Title: Neuronal androgen receptor is required for activity dependent enhancement of peripheral nerve regeneration.

Running title: Androgens facilitate axon regeneration

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Abstract

Neuronal activity after nerve injury can enhance axon regeneration and the restoration of function. The mechanism for this enhancement relies in part on hormone receptors, and we...
previously demonstrated that systemic androgen receptor antagonism blocked the effect of exercise or electrical stimulation on enhancing axon regeneration after nerve injury in both sexes. Here, we tested the hypothesis that the site of this androgen receptor signaling is both neuronal and involves the classical, genomic signaling pathway. In vivo, dorsal root ganglion neurons successfully regenerate in response to activity-dependent neuronal activation, and conditional deletion of the DNA-binding domain of the androgen receptor in adults blocks this effect in males and females. Motoneurons in males and females also respond in this manner, but we also observed a sex difference. In vitro, cultured sensory dorsal root ganglion neurons respond to androgens via traditional androgen receptor signaling mechanisms leading to enhanced neurite growth and did not respond to a testosterone conjugate that is unable to cross the cell membrane. Given our previous observation of a requirement for activity-dependent androgen receptor signaling to promote regeneration in both sexes, we interpret our results to indicate that genomic neuronal androgen receptor signaling is required for activity-dependent axon regeneration in both sexes.

Keywords: androgen receptor, testosterone, electrical stimulation, axon regeneration, nerve injury, motoneuron, dorsal root ganglia

Introduction

In the United States alone, twenty million people are affected by nerve injuries resulting from trauma or medical disorders (NIH Publication No. 18-NS-4853). Nerve injury causes muscle paralysis and loss of sensation from the affected areas. Axons in peripheral nerves exhibit limited spontaneous regeneration that can be enhanced with interventions experimentally, but the lack of full functional recovery in humans and lack of an approved, standard therapeutic treatment after surgery for nerve injury patients calls for continued mechanistic studies of axonal injury and regeneration. Exercise and healthy active life-styles are becoming increasingly recognized as important factors for recovery from various neurological injuries, and we have shown that neuronal activity – in the forms of exercise, optical stimulation, or electrical stimulation – enhances axon regeneration following peripheral nerve injuries (English, Cucoranu, Mulligan, & Sabatier, 2009; Jaiswal, Tung, Gross, & English, 2017; Thompson, Sengelaub, & English, 2014; Ward, Clanton, & English, 2018; Ward et al., 2016).

Exercise has broad effects in both men and women, and sexual dimorphism exists in a number of tissues, including the nervous system. We previously found that after nerve injury, male and
female mice respond differentially to interval and continuous exercise training paradigms (Wood et al., 2012). When evaluating regeneration two weeks after injury, a continuous treadmill running paradigm for one hour each day is effective in males but not in females; a higher speed interval treadmill training paradigm (four intervals of two minutes running followed by five minutes rest) effectively enhances axon regeneration in females but not in males. Enhancement of axon regeneration after nerve injury, either through exercise or one hour of 20 Hz electrical stimulation (Al-Majed, Neumann, Brushart, & Gordon, 2000), requires signaling through the androgen receptor in both males and females. In males, the primary source of gonadal androgens are the testes, and castration of male mice blocked the regeneration-enhancing effect of exercise (Wood et al., 2012). In unexercised female mice, axon regeneration was promoted when testosterone conversion to estradiol was inhibited by the aromatase inhibitor anastrozole (Wood et al., 2012). Blocking systemic androgen receptor signaling with the antagonist flutamide prevented the effects of both exercise and electrical stimulation on nerve regeneration in both males and females (Thompson et al., 2014). Furthermore, exercise training increased serum testosterone in males but not females, and anastrozole treatment did not increase serum testosterone in females (Wood et al., 2012), which suggests that local cellular steroidogenesis and androgen receptor signaling is occurring in females. In support of this idea, it has been shown that local steroid synthesis occurs in response to treadmill exercise that is required for adult hippocampal neurogenesis (Okamoto et al., 2012).

The androgen receptor is a ligand-dependent nuclear transcription factor in the steroid hormone nuclear receptor family. The mechanism of androgen receptor signaling is complex and incompletely understood, especially in non-reproductive tissues. The androgen receptor has three functional domains: a ligand-binding domain, DNA-binding domain, and an N-terminal transcriptional regulation domain. Androgen receptor mediated actions can occur through three mechanisms: DNA-binding dependent, non-DNA-binding dependent, and ligand-independent actions (see review (Davey & Grossmann, 2016)). Here, we investigated whether the genomic (classical, DNA-binding) action of the androgen receptor was required for enhancing axon regeneration after 20 Hz electrical stimulation using a neuron-specific androgen receptor mouse mutant in which an in-frame deletion of the second zinc finger of the DNA-binding domain abolishes genomic AR signaling (MacLean et al., 2010). We also examined whether the non-DNA binding action of the androgen receptor could be involved in this activity-dependent
enhancement in cultured dorsal root ganglion neurons by utilizing a testosterone conjugate that is unable to cross the cell membrane (Gu et al., 2013). A preliminary report of some of these findings has been made.

**Materials and Methods**

*Animals and Surgeries*

All procedures were approved by the Institutional Animal Care and Use Committee of Emory University and conformed to the Guidelines of the Office of Laboratory Animal Welfare of the National Institutes of Health. The mice were group housed (12:12 light:dark cycle) with ad libitum access to food and water. The mice were bred and maintained at the Division of Animal Resources at Emory University.

For cell culture experiments, wild-type C57BL/6J mice of each sex were used. The androgen receptor (AR) knockout mice were developed using the Cre-lox system with an animal known as Single-neuron Labeling with Inducible Cre-mediated Knockout or SLICK. The A strain of SLICK mice was used throughout. Neurons expressing tamoxifen-inducible Cre also express Enhanced Yellow Fluorescent Protein (eYFP). Thus, SLICK-A mice co-express a drug-inducible form of Cre recombinase and eYFP protein in small subsets of neurons which facilitates conditional genetic manipulation and fluorescent labeling of single neurons for imaging (Young et al., 2008). Mice expressing a Cre-dependent floxed allele of exon three of the androgen receptor gene (Notini, Davey, McManus, Bate, & Zajac, 2005) were bred with SLICK mice (strain A) expressing a tamoxifen-inducible Cre recombinase (Jackson Laboratories, B6.Cg-Tg(Thy1-cre/ERT2,-EYFP)AGfng/J; SLICK-A; stock no. 007606). The androgen receptor gene is X-linked and requires both X chromosome copies of the androgen receptor gene to be floxed (fl) in female mice. Male mice only carry one copy of the androgen receptor gene. Thus, the resulting genotypes are SLICK-AR^{fl/y} if male or SLICK-AR^{fl/fl} if female. Note that this nomenclature refers to knockout of exon 3 and not the entire AR gene.

To induce Cre recombinase expression, 6–8 week old animals expressing both Cre (SLICK-A) and AR floxed genotypes were treated with tamoxifen (Sigma-Aldrich, St. Louis, MO, USA). To ensure that the Cre recombinase enzyme was successfully and sufficiently activated, we treated mice with two oral treatments of three consecutive days of tamoxifen, and the two treatments were separated by two weeks (six oral treatments in total, three days in each week). The efficacy of this tamoxifen treatment protocol to induce Cre recombinase expression has been validated.
several times in our hands (English, Liu, Nicolini, Mulligan, & Ye, 2013; Wilhelm et al., 2012; Zhu, Ward, & English, 2016). Once Cre is expressed, the DNA binding domain of the androgen receptor is excised and permanently deleted. When crossed with a global Cre expressing line, we observe a robust phenotype in which males fail to sexually differentiate and display androgen insensitivity (Notini et al., 2005). Male mice expressing the floxed exon 3 of the androgen receptor crossed with the global Cre exhibit a female phenotype, and molecular analysis revealed that the mutant androgen receptor is expressed at levels similar to wild-type androgen receptor in tail, kidney, skeletal muscle, liver, heart and spleen. We have also detected physiological changes in females lacking exon 3 of the androgen receptor, including decreased heart and kidney mass and decreased cortical bone growth (MacLean et al., 2010). With our colleagues and collaborators, many studies have validated this mouse line (Lee, Skinner, Zajac, & MacLean, 2011; MacLean et al., 2008; McManus et al., 2020; Pang et al., 2012; Rana, Clarke, Zajac, Davey, & MacLean, 2014; Rana et al., 2011). The ligand-binding activity and non-DNA binding dependent activity of the androgen receptor remain (Davey & Grossmann, 2016; Notini et al., 2005). Tamoxifen-treated SLICK-AR fl/y male and SLICK-AR fl/fl female mice were used in experiments two to four weeks after the end of the second round of tamoxifen treatments. The term SLICK-AR is used to denote male and female mice in which the DNA binding domain of the androgen receptor is deleted.

All experiments were conducted on adult mice weighing between 18–28 grams. A mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) was used to induce anesthesia. Additional ketamine was administered as required throughout the surgical procedure. The sciatic nerve was exposed unilaterally and draped onto a small rectangle of SILASTIC film (Dow Corning 501–1, Thermo Fisher, Norcross, GA, USA). The sciatic nerve was cut with sharp micro-scissors just proximal to the branching of the tibial, common fibular, and sural nerves. The aligned proximal and distal nerve stumps were secured in place using fibrin glue: a mixture of fibrinogen, fibronectin, and thrombin (Sigma-Aldrich, St. Louis, MO, USA). This solution cures to form a clot and mechanically secure the nerve stumps (Akhter, Rotterman, English, & Alvarez, 2019). The post-operative analgesic, meloxicam, was administered subcutaneously after the animals awoke from anesthesia. Nerve grafting experiments were similarly performed, with the exception that only the common fibular branch was transected. The common fibular nerve was then
repaired with a donor nerve from a wild-type, age- and sex-matched mouse. The donor nerve provides a dark background through which the regenerating eYFP axon profiles can be visualized and quantified for elongation (Jaiswal, Mistretta, Ward, & English, 2018; Thompson et al., 2014; Wilhelm et al., 2012).

Electrical Stimulation and Axon Elongation in Nerve Grafts

Mice of each genotype (SLICK-ARfl/y, SLICK-ARfl/fl, or SLICK) and sex were randomly assigned to one of two treatments: transected/repaired and untreated or transected/repaired and treated with electrical stimulation for 1 hour. For electrical stimulation, a small bipolar cuff electrode was constructed from an incised piece of silastic tubing with two sets of fine stainless steel wires sewn into the inner diameter of tubing (Ward & English, 2019). At the time of surgery, nerves were supramaximally stimulated continuously for one hour with 0.1 msec duration constant voltage pulses delivered through a stimulus isolation unit at 20 Hz. Stimulation can be performed prior to or after (Foecking et al., 2012; Gordon, Amirjani, Edwards, & Chan, 2010) cutting the nerve; stimulation of the soma is necessary for the enhancing effects of stimulation (Al-Majed, Neumann, et al., 2000). Stimulating immediately prior to nerve transection provides a useful visual, physical readout of the stimulation. Results from longer or repeated periods of stimulation have been mixed and may inhibit regeneration and alter central circuitry (Asensio-Pinilla, Udina, Jaramillo, & Navarro, 2009; Geremia, Gordon, Brushart, Al-Majed, & Verge, 2007; Koo et al., 2018; Park, Liu, Ward, Jaiswal, & English, 2019). Thus, we chose to limit the stimulation to 1 hour at the time of nerve surgery.

To measure axon profile lengths, nerve grafts (proximal and distal stumps) were harvested at two weeks post-transection and repair as we have performed previously (Jaiswal et al., 2018; Thompson et al., 2014; Wilhelm et al., 2012). Briefly, the common fibular nerves were mounted onto slides using VectaShield (Vector Labs) and stored at 4°C. The nerves were imaged at a low magnification using a Leica SP8 confocal microscope. Stacks of serial optical sections (10 μm thick) were then reconstructed using a stitching function to visualize the entire length of the repaired nerve and graft. The lengths of eYFP+ axon profiles were quantified using FIJI software. More than 1500 axon lengths were quantified with 2-4 animals represented in each treatment group and sex. We compared the effects of electrical stimulation and knockout of the DNA-binding domain of the androgen receptor on axon elongation in males and females.

Cell Culture

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Adult wild-type mice of both sexes were euthanized with an overdose of isoflurane. The vertebral columns were removed, and dorsal root ganglia (DRGs) were gently and quickly dissected and placed in cold Hank’s Balanced Salt Solution (HBSS, Corning, Corning, NY). DRGs were incubated in dispase (2.5 u/mL Sigma-Aldrich) and collagenase (200 u/mL Worthington Biochemical, Lakewood, NJ) in a 37°C bead bath for 45 minutes with gentle agitation applied every 15 minutes. DRGs were then treated in 37°C DNase (Worthington Biochemical) for 2.5 minutes before addition of room temperature HBSS. Cells were triturated using a fire-polished glass pipette and centrifuged for 3 minutes at 3000 rpm. The HBSS was removed and cells were resuspended in neurobasal medium A (NB-A, Invitrogen, Carlsbad, CA) containing 2% B-27 (Invitrogen), 1% penicillin/streptomycin (Lonza Biowhittaker), and 1% Glutamax (Invitrogen). Cells were seeded at a density of 1000 cells/well in 24-well Nunc plates containing a glass coverslip pre-coated in laminin (0.2 mg/mL, Thermo Fisher Scientific, Waltham, MA) and poly-l-lysine (2 mg/mL, Sigma-Aldrich).

Twenty-four hours after plating, the NB-A solution was replaced with treatments diluted in NB-A. Treatments were 100 µM testosterone, 100 µM testosterone conjugated to bovine serum albumin, testosterone with flutamide, or vehicle. Cells were fixed with 4°C paraformaldehyde fixative seventy-two hours after plating, i.e., 48 hours after treatment. To visualize the neurons, anti-tubulin β-3 antibody was added to each well overnight (1:1000, 14-16 hours, Biolegend, San Diego, CA) followed by application of a secondary, goat anti-mouse antibody conjugated to Alexafluor 555 (Invitrogen). Washes were performed with 0.1 M PBS. All glass coverslips were then removed from the wells and mounted on slides with Vectashield. The longest process from each neuron was measured from these images using Fiji. Between 30-75 neurons per treatment were measured for each animal with 3-9 animals represented in each treatment group for each sex.

Retrograde Labeling of Regenerating Axons

Mice of each genotype (SLICK-AR^{fl/y}, SLICK-AR^{fl/fl}) and sex were randomly assigned to one of two treatments: transected/repaired and untreated or transected/repaired and treated with electrical stimulation for 1 hour as described above. Two weeks after nerve repair, mice were re-anesthetized, and the nerve repair site was re-exposed. The nerve was transected approximately 5 mm below the original transection and repair site. To measure the number of neurons whose axons had regenerated successfully, spinal motoneurons and dorsal root ganglia primary sensory
neurons in these animals were labeled by application of the retrograde fluorescent tracer, dextran (MW 10,000) conjugated to a red fluorophore (Alexafluor 555, Invitrogen, Eugene, OR).

The proximal nerve stump was surrounded by a well of vacuum grease formed on a small square of Parafilm. The cut nerve was then soaked in saline for five minutes. If the well of vacuum grease did not leak, the saline was then removed and replaced with crystals of dextran amines (10,000 MW, fixable) conjugated to Alexafluor 555. Once in place, the crystals were hydrated with a small drop of saline solution. The tracers were left in place for 60 minutes with constant observation to ensure that the nerve stump did not dry out. Next, the crystals were removed by irrigating the entire surgical field three times with normal saline solution. The surgical wounds were closed in layers, and the animals were returned to their cages.

Animals survived four days after tracer application before being euthanized and perfused with aldehyde fixative. The L2-L5 segments of spinal cord and the fourth lumbar dorsal root ganglion (ipsilateral to the nerve injury) were removed and stored overnight in 20% sucrose solution at 4°C for cryoprotection. Serial transverse sections were cut on a cryostat and mounted directly onto Superfrost Plus slides. Section thickness was 30 and 14 μm for spinal cords and dorsal root ganglia, respectively. High resolution RGB images (20X) were obtained with a Leica DM6000 upright microscope, a low-light camera, and HCImage software. Neurons were then manually scored as retrogradely-labeled if the label filled the neuron soma and also contained a visible nuclear area devoid of label. To avoid double counting, care was taken to identify any fragments of each labeled neuron on adjacent serial sections. We assumed that retrogradely labeled neurons represent neurons whose cut axons had regenerated at least five mm by two weeks post-transection. Neurons that were retrogradely labeled and also expressed eYFP in the SLICK-AR<sup>fl/y</sup> and SLICK-AR<sup>fl/fl</sup> mice that had been treated with tamoxifen were assumed to be AR knockout neurons. Neurons that were retrogradely labeled and eYFP negative were assumed to be wild-type neurons. The total number of retrogradely-labeled neurons was compared between untreated and electrically stimulated groups with and without deletion of the DNA-binding domain of the androgen receptor (4-6 animals in each treatment group and sex).

**Euthanasia**

In all nerve repair experiments, animals were euthanized at two weeks post-transection. Euthanasia was performed via overdose of pentobarbital (150 mg/kg, IP) and mice were then perfused transcardially with saline followed by paraformaldehyde fixative.

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Statistics
We designed the statistics to test for a sex difference by using 2-way ANOVA (sex and treatment) on unpooled data. When a statistically significant sex difference was detected (as in Figure 1 and 2), we show the relevant unpooled data as in Figures 1E, 1F, and 2D and provide the post-hoc tests. For mean longest neurite lengths and numbers of retrogradely labeled neurons, we performed a two-way (sex and treatment) analysis of variance (ANOVA) followed by Fisher’s least significant difference post-hoc testing where appropriate. Distributions of axon profile lengths were not normally distributed and thus were analyzed using the two sample nonparametric Kolmogorov-Smirnov test. Probabilities of less than 0.05 were considered statistically significant. All statistical comparisons were performed using GraphPad-Prism. Researchers were blinded to the experimental conditions applied until the quantitative analyses had been completed.

Results
Neuronal activity enhances axon elongation and this effect is blocked by disabling genomic androgen-receptor signaling in male but not female mice in vivo
We had previously shown that systemic pharmacologic androgen receptor antagonism blocked activity-dependent enhancement of axon regeneration in both males and females (Thompson et al., 2014). Here, we tested whether the local site of the androgen receptor activity required for enhancement was neuronal. In SLICK-AR\(^f/y\) male and SLICK-AR\(^f/f\) female mice that had been tamoxifen-treated or SLICK controls, peripheral nerves were cut and repaired with grafts harvested from non-fluorescent wild-type mice. The host mice received either electrical stimulation or were untreated. Two weeks later, grafts were harvested for confocal imaging. Lengths of profiles of fluorescent regenerating axons were measured in optical sections (Figure 1A-D). The distributions of axon profile lengths measured in these mice are shown as cumulative histograms in Figures 1E and F for males and females, respectively. The unpaired Kolmogorov-Smirnov test was used to compare the cumulative distributions between groups. Brief electrical stimulation significantly shifted the distribution of regenerating eYFP+ axon profiles to the right (increasing length) from that of SLICK untreated controls in both males (D(6)=0.2868, p<0.001) and females (D(5)=0.2749, p<0.001) (Figure 1E, F, gray trace). This result indicates that axon
elongation after nerve cut and repair was enhanced by the electrical stimulation in SLICK mice and agrees with previous results. This effect was not observed in tamoxifen-treated SLICK-AR\textsuperscript{fl/y} male mice that received electrical stimulation, e.g., the distribution of axon profile lengths from electrically stimulated SLICK-AR\textsuperscript{fl/y} male mice was similar to that of untreated SLICK male mice (D(6)=0.1698, p=0.16) (Figure 1E, yellow trace). We interpret this finding to indicate that electrical stimulation did not significantly enhance axon elongation in male mice in which genomic androgen receptor signaling had been conditionally deleted in neurons.

In tamoxifen-treated SLICK-AR\textsuperscript{fl/fl} female mice in which ES was applied at the time of nerve repair, the distribution of regenerating eYFP+ axon profiles was significantly shifted to the right vs SLICK-AR\textsuperscript{fl/fl} untreated (D(5)=0.5243, p<0.0001) and SLICK untreated female mice (D(6)=0.3591, p<0.0001) (Figure 1F, yellow trace). A similar shift in the distribution of lengths of regenerating axon profiles was found in electrically stimulated SLICK mice (D(5)=0.2749, p<0.001) (Figure 1F, grey trace). Electrical stimulation thus promoted axon elongation in both SLICK females with wild type androgen receptor and SLICK-AR females with genomic androgen receptor signaling deletion.

Cell-type specificity of activity-dependent treatment via DNA-binding androgen receptor signaling

Next, we further investigated the requirement for androgen receptor signaling in two neuronal types. For this experiment we used tamoxifen treated SLICK-AR\textsuperscript{fl/y} and SLICK-AR\textsuperscript{fl/fl} mice that received either ES or no treatment after nerve transection. We performed retrograde labeling two weeks after nerve repair and sectioned and imaged the ventral spinal cord to determine the numbers of motoneurons whose axons had regenerated successfully (Figure 2A). We anticipated that electrical stimulation would promote motor axon regeneration leading to an increase the number of retrogradely labeled motoneurons. Because not all neurons express Cre-eYFP in SLICK-AR mice, we were able to distinguish regenerating neurons expressing wildtype androgen receptor (single-labeled) from neurons in which the DNA binding domain of the androgen receptor had been deleted (double-labeled). There was a significant effect of treatment on the number of single-labeled motoneurons (F(1,14)=10.90, p=0.0052). Sex and the interaction were not significant (F(1,14)=0.4989, p=0.4916; F(1,14)=0.006, p=0.98, respectively). We pooled the data by sex and performed a two-tailed post-hoc unpaired t-test on the variable treatment. The mean counts of single-labeled motoneurons from the electrically stimulated group...
was significantly different from untreated ($t_{16}=3.493$, $p=0.003$). Thus, electrical stimulation resulted in greater numbers of regenerating motoneurons expressing wildtype androgen receptor in males and females (Figure 2B). Next, we analyzed the numbers of double-labeled (DNA binding domain androgen receptor knockout) motoneurons. Treatment and the interaction between treatment and sex were not significant ($F(1,14)=0.9762$, $p=0.34$), ($F(1,14)=0.7665$, $p=0.3961$, respectively). However, sex was a significant factor among motoneurons lacking the DNA-binding domain of the androgen receptor ($F(1,14)=7.932$, $p=0.0137$). We pooled the data by treatment. Because the variances differed significantly, we performed an unpaired, two-tailed nonparametric t-test. The mean count of double-labeled motoneurons from the female group was significantly different from the male group ($D(18)=0.625$, $p=0.015$) (Figure 2D). We interpret this finding to indicate that electrical stimulation was not effective at promoting the regeneration of axons of motoneurons lacking the DNA-binding domain of the androgen receptor (Figure 2C). However, more motoneurons lacking the DNA-binding domain of the androgen receptor regenerated in females compared to males (Figure 2D).

We next investigated the numbers of successful regenerating sensory neurons in tamoxifen treated SLICK-AR$^{fl/y}$ and SLICK-AR$^{fl/fl}$ mice that received either ES or no treatment after sciatic nerve transection and repair. We performed retrograde labeling two weeks after nerve repair and sectioned and imaged the lumbar level 4 DRGs to determine the numbers sensory neurons whose axons had regenerated successfully (Figure 3A). As above, we anticipated that electrical stimulation would promote sensory axon regeneration leading to an increase in the number of retrogradely labeled (single-labeled = wildtype androgen receptor) DRG neurons in SLICK-AR mice. There was a significant effect of treatment on the number of single-labeled DRG neurons ($F(1,14)=14.41$, $p=0.002$). Sex and the interaction term were not significant ($F(1,14)=1.295$, $p=0.274$), ($F(1,14)=1.786$, $p=0.203$, respectively). We pooled the data by sex. Because the variances differed significantly, we performed an unpaired, two-tailed nonparametric t-test. The mean counts of single-labeled DRG neurons from the electrically stimulated group were significantly different from those of untreated mice ($D(18)=0.7778$, $p=0.0049$). Thus, electrical stimulation resulted in greater numbers of regenerating DRG neurons expressing wild type AR (Figure 3B). Next, we analyzed the numbers of double-labeled (genomic AR signaling knockout) DRG neurons. Sex, treatment, and the interaction between treatment and sex were not significant among DRG neurons lacking the DNA-binding domain of the androgen receptor.
Testosterone enhances neurite outgrowth in dorsal root ganglia cultures from males and females via classical ligand-dependent, DNA-binding dependent androgen receptor action

In a variety of cell types, it has been demonstrated that the androgen receptor complex can signal through non-DNA binding-dependent pathways that are activated within seconds to minutes of androgen treatment. This time frame is thought to be too short to be due to the classical DNA-binding dependent androgen receptor action involving gene transcription and translation. Membrane bound androgen receptor activity has also been described in vivo (Gu et al., 2013; Peterziel et al., 1999) and importantly within the neuronal plasma membrane (Garza-Contreras, Duong, Snyder, Schreihofer, & Cunningham, 2017).

To probe some of these possibilities, we studied growing neurites from dissociated primary sensory neurons from male and female wild type mice (Figure 4). Neurons collected from male and female adult mice were grown in culture for 72 hours followed by fixation, imaging, and measuring the longest single neurite from 30-70 neurons per treatment. The following reagents were added to the culture media: testosterone, testosterone conjugated to bovine serum albumin, testosterone plus flutamide, and testosterone plus 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole. Sex and the interaction terms were not significant (F(4,40)=0.7684, p=0.39; F(4,40)=0.5363, p=0.71), but the effect of treatment was significant F(4,40)=4.244, p<0.006). The addition of testosterone to the media increased the mean longest neurite length compared to vehicle controls (dashed line) (Figure 4A, F) (p<0.0049). This observation agrees with in vivo studies from our lab and others (Sharma et al., 2009; Sharma, Marzo, Jones, & Foecking, 2010; Thompson et al., 2014) demonstrating the regeneration enhancing effects of androgens. Next, we tested the possibility that membrane bound androgen receptor activity could promote enhanced neurite elongation. Testosterone conjugated to bovine serum albumin (T-BSA) is a cell impermeable androgen. However, there was no significant change in longest neurite length after the addition of T-BSA to dissociated DRG cultures from males or females. Next, we tested the ligand- and transcriptional-dependency of neurite growth by the addition of testosterone plus flutamide or testosterone plus 5,6-dichloro-1-beta-D-
ribofuranosylbenzimidazole (DRB), respectively. Flutamide is an anti-androgen that blocks androgen receptors (Thompson et al., 2014). DRB halts transcription via inhibition of RNA polymerase. There was no difference in longest neurite length after the addition of testosterone plus flutamide or testosterone plus DRB to dissociated DRG cultures from males and females (Figure 4) (both p>0.05). Antagonism with flutamide prevented testosterone’s neurite enhancing effect. Similarly, the effect of testosterone on neurite length was blocked by transcriptional inhibition with DRB. These results are consistent with an interpretation that androgens signal via the classical ligand-dependent, DNA-binding dependent mechanism to enhance axon regeneration in both males and females.

Discussion

Androgen receptors are widely expressed in different cell types throughout the nervous system (Menard & Harlan, 1993; Sarkey, Azcoitia, Garcia-Segura, Garcia-Ovejero, & DonCarlos, 2008). Jones and colleagues have shown that testosterone enhances the regeneration of motor axons (Sharma et al., 2009; Sharma et al., 2010). We investigated the cell type specificity of androgen receptor action and whether this action was via non-genomic or classical, DNA-binding genomic activity of the androgen receptor for axon regeneration. We utilized a testosterone conjugate that is membrane delimited (Gu et al., 2013) and a neuron-specific androgen receptor knockout mouse line in which a DNA-binding domain deletion abolishes genomic androgen receptor signaling (MacLean et al., 2010). We performed in vivo conditional deletion of the DNA-binding dependent domain of the androgen receptor from subsets of motoneurons and DRG neurons. Brief neuronal activity in the form of 1 hour of electrical stimulation promoted axon elongation in wild-type neurons from male and female mice (Figure 1), which agrees with many in vivo studies from our lab and others demonstrating the regeneration enhancing effect of brief neuronal activity (Al-Majed, Brushart, & Gordon, 2000; Gordon et al., 2010; Jaiswal et al., 2018; Ward et al., 2018; Ward & English, 2019; Zuo et al., 2020). When this same treatment was applied in mice with selective deletion of the DNA-binding dependent domain of the androgen receptor, the axon elongation enhancing effect of electrical stimulation was prevented in males (but not females). In support of the axon...
profile data quantifying elongation, retrograde tracing resulted in greater numbers of regenerating wild-type DRG and motoneurons from male and female mice after 1 hour of electrical stimulation (Figure 2B and 3B). The numbers of regenerating AR-KO motoneurons and DRG neurons from male and female mice was not enhanced after 1 hour of electrical stimulation; however, female mice had more regenerating AR-KO motoneurons compared to male mice. Since we have previously demonstrated a complete blockade of activity-dependent regeneration in females in the presence of the systemic androgen receptor blocker flutamide (Thompson et al., 2014), one could conclude that the site of androgen receptor action within the spinal cord of female mice involves a different cell type, such as glia (Bielecki et al., 2016; Cerghet et al., 2006; Garcia-Ovejero, Veiga, Garcia-Segura, & DonCarlos, 2002; Hosli, Jurasin, Ruhl, Luthy, & Hosli, 2001; Li, Penderis, Zhao, Schumacher, & Franklin, 2006). Alternatively, a limitation is that due to the small number of neurons expressing Cre, it was not confirmed that exon 3 was deleted, and our results may reflect incomplete or failure of deletion in females.

Using cultured DRG neurons, we were able to test some of the modes of androgen receptor signaling. The fast effects of androgen receptor signaling in neurons can promote intracellular calcium signaling, which is a known requirement for axon regeneration (Estrada, Uhlen, & Ehrlich, 2006; Holmes, Abbassi, Su, Singh, & Cunningham, 2013). Testosterone conjugated to bovine serum albumin (T-BSA) is a cell impermeable androgen that also elicits calcium signaling (Estrada, Espinosa, Muller, & Jaimovich, 2003; Estrada et al., 2006). However, T-BSA was not sufficient to enhance neurite growth. We interpret our data to indicate that testosterone promotes neurite growth and regeneration via the classical ligand-dependent, DNA-binding dependent mechanism (via the androgen receptor acting at the genomic level as a transcription factor) at least in cultured DRG neurons.

Importantly, estrogen receptors are also important for axon regeneration (Islamov et al., 2002; Islamov et al., 2003). Pharmacological blockade of estrogen receptors blocks the enhancing effect of treadmill exercise on the numbers of motoneurons participating in regeneration in both sexes (Wilhelm et al., 2012). Thus, there is a complex interplay between estrogen and androgen signaling and activity-dependent therapies. Importantly, testosterone can be converted to estrogen by aromatization. Supporting this interaction, several studies have found that treating mice with a non-aromatizable form of testosterone produces less enhancement of axon regeneration.
elongation than treating mice with the aromatizable form of testosterone, suggesting that androgens work synergistically with estrogen signaling to enhance axon regeneration. Based on our current findings, we believe that neuronal genomic androgen receptor signaling is a required mechanistic component of the enhancement of axon regeneration produced by activity-dependent experimental therapies in both sexes. Given that circulating testosterone decreases with aging, these mechanisms may in part underlie the generally poor regenerative capacity of the nervous system in aged mammals (Baulieu, 1997; Buttner et al., 2018; Le, Aszmann, Chen, Royall, & Brashart, 2001; Scheib & Hoke, 2016). In female mice, motoneurons lacking genomic androgen receptor signaling retained an enhanced regenerative capacity compared to males. Given the difficulty of culturing adult motoneurons, future studies could consider culturing stem cell derived motoneurons to evaluate the role of non-traditional androgen receptor signaling in females. Future studies should also more closely consider the role of aromatization, second-messenger signaling, and the role of androgen receptor signaling in glia or immune cells surrounding the motoneurons after injury, especially in females.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon request.

Author Contributions: PJW participated in study conception/design, data collection, data analysis and interpretation. RAD and JDZ participated in data interpretation and developed the floxed exon 3 androgen receptor mouse line. AWE participated in study conception/design and data interpretation. All authors participated in drafting and revising the manuscript and have approved the final version for publication.


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nerve crush injury in ovariectomized mice. *Brain Res, 966*(1), 65-75. doi:10.1016/s0006-8993(02)04191-4


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The data that support the findings of this study are available from the corresponding author upon reasonable request.

Figure Legends

Figure 1. A) Images from optical sections through repaired nerves from SLICK or SLICK-AR mice harvested two weeks after injury. White lines indicate site of sciatic nerve transection and repair. The dashed box is enlarged in B-D. Note that female mice with genomic AR deletion (D) exhibit many regenerating axons in the distal stump. E-F) The distributions of regenerating axon profile lengths in males and females, respectively. Dashed lines = medians. ES = electrical stimulation. Two-sample nonparametric Kolmogorov-Smirnov.

Figure 2. A) Examples of single (red or green arrow) and double (yellow arrow) labeled motoneurons in the spinal cord. B) Mean number of retrogradely labeled wild-type motoneurons two weeks after sciatic nerve transection and repair. C) Mean number of retrogradely labeled AR knockout motoneurons. D) Comparison of mean number of retrogradely labeled AR knockout motoneurons between males and females. YFP = yellow fluorescent protein. AF555 = AlexaFluor 555. ES = electrical stimulation. Individually graphed points represent biological replicates. Two-way ANOVA. Error bars = SEM.

Figure 3. Examples of single (red or green arrow) and double (yellow arrow) labeled neurons in the L4 dorsal root ganglia. B) Mean number of retrogradely labeled wild-type neurons two weeks after sciatic nerve transection and repair. C) Mean number of retrogradely labeled AR knockout motoneurons. ES = electrical stimulation. Individually graphed points represent biological replicates. Two-way ANOVA. Error bars = SEM.

Figure 4. Examples dorsal root ganglia neurons cultured with A) T = testosterone; B) T-BSA = testosterone conjugated to bovine serum albumin; C) T+Flut = testosterone and flutamide; D) T+DRB = testosterone and 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole; E) vehicle. F) Neurite length as percentage of vehicle. Dashed line = vehicle control. Individually graphed points represent biological replicates. Two-way ANOVA. Error bars = SEM.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Single or Double</th>
<th>Cell Type</th>
<th>Treatment</th>
<th>Mean ± SD (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLICK AR&lt;sup&gt;f/y&lt;/sup&gt;</td>
<td>Single</td>
<td>Motoneuron</td>
<td>Untreated</td>
<td>59.2 ± 48.8 (5)</td>
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<tr>
<td>SLICK AR&lt;sup&gt;f/y&lt;/sup&gt;</td>
<td>Single</td>
<td>Motoneuron</td>
<td>Electrical Stim</td>
<td>115 ± 39.8 (5)</td>
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<tr>
<td>SLICK AR&lt;sup&gt;f/f&lt;/sup&gt;</td>
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<td>Motoneuron</td>
<td>Untreated</td>
<td>47.8 ± 6.1 (4)</td>
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<tr>
<td>SLICK AR&lt;sup&gt;f/f&lt;/sup&gt;</td>
<td>Single</td>
<td>Motoneuron</td>
<td>Electrical Stim</td>
<td>102.8 ± 22.6 (4)</td>
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<tr>
<td>SLICK AR&lt;sup&gt;f/y&lt;/sup&gt;</td>
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<td>Motoneuron</td>
<td>Untreated</td>
<td>7.2 ± 5.8 (5)</td>
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<tr>
<td>SLICK AR&lt;sup&gt;f/y&lt;/sup&gt;</td>
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<td>Motoneuron</td>
<td>Electrical Stim</td>
<td>8 ± 2.2 (5)</td>
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<td>21 ± 18.8 (4)</td>
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<tr>
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<td>Double</td>
<td>Motoneuron</td>
<td>Electrical Stim</td>
<td>34.3 ± 25.4 (4)</td>
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<tr>
<td>SLICK AR&lt;sup&gt;f/y&lt;/sup&gt;</td>
<td>Single</td>
<td>DRG neuron</td>
<td>Untreated</td>
<td>54.0 ± 22.3 (5)</td>
</tr>
<tr>
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<td>Single</td>
<td>DRG neuron</td>
<td>Electrical Stim</td>
<td>196.4 ± 93.5 (5)</td>
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<tr>
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<td>Single</td>
<td>DRG neuron</td>
<td>Untreated</td>
<td>59.5 ± 32.0 (4)</td>
</tr>
<tr>
<td>SLICK AR&lt;sup&gt;f/f&lt;/sup&gt;</td>
<td>Single</td>
<td>DRG neuron</td>
<td>Electrical Stim</td>
<td>127.8 ± 51.3 (4)</td>
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<tr>
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<td>Double</td>
<td>DRG neuron</td>
<td>Untreated</td>
<td>6.4 ± 4.3 (5)</td>
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<tr>
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<td>DRG neuron</td>
<td>Electrical Stim</td>
<td>6.4 ± 4.3 (5)</td>
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<td>DRG neuron</td>
<td>Electrical Stim</td>
<td>8.5 ± 5.9 (4)</td>
</tr>
</tbody>
</table>

Table 1. Results from the retrograde labeling studies. Retrograde labeling was performed two weeks after nerve repair to identify regenerating neurons. Single-labeled neurons were assumed to be wild-type. Double-labeled neurons were assumed to have a deletion of exon 3 – the DNA-binding domain of the androgen receptor.
Author/s:
Ward, PJ; Davey, RA; Zajac, JD; English, AW

Title:
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Date:
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