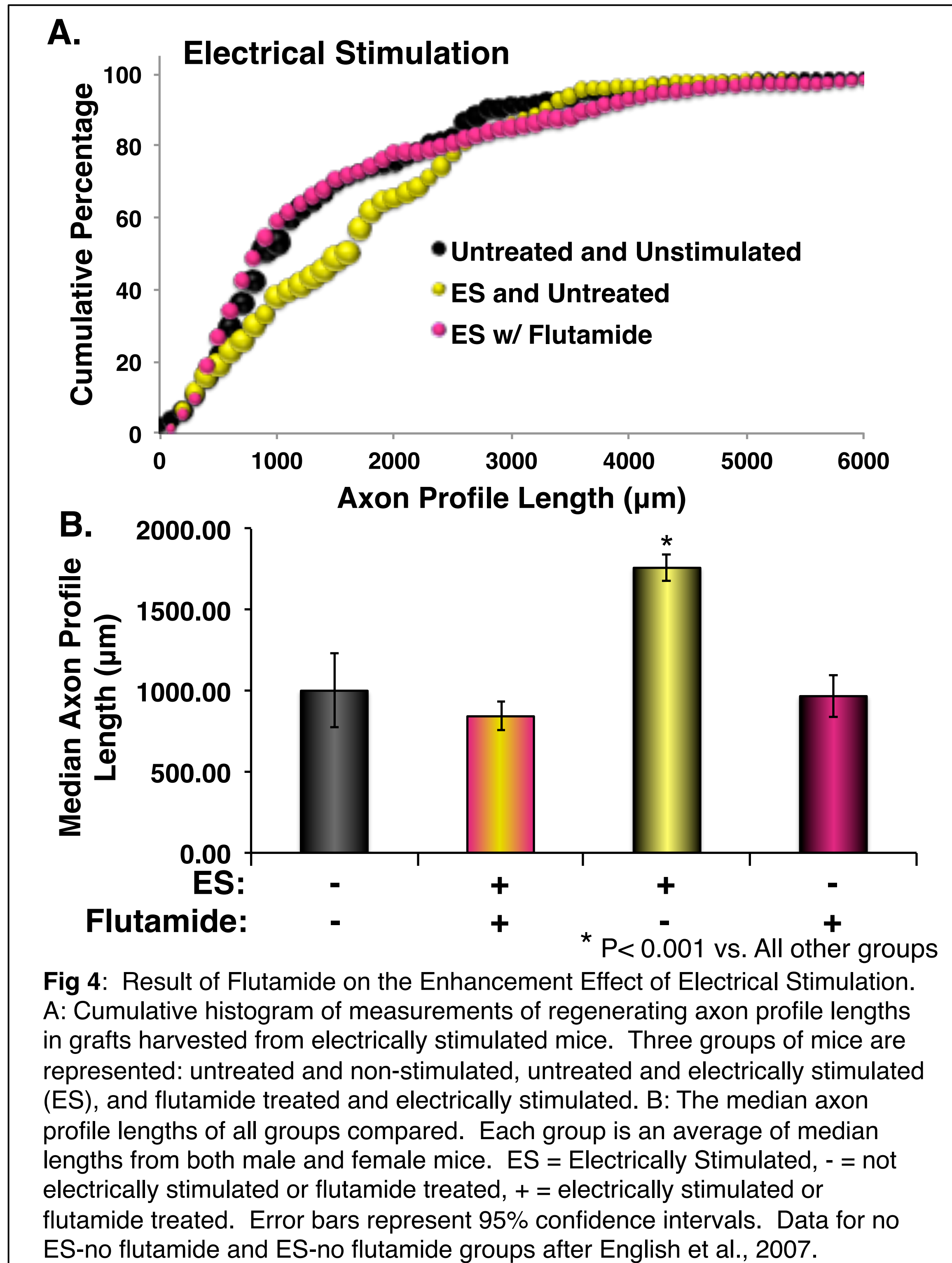

Fig 4: Result of Flutamide on the Enhancement Effect of Electrical Stimulation. A: Cumulative histogram of measurements of regenerating axon profile lengths in grafts harvested from electrically stimulated mice. Three groups of mice are represented: untreated and non-stimulated, untreated and electrically stimulated (ES), and flutamide treated and electrically stimulated. B: The median axon profile lengths of all groups compared. Each group is an average of median lengths from both male and female mice. ES = Electrically Stimulated, - = not electrically stimulated or flutamide treated, + = electrically stimulated or flutamide treated. Error bars represent 95% confidence intervals. Data for no ES-no flutamide and ES-no flutamide groups after English et al., 2007.

flutamide treated and electrically stimulated mice, the median lengths of

regenerating axon profiles were not significantly different from unstimulated and

untreated male ($p=0.3399$) or female ($p=0.1546$) mice, nor flutamide treated and unstimulated male ($p=0.6385$) or female ($p=0.05704$) controls. However, the median axon profile length of electrically stimulated and flutamide treated mice were significantly lower than the median lengths of electrically stimulated, untreated mice for both males (LSD, $p< 0.00006$) and females (LSD, $p<0.00000003$). Thus, the enhancement of axon regeneration after peripheral nerve injury produced by brief electrical stimulation is dependent on androgen receptor signaling.

Discussion

Complete functional recovery after peripheral nerve injury is rare, despite the inherent capacity for regeneration (Frostick *et al.*, 1998; Scholz *et al.*, 2009). The slow speed of axon regeneration is a major reason for this poor clinical outcome (Gordon *et al.*, 2009). Exercise in the form of treadmill training has been shown to increase axonal elongation significantly in the weeks following axotomy (Sabatier *et al.*, 2008). Androgens were suspected to be involved in the enhancement of axon regeneration due to treadmill training because of a sex difference we previously identified, in which different regimens of exercise lead to dissimilar results of axon regeneration between male and female mice (Wood *et al.*, 2012). In this study, we explored the involvement of androgen receptor signaling in the enhancement of axon regeneration produced by treadmill training by using flutamide, an androgen receptor antagonist. Both male and female mice were given a sustained dose of flutamide in order to inhibit androgen receptor signaling and then exercised to determine the importance of signaling through this receptor in the enhancement. In both sexes, the enhancement effect of exercise was eliminated upon flutamide treatment, demonstrating that androgen receptor signaling is necessary for the increase in axon regeneration due to treadmill training (Figure 3). Because there was no significant difference between flutamide treated, untrained groups and the untreated, untrained controls, androgen receptor signaling is necessary for the enhancement of axon

regeneration, but it does not affect the basal rate of regeneration found in unexercised mice.

The effectiveness of sustained release flutamide treatments was evaluated by comparing the weights of seminal vesicles and bulbospongiosis muscles in males and of uterus weights in females between treated and untreated animals. As predicted, bulbospongiosis muscle weights were significantly lower in flutamide treated males compared to untreated male mice, demonstrating that the flutamide treatment employed in this study effectively inhibited androgen receptor signaling (Figure 1C). It was surprising that the flutamide treatment eliminated the enhancement of axon regeneration due to exercise/electrical stimulation and significantly decreased bulbospongiosis muscle weight, but did not significantly lower seminal vesicle or uterus weights. It is possible that seminal vesicle and uterus weights are less sensitive to changes in androgen receptor signaling than bulbospongiosis muscle weight or the enhancements of axon regeneration due to exercise or electrical stimulation. Inter-animal variability in the seminal vesicle and uterus assays was high, while the number of animals in each group was small. It is possible that a significant difference will be found with a larger sample size. However, given the significant change in both bulbospongiosis muscle weight and axon regeneration following flutamide treatment, we believe that the androgen receptor signaling was sufficiently inhibited with flutamide.

The androgen receptor dependence of the enhancement of axon regeneration due to exercise is in keeping with previous findings from the English laboratory. In male mice, continuous training increased serum testosterone levels nearly eightfold and castration eliminated both this increase and the enhancement effect of continuous training (Wood *et al.*, 2012). The results presented above are consistent with a requirement for androgens in the enhancement of axon regeneration due to exercise in male mice. For female mice, the involvement of androgens has been less obvious. No increase in serum testosterone due to either continuous or interval training was found in females (Wood *et al.*, 2012). However, interval training has been shown to increase skeletal muscle androgen receptor expression (Aizawa *et al.*, 2010) and decrease muscle cytochrome C P450 aromatase mRNA levels (Aizawa *et al.*, 2008). Aromatase converts testosterone or its precursors into estradiol. Decreased aromatase activity would be expected to result in greater availability of testosterone. Pharmacologic inhibition of aromatase in unexercised female mice increased axon regeneration without increasing serum testosterone (Wood *et al.*, 2012), giving rise to the speculation that local inhibition of aromatase might underlie the effect of interval training in females. The finding presented above, that flutamide treatment eliminates the effect of interval training on the enhancement of axon regenertion in female mice is consistent with this hypothesized increase in local androgen availability. Determining whether interval training in female mice results in a

decrease in aromatase activity in the spinal cord or/and dorsal root ganglia would constitute a potent future test of this hypothesis.

Brain-derived neurotrophic factor (BDNF) has been identified as an important factor in axon regeneration after peripheral nerve injury (Gordon, 2009). Both Schwann Cell- and neuron-derived BDNF facilitate axon growth post axotomy (Funakoshi *et al.*, 1993) but neuron-derived BDNF is required for enhancement due to exercise (Wilhelm *et al.*, 2012). It has been suggested that neuronal BDNF could provide the link between exercise and axon regeneration, where exercise leads to an increase in androgens, which in turn increases androgen receptor signaling and promotes neuronal BDNF expression (Wood *et al.*, 2012). Supporting this connection, in castrated male rats supplemented with testosterone propionate, a long-term increase in BDNF mRNA was noted (Sharma *et al.*, 2010).

Electrical stimulation for as little as one hour at the time nerve injury has also been shown to enhance axon regeneration both alone (Al-Majed *et al.*, 2000b) and in conjunction with treadmill exercise (Asensio-Pinilla *et al.*, 2009). To test if the enhancement due to electrical stimulation is dependent on androgen receptor signaling, male and female mice were flutamide treated and then electrically stimulated prior to axotomy. We were surprised to find that inhibition of androgen receptor signaling with flutamide completely eliminated the enhancement of axon regeneration due to brief electrical stimulation in both males and females (Figure 4). Prior to this study, little evidence existed that androgens and the

enhancement effects of electrical stimulation are connected. Electrical stimulation increases mRNA of BDNF and its receptor, trkB, in motoneurons in both gonadally intact female rats (Al-Majed *et al.*, 2000a) and castrated male rats (Sharma *et al.*, 2010). Unless non-gonadally produced androgens were acting to increase BDNF expression in these castrated male rats, the increase in BDNF mRNA after electrical stimulation occurred independently of androgen receptor signaling. However, if BDNF mRNA is increased after electrical stimulation to the same extent in our chemically castrated, flutamide treated mice as in physically castrated rats, we would be compelled to conclude that the increase in BDNF is not sufficient to stimulate an enhancement of axon regeneration. Further investigation is needed to assess the effect of electrical stimulation on BDNF mRNA levels in flutamide treated mice.

A similar conclusion can be reached upon examination of the relationship between treadmill training and spinal cord BDNF expression. In male mice, a clear correlation exists between BDNF and treadmill training, as continuous training induces a significant increase in both spinal cord BDNF mRNA and axon regeneration. Interval trained males had neither an increase in spinal cord BDNF mRNA nor an increase in axon regeneration (Wood *et al.*, 2012). However, for female mice, no clear trend exists. Continuous training does not promote an enhancement of axon regeneration in female mice, yet a small but significant increase in spinal cord BDNF mRNA was found. A similar increase was found in interval trained female mice where axon regeneration was enhanced significantly.

It is possible that the detectable increase in spinal cord BDNF found in continuously trained female mice is somehow not sufficient to stimulate the enhancement of axon regeneration. Additionally, given the confusing relationship between electrical stimulation, androgens and axon regeneration previously discussed, it should not be assumed that the increase in testosterone seen in continuously trained, untreated male mice is the sole cause of the associated increase in spinal cord BDNF mRNA and axon regeneration, as previously proposed (Wood *et al.*, 2012). Measurement of spinal cord and/or neuronal BDNF mRNA in flutamide treated, continuously trained male mice should be completed to determine if BDNF is acting downstream of treadmill exercise and/or androgen receptor signaling.

With the knowledge that both neuronal BDNF and androgen receptor signaling are necessary for an enhancement of axon regeneration due to exercise or electrical stimulation after peripheral nerve injury, we propose three different models. The first model is a linear mechanism in which BDNF is downstream from androgen receptor signaling in a single neuron. Increased activity in the neuron, induced by exercise or electrical stimulation, would upregulate testosterone availability on a local or serum level, which in turn increases AR signaling and then BDNF levels to enhance axon regeneration. Given that BDNF has been found to increase in the motorneurons of castrated male rats that received electrical stimulation (Sharma *et al.*, 2010) and that castration eliminated the enhancement of axon regeneration due to continuous

training in male mice (Wood *et al.*, 2012), this explanation seems unlikely, and too simplistic. The second model is a parallel mechanism in which androgen receptor signaling works in conjunction with an increase in BDNF. Androgen receptor signaling could recruit a molecule or protein that is distinct from BDNF, but is also necessary for the increase in axon regeneration due to electrical stimulation or exercise. The neuronally produced BDNF would work in conjunction with the downstream target molecule of the androgen receptor signaling to enhance axon regeneration. The third model involves convergence of androgen receptor signaling with increased activity of the neuron to produce a marked increase in BDNF. BDNF produced separately by both androgen receptor and neuronal activity would compound to overcome a BDNF threshold, which then leads to enhanced axon regeneration. However, the neuronal BDNF produced by an increase in neuron activity is not at a high enough level to pass this threshold, and so if androgen receptor signaling (and its accompanied increase in BDNF) is blocked, axon regeneration would not be enhanced.

The second and third models, while more complicated, seem most compatible with the current literature, as they can explain an increase in activity-induced BDNF in motorneurons, as seen in electrically stimulated, castrated male rats (Sharma *et al.*, 2010), with the lack of enhanced axon regeneration we found in flutamide treated and electrically stimulated or treadmill trained mice. To determine the best candidate between the three models, one could treat mice with flutamide and then give them a large dose of small-molecule BDNF

analogues. Small-molecule BDNF analogues have been shown to increase axon regeneration in mice (English Lab – unpublished data). If small-molecule BDNF analogues do not increase axon regeneration in mice that have androgen receptor signaling blocked by flutamide, then the second model, in which androgen receptor works in parallel with BDNF, seems most likely. If axon regeneration increases in this experiment, then either the first or third models are better candidates. A second experiment, in which one measures BDNF mRNA in the spinal cord and motoneurons of flutamide treated, exercised/electrically stimulated mice could be performed. The third mechanism would predict a small, but significant increase in neuronal BDNF in these mice, produced by increased neuronal activity, while no increase in BDNF would be expected from the first, linear model.

In either of the latter two models, it is also possible that a local cell distinct from the BDNF producing neuron, such as an oligodendrocyte or Schwann Cell, is producing the androgens and/or androgen receptor signaling. Furthermore, if the locally produced androgens were immediately metabolized and promoted androgen receptor signaling in either the BDNF producing neuron or a nearby cell, it would explain why no increase in testosterone serum level or spinal cord BDNF was detected in interval trained, female mice, compared to continuous trained, female mice (Wood *et al.*, 2012). To test which cells use androgen receptor signaling and increase axon regeneration, cell-type specific androgen receptor knockout mice could be employed. Knocking out androgen receptors in

motorneurons, sensory neurons, or the cells surrounding neurons such as oligodendrocytes, would elucidate this pathway further.

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